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Signal transduction of abscisic acid in *Arabidopsis thaliana*:

Identification and characterisation of protein interaction partners of ABI2

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Abbreviations:

aaRS	aminoacyl-tRNA synthetase
ABA	abscisic acid
abi1	abscisic acid-insensitive-1
ABRE	ABA response element
AC	accession number
Amp	ampicillin
AP	alkaline phosphatase
AtGluRS	Arabidopsis glutamyl tRNA synthetase
Ath	*Arabidospsis thaliana* (L.) Heynh
ATP	adenosine 5'-triphosphate
BAC	bacterial artificial chromosome
BAP	6-benzylamino-9-methylpurin
BCIP	5-bromo-4-chloro-3-indolyphosphate
bp	base pair
BSA	bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
CE	coupling element
cfu	colony forming units
CIP	calf intestine phosphatase, alkaline
CTAB	cetyltrimethylammoniumbromid
DAPI	4',6'-diamidino-2-phenyl-indol-dihydrochlorid
Dig	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiotreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis (β-aminorthylether)-N,N,N',N',-tetracetic acid
FG	fresh weight
FPR-GUS	β-D-glucuronidase gene under the control of fibrillin promoter
FPR-LUC	luciferase gene under the control of fibrillin promoter
GPR-LUC	luciferase gene under the control of AtGluRS promoter
GPR-GUS	β-D-glucuronidase gene under the control of AtGluRS promoter
GST	glutathione S-transferase
GUS	β-D-glucuronidase
h	hour
HB6	homeodomain protein in *Arabidopsis thaliana*
IPTG	isopropyl β-D-thiogalactoside
Kan	kanamycin
kb	kilobase
kDa	kilo Dalton
LTI	low temperature induced protein

LTI-LUC	luciferase gene under the control of LTI promoter
Lu	light units
LUC	luciferase
MES	2-morpholinoethansulfone acid
min	minute
MU	4-methyumbelliferon
MUG	4-methylumbelliferon-β-D-glucuronid
MUP	methylumbelliferyl phosphate
NBT	nitroblue tetrazolium
NCED	9-*cis*-epoxycarotenoid dioxygenase
NOS	napaline synthase
OD	optimal density
ORF	open reading frame
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
Rab18	rab-related protein (dehydrin gene family)
Rab18-LUC	luciferase gene under the control of Rab18 promoter
RFU	relative fluorescence units
Rif	rifampicine
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
SSC	standard saline citrate
TBE	tris-borate-EDTA buffer
TEMED	N,N,N,N'-tetramethylethylene diamine
Tris	Tris (hydroxymethyl) aminomethane
β-gal	β-galactosidase
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranosid
ΔABI1 and Δabi1	N-terminal deleted version of ABI1 and abi1 (121-434 .a.a.)
ΔABI2 and Δabi2	N-terminal deleted version of ABI2 and abi2 (95-423 .a.a.)
ΔAtGluRS	N-terminal domain of AtGluRS (1-262 amino acids)
35S-GUS	β-D-glucuronidase gene under the control of constitutive 35S promoter
35S-LUC	luciferase gene under the control of constitutive 35S promoter

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

Abscisic acid (ABA) plays a major role in regulating plant growth and development including seed maturation, germination and dormancy, as well as mediating adaptations to environmental stresses such as cold, drought and salinity (Zeevaart and Creelman, 1988; Leung and Giraudat, 2000). Key regulators of the diverse ABA-mediated responses are ABI1 and ABI2. Both proteins are homologous protein phosphatases 2C (PP2Cs) that act to a large extent in a redundant manner as negative regulators of ABA signalling (Rodriguez 1998; Gosti et al., 2001).

The establishment of the yeast two-hybrid system (Fields and Song, 1989) provides genetic means to identify proteins that physically interact *in vivo* as well as an efficient tool to screen libraries in order to isolate and identify genes that encode interacting partners for a protein of interests (Chien et al., 1991).

In this work, 13 potential interacting candidates of ABI2 were identified from *Arabidopsis* cDNA libraries using the yeast two-hybrid system. Analysis of DNA and protein sequences revealed that the primary interacting partners of ABI2 included: catalase (ATU43340), fibrillin (AF075598), Erd15 (ATHERD15), RibA (ATHJ0053), glutamyl tRNA synthetase (AF067773), AtHB-6 (ATH5J17), Cab binding protein (CAA39534), hypothetical protein (AF007271), a mitochondrial protein (MIATGENB), peroxiredoxin (CAA71503), and unknown proteins (ATAC006587, AB025622 and AC023628) of *Arabidopsis thaliana.* Further analysis demonstrated that most candidates revealed interaction with ABI2 as well as an even stronger interaction with ABI1. ABI1 and ABI2 have the same characteristic modular architecture and catalytic activity, and both PP2Cs seem to share interacting proteins.

Of the 13 potential interacting candidates, fibrillin and glutamyl tRNA were selected to characterize their functions in ABA signal transduction pathway in detail.

Fibrillin was identified as a protein physically interacting with ABI2. Interaction analysis between fibrillin and different truncated (deleted) and single point mutated versions of ABI2 demonstrated that fibrillin interacted only with ABI2 full-length protein, indicating

that the binding of ABI2 to fibrillin was dependent on both PP2C activity and the N-terminal sequence of ABI2. Thus, fibrillin interacted with ABI2 but not with the catalytically deficient mutant form abi2. In addition, fibrillin expression was up-regulated by ABA in an ABI1-dependent manner. Promoter-reporter fusions revealed stress-dependent activation of fibrillin expression in plant leaves and a strong constitutive expression in reproductive organs, particularly in tapetum and stigma. Cellular localisation studies indicated that the fibrillin-GUS fusion protein is present in the cytoplasm as fibrillar-like structures which were greatly decreased in number and in length in the presence of exogenous ABA or osmoticum.

RNA interference analysis in transgenic plants revealed that down-regulation of fibrillin transcript levels led to ABA-hypersensitivity in the germination as well as in stomatal response. The data support a critical function of fibrillin in plant's adjustment to ABA-induced responses. Fibrillin is likely to act as a negative regulator in ABA signal transduction pathway or represents a necessary component to compensate ABA-dependent stresses.

Glutamyl tRNA synthetase (AtGluRS), being also identified as an interacting partner of ABI2, revealed a strong preference for ABI1 and abi1 compared to ABI2. In addition, the N-terminal deleted ABI1 and catalytically inactive versions also interacted with AtGluRS, suggesting that PP2C activity as well as N-terminus of ABI1 is not critical for binding but minor structural difference between ABI1 and its homologue ABI2.

Analysis of transcript levels revealed that AtGluRS expression was not regulated by ABA. Its expression was the same in wild-type plant with or without ABA. However, its expression was greatly decreased in *abi1* and *abi2* mutant plants and normalized by application of exogenous ABA. Hence, its expression is dependent on both ABI1 and ABI2. In addition, AtGluRS seems to be involved in the processing of ABI1 and ABI2 by regulating splicing of the pre-mRNA to mature mRNA. AtGluRS overexpression inhibited this process. After application of exogenous ABA, correct splicing was observed. On the other side, some ABA-regulated genes, for example, *HB6* and *NCED3* in *Arabidopsis thaliana*, were found to be under-expressed if AtGluRS expression was down-regulated. NCED3 is a key enzyme in the biosynthesis of ABA and HB6 a key regulator of ABA signalling, indicating that AtGluRS perhaps modulates ABA biosynthesis and signalling.

Ectopic expression of AtGluRS in *Arabidopsis* resulted in ABA-hypersensitivity during germination and in the stomatal regulation. The findings supported a role for AtGluRS as a positive regulator in ABA signalling. Enzymatic analysis *in vitro* supported that concept since AtGluRS inhibited the PP2C activity of the negative regulator ABI1. The possible mechanisms of PP2Cs inhibition by AtGluRS *in vivo* is the following: AtGluRS directly inhibits either the enzymatic activity of PP2Cs or AtGluRS is involved in the processing of ABI1 and ABI2 transcripts by impeding the splicing of pre-mRNA to mRNA thereby leading to decreased PP2C expression. As a consequence, AtGluRS antagonizes ABI1/ABI2 actions.

1 Introduction

1.1 Abscisic Acid (ABA)

Abscisic acid (ABA) was originally called abscisin II because it promotes abscission of cotton fruit (Ohkuma et al., 1963). At the same time, a substance that promotes bud dormancy was purified from sycamore leaves and was called dormin (Cornforth et al., 1965). When dormin was chemically identified, it was found to be similar to abscisin II. Because of its involvement in the abscission process, the compound was renamed as abscisic acid (ABA) (Addicott et al., 1968; 1983).

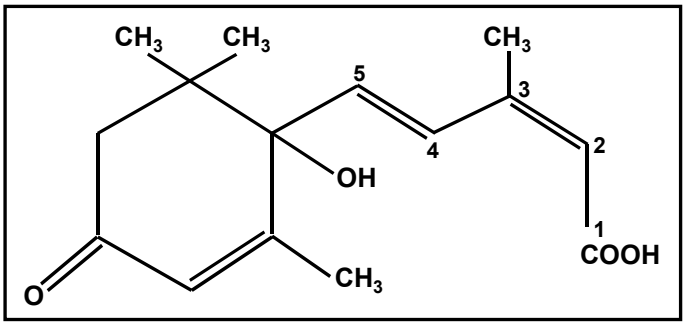


Figure 1. Abscisic Acid (ABA)

ABA is widely distributed in plants and some fungi. Within a plant, ABA has been detected in every organ or living tissues from root cap to the apical bud (Milborrow, 1984). ABA is synthesized almost in all cells containing chloroplasts or amyloplasts.

The structure of ABA is similar to the terminal portion of some carotenoid molecules. The 15 carbon atoms of ABA configure an aliphatic ring with one double bond, two methyl groups, and an unsaturated chain with a terminal carboxyl group (Figure 1). The position of the protons at C-2 and C-4 and the ensuring orientation of carboxyl group at C-2 determine the *cis* and *trans* isomers of ABA. Almost all the naturally occurring ABA is in the *cis* form. ABA also has an asymmetric carbon atom at the ring resulting in the (+) and (-) (or S and R, respectively) entantiomers (Milborrow et al., 1970; Taylor et al, 1973).

The amount of ABA in tissues depends on its biosynthesis, metabolism and transport. Like other plant hormones, the regulatory effect of ABA depends on its concentration in the tissues. Endogenous concentrations of ABA vary with growth and environmental conditions. ABA concentration in tissues is determined by the balance between biosynthesis and catabolism. A major cause of inactivation of free ABA is oxidation, but there is also conjugation. ABA is transported in both the xylem and the phloem, but is much more abundant in the phloem sap (Taiz and Zeiger, 1991).

The distribution of ABA is affected by compartmentation. ABA is a weak acid with a pKa 4.7 and its dissociation depends on the pH of each cellular compartment. The protonated form of ABA permeates freely across membranes, but the dissociated anion does not. As a result, the distribution of ABA between different compartments depends on their pH values, the more alkaline a compartment, the more ABA it accumulates (Cowan et al., 1982). Because of these properties, ABA concentration in the chloroplast increases in light, whereas the apoplast concentration increases in the dark (Kaiser et al., 1985).

The biosynthetic pathway of ABA comprises cleavage of a 40-carbon carotenoid to form the C_{15} compound xanthoxin. Xanthoxin is a neutral growth inhibitor with physiological properties similar to those of ABA. Xanthoxin is converted to ABA by oxidation (Seo and Koshiba 2002).

ABA biosynthesis is confined to the chloroplasts where pyruvate and glyceraldehyde phosphate are combined and rearranged (Figure 2), via deoxy-xylulosephosphate (DXP), to give isopentenyl diphosphate (IPP). In some tissues, mevalonate is incorporated into IPP in the cytosol (Mitsunori et al., 2001). Some of the IPP can then enter the chloroplasts where it is elaborated into carotenoids and can give rise to ABA (Rohmer et al., 1993; Rohmer 1999). IPP is converted to a C20 product, geranylgeranyl pyro-phosphate (GGPP). As the first committed and rate-limiting step in carotenoid synthesis, GGPP is converted to a C40 carotenoid phytoene, catalyzed by phytoene synthase (PSY). Then phytoene is subsequently converted to zeaxanthin through several steps, for example, ζ-carotene, lycopene and β-carotene. Zeaxanthin epoxidase (ZEP), the first enzyme to be identified as an ABA biosynthetic enzyme (Marin et al., 1996), catalyzes the formation of all-trans-violaxthin from zeaxanthin. NCED (9-cis-epoxy-carotenoid dioxygenaes) is involved the conversion of xanthoxin. The gene coding NCED was first identified during the characterization of viviparous 14 (*vp14*) mutant in maize (Tan et al., 1997). At last xanthoxin is converted to abscisic acid in the cytosol (Seo and Koshiba 2002).

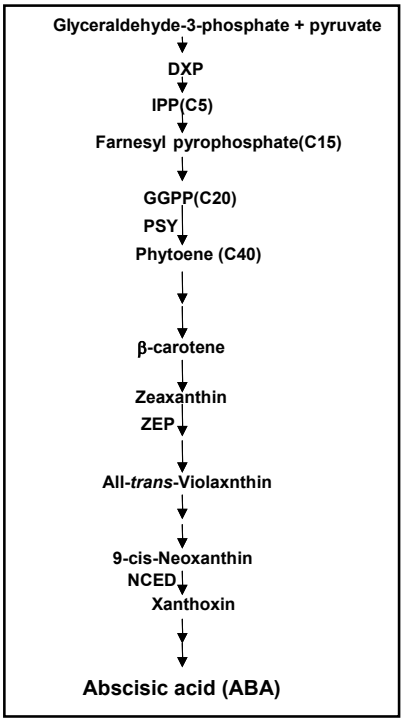


Figure 2. ABA biosynthetic pathway in plants
[According to Seo and Koshiba, 2002]

ABA is involved in regulation of many physiological responses in higher plants, e.g. bud dormancy, seed dormancy, growth, stress and stomatal closure. In woody species, dormancy is an important adaptive feature in cold climates. ABA was originally suggested as a dormancy-inducing hormone (Wareing, 1982). A characteristic of seeds is their ability to suspend developmental progress until the conditions necessary for germination are met (Bewley and Black, 1982). ABA appears to act as the dormancy-inducing hormone. A role of ABA in dormancy is also indicated by the observation that exogenous ABA prevents germination and maintains dormancy. Exogenous ABA also reimposes dormancy on embryos kept in culture, often causing them to continue to grow and to accumulate reserves (Zeevaart and Creelman, 1988). Auxin-induced growth of seedlings is inhibited by ABA, and for this reason ABA is also called a growth inhibitor hormone (Taiz and Zeiger., 1991). The role of ABA in response to cold, drought and salinity has led to the characterization of ABA as the stress hormone. Under drought conditions, the leaf ABA concentration can increase up to 40 times, which is a most dramatic change in concentration reported for any hormone in response to an environmental signal. ABA is very effective in causing stomatal closure, and its accumulation in stressed leaves plays an important role in the reduction of water loss by transpiration under water stress condition (Raschke, 1987). Mutants that lack the ability to synthesize ABA show permanent wilting because of their inability to close stomata. Application of exogenous ABA to such mutants causes stomatal closure and turgor buildup (Tal and Imber, 1971).

Significant progress has been made in understanding how ABA controls diverse, essential physiological progresses since the 1960s (Zeevaart and Creelman, 1988; Giraudat et al., 1994). Many of the actions of ABA, in both seeds and vegetative tissues, involve modification of gene expression at the transcriptional level. Many ABA-responsive genes have been isolated, and their analysis has provided insights into the biological function of the encoded proteins, as well as into the nature of the *cis*- and *trans*-acting factors involved in ABA responsiveness (Ingram and Bartels, 1996).

1.2 Abscisic acid signal transduction

ABA plays a major role in plant growth and development, including seed maturation, germination and dormancy, as well as in adaptation to abiotic environmental stresses such as cold, drought or salinity (Zeevaart and Creelman, 1988). Significant progress in understanding the regulation of growth and development of plants by ABA has been made by characterizing several mutants that are either defective in ABA biosynthesis or ABA responsiveness. In mutants *aba1*, *aba2* and *aba3* the pathway of ABA biosynthesis is blocked at different steps and their phenotypes can be reversed to wild type by exogenous

addition of ABA (Koornneef et al., 1982; Marin et al., 1996; Schwartz et al., 1997). The other mutants, ABA-insensitive (*abi1-abi5*) and enhanced response to ABA (*era1-era3*), have an altered responsiveness to ABA. They do not have a reduced endogenous ABA content and their phenotypes can not be reversed to wild-type by exogenous supply of ABA (Koornneef et al., 1984; Finkelstein, 1994; Cutler et al., 1996). Thus, such mutants can be used to study components of ABA signal transduction cascades and to unravel their pathways (Grill and Himmelbach, 1998).

1.2.1. Genetic analysis of ABA signal transduction

Phenotypically, the *abi1*, *abi2* and *abi3* mutants display significant reductions in seed dormancy, and *abi3*, *abi4* and *abi5* mutants exhibit defects in various aspects of seed maturation and *abi3* mutants also show a numerous defects during embryo development (Koornneef et al., 1984; Finkelstein, 1994; Nambara et al., 1995; 2000). However, seed dormancy is increased in *era1* mutants (Culter et al., 1996). Aside from the seed-specific defects, the *abi1* and *abi2* mutants also clearly affect ABA responses in vegetative tissues. Particularly, both mutants display abnormal stomatal regulation and defects in various ABA-mediated morphological and molecular responses to stress (Koornneef et al., 1984; Finkelstein et al., 1990 and 1994; Giraudat et al., 1994).

A two-pathway model is provided to explain the developmental variations among ABA-insensitive mutants (Merlot and Giraudat, 1997). As shown in Figure 3-A, the characteristic ABI3-dependent developmental processes are postulated to be under strict ABA control. The ABI1 and ABI2 loci would then act in a different ABA signalling pathway from ABI3, ABI4 and ABI5. In Figure 3-B, these developmental process are postulated to be jointly regulated by ABA and by additional developmental factors, with both types of signals being mediated by ABI3 and possibly ABI5. The ABI1/ABI2 pathway may then mediate ABA signalling in seeds as in vegetative tissues, but only in seeds would this cascade recruit the seed-specific ABI3, ABI4 and ABI5 proteins. Otherwise, ABI1 and ABI2 proteins have in fact partially redundant functions. As shown in Figure 3-C, evidence indicates that the homologous proteins ABI1 and ABI2 act as redundant signalling elements for certain ABA responses but that each of these proteins has additional specific roles in mediating other ABA actions.

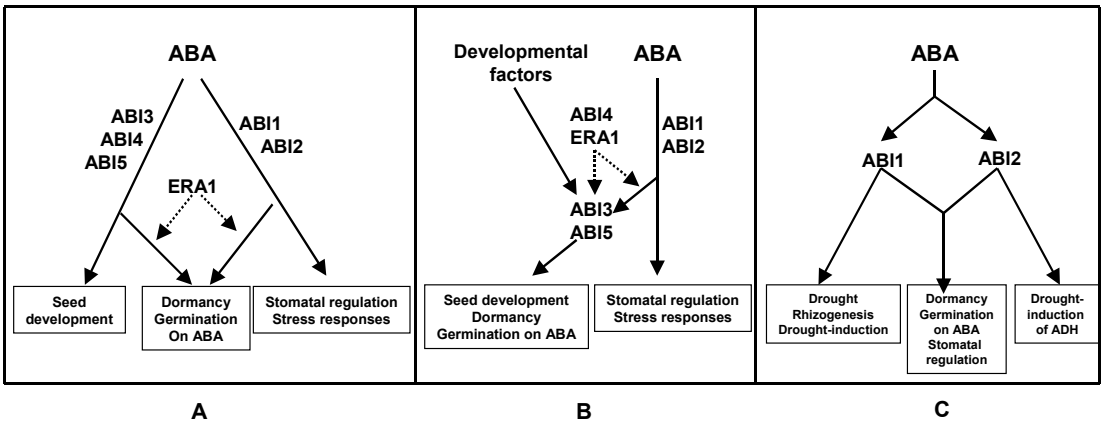


Figure 3. Hypothetical models to explain the develpomental variations among ABA-insensitive mutants.
A and B: two pathways model for explaining the phenotypic differences between *abi1*/*abi2* and *abi3* mutants;
C: a model of the partially redundant roles of ABI1 and ABI2 in ABA signaling. [Merlot and Giraudat, 1997]

The *ABI1* to *ABI5* genes have already been cloned. The *ABI1* and *ABI2* genes encode similar class 2C serine/threonine protein phosphatases (Leung et al., 1994 and 1997; Meyer et al., 1994; Rodriguez et al., 1998). *ABI3*, *ABI4* and *ABI5* encode putative transcriptional regulators. ABI3 is orthologous to the maize Viviparous 1 protein (McCarty et al., 1991; Giraudat et al., 1992). ABI4 contains an APETALA2-like DNA binding domain (Finkelstein et al., 1998) and ABI5 is a member of a basic leucine zipper transcription factor family (Finkelstein and Lynch, 2000).

Biochemical and molecular studies in *Arabidopsis* have identified PP2C enzymes as a key factors in plant signal transduction process. Two corresponding genes, *ABI1* and *ABI2*, encode type 2C protein phosphatases, and the dominant mutant alleles *abi1-1* and *abi2-1* which confer impaired ABA-induced stomatal closure (Koornneef et al., 1984). The corresponding proteins have point mutations altering a conserved glycine amino acid into aspartic acid (Meyer et al., 1994; Leung et al., 1994, 1997; Rodriguez et al., 1998). ABI1 and ABI2 proteins display the same characteristic modular architecture and their C-terminal domains share 86% amino acid identity as shown in Figure 4. However, the N-terminal domains of ABI1 and ABI2 are less conserved (49% identity) and display no extensive similarity to other known proteins (Meyer et al., 1994; Leung et al., 1994, 1997). A calcium-binding domain known as EF hand (Moncrief et al., 1990) is found in the N-terminal extension of ABI1 contains but not in that of ABI2. However, no evidence is provided that EF hand of ABI1 binds calcium. Phenotypic complementation of a yeast PP2C mutant and *in vitro* enzymatic assays using recombinant proteins support the idea that both C-terminal domains of ABI1 and ABI2 have PP2C catalytic activity (Meyer et al., 1994; Bertauche et al., 1996; Leung et al., 1997). In both mutants the phosphatase activity of abi1 and abi2 proteins is markedly reduced because of the substitutions of Gly to Asp at

the equivalent positions in ABI1 (Gly-180) and ABI2 (Gly-168) phosphatase domains, respectively (Meyer et al., 1994; Leung et al., 1997; Rodriguez et al., 1998). The Gly to Asp substitutions probably preferences with two neighboring invariant residues (Asp-177 and Gly-178 in ABI1 and Asp-165 and Gly-166 in ABI2), which are essential for metal coordination and phosphatase activity (Das et al., 1996).

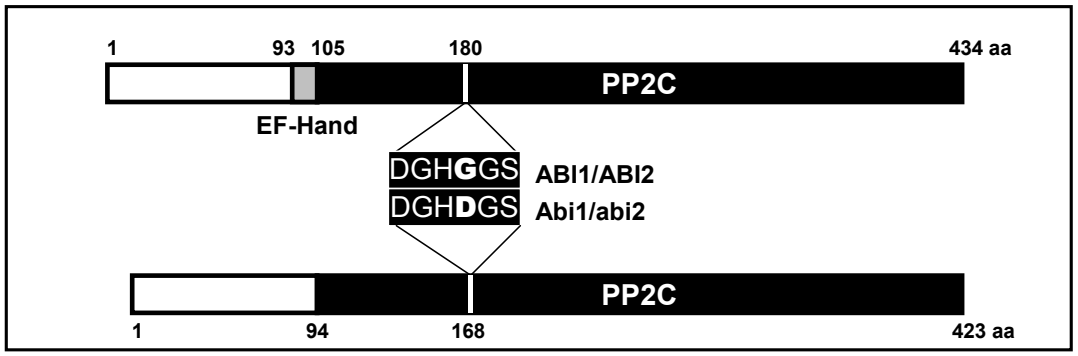


Figure 4. Schematic diagram of two homologous protein phosphatases—ABI1 and ABI2.

The diagram represents the *Arabidopsis* ABI1 and ABI2 proteins. Full length ABI1 has 434 and full length ABI2 has 423 amino acids. ABI1 and ABI2 have 86% identity in C-terminal domains (solid boxes) that contain the PP2C but less identity (49%) in N-terminal extensions (open boxes). ABI1 has a potential Ca^{2+} binding site (EF-hand). The Gly residue is substituted by Asp in both mutants abi1 ($180^{G\ to\ D}$) and abi2 ($168^{G\ to\ D}$) in the phosphatase domain.

Due to the less conserved N-terminal extensions of ABI1 and ABI2, it may be that it provides the domain to interact with different cellular targets. For example, drought rhizogenesis is inhibited in *abi1-1* but not in *abi2-1* (Vartanian et al., 1994). Differences are also demonstrated in the expression of certain ABA-, cold-, drought- or salt-inducible genes (Merlot and Giraudat, 1997; Rodriguez, 1998).

Several downstream responses to ABA are impaired in these *Arabidopsis* mutants including K^+_{out} and K^+_{in} channel regulation (Armstrong et al., 1995) and anion channel activation (Pei et al., 1997). These mutations also impair ABA-induced $[Ca^{2+}]_{cyt}$ increases (Allen et al., 1999). Furthermore, experimental elevation in $[Ca^{2+}]_{cyt}$ causes anion channel activation and stomatal closure in *abi1-1* and *abi2-1*, thus bypassing the effects of the *abi1-1* and *abi2-1* mutations (Allen et al., 1999). These data demonstrate that the dominant PP2C mutants interfere with very early ABA signalling events that act upstream of $[Ca^{2+}]_{cyt}$ (Allen et al.,1999).

Intragenic revertants of the *abi1*-1 and *abi2*-1 mutants were isolated and shown to have reduced or no detectable phosphatase activity *in vitro* (Gosti et al., 1999; Merlot et al., 2001). Because a double mutant of both revertants shows hypersensitivity to ABA, ABI1

and ABI2 are likely negative regulators of ABA signalling (Merlot et al., 2001). In correlation, over-expression of wild-type ABI1 in maize mesophyll protoplasts blocks ABA induction of gene expression (Sheen, 1998; Hoffmann, 2001).

Table 1. Mutants that affect ABA sensitivity or related physiological responses in *Arabidopsis thaliana*

Mutation/alles	phenotype	Gene product	References
abi1	Pleiotropic ABA insensitive	Protein phosphatase 2C	Koornneef et al (1984); Leube et al (1998); Gosti et al (1999)
abi2	Pleiotropic ABA insensitive	Protein phosphatase 2C	Koornneef et al (1984); Leung et al (1997); Rodriguez et al (1998)
abi3	ABA insensitive in seeds; altered plastid develpment and flowering	Vp1-like transcription factor	Koornneef et al (1984);Giraudat et al (1992); Kurup et al (2000)
abi4	ABA insensitive in seeds; sugar and salt insensitive	APETELA2-like transcription factor	Finkelstein (1994); Finkelstein et al (1998)
abi5	ABA insensitive in seeds;	bZIP transcription factor	Finkelstein (1994); Finkelstein & Lynch (2000);
era1	ABA hypersensitive	β-subunit of farnesyl tranferase	Cutler et al (1996); Pei et al (1998)
era2/3	ABA hypersensitive in seed		Cutler et al (1996)
gca1/2	Pleiotropic ABA insensitive		Himmelbach et al (1998)
gca3/8	Insensitive in ABA inhibition of root growth		Himmelbach et al (1998)
abh1	Recessive ABA hypersensitive	mRNA cap binding protein	Hugouvieux et al (2001)
ahr1	Recessive ABA hypersensitive		Wang et al (unpublished)

Modified from Rock (2000)

Another type of ABA insensitive mutants *gca1* to *gca8* were identified (Himmelbach et al., 1998). Two mutants, *gca1* and *gca2*, show a pleiotropic ABA insensitive phenotype like *abi1* and *abi2*. However, all the other mutants show that both ABA and ethylene affect their growth (Himmelbach et al., 1998), suggesting cross-talk between the pathways of ABA and ethylene.

Mutations in the *ERA1* to *ERA3* (**E**nhanced **R**esponse to **A**BA) loci of *Arabidopsis thaliana* were identified by a lack of seed germination at low concentrations of ABA (0.3 μM) that do not inhibit the wild type (Culter et al., 1996). The *era1* mutations also markedly increase seed dormancy. The *ERA1* gene encodes the β subunit of a farnesyl transferase, which may possibly function as a negative regulator of ABA signalling by modifying signal transduction proteins for membrane localization (Culter et al., 1996). Desiccation of plants during drought can be detrimental to agricultural production. Deletion of the *Arabidopsis* farnesyltransferase gene *ERA1* or application of farnesyltransferase inhibitors resulted in ABA hypersensitivity of guard cell anion-channel activation and of stomatal closing. *ERA1* was expressed in guard cells. Double-mutant analyses of *era1* with the ABA-insensitive mutants *abi1* and *abi2* showed that era1 suppresses the ABA-insensitive phenotypes. Moreover, *era1* plants exhibited a reduction in transpirational water loss during drought treatment (Pei et al., 1998)

The lately isolated recessive ABA hypersensitive mutant, *abh1*, shows ABA-hypersensitive stomatal closing and reduced wilting during drought (Hugouvieux et al., 2001). *ABH1* encodes the *Arabidopsis* homolog of a nuclear mRNA cap binding protein and functions in a heterodimeric complex to bind the mRNA cap structure. Interestingly, ABA-hypersensitive cytosolic calcium increases in *abh1* guard cells demonstrate amplification of an early ABA signalling step. Thus, ABH1 represents a modulator of ABA signalling proposed to function by transcript alteration of early ABA signalling elements (Hugouvieux et al., 2001). Another dominant mutant, *ahr1*, was identified as being hypersensitive to ABA responses (Wang and Grill, unpublished).

Mutants that affect ABA sensitivity and related physiological responses in *Arabidopsis thaliana* are summarized in Table 1.

1.2.2. Analysis of secondary messengers of ABA signal transduction

ABA induces the rapid depolarization of the plasma membrane, which in turn triggers a massive redistribution of ions and solutes from the vacuole and cytoplasm to the apoplast, resulting in stomatal closure by loss of turgor and cell volume. ABA also inhibits stomatal opening. Cytosolic free [Ca^{2+}] and pH are thought to be intracellular second messengers of

the response (Irving et al., 1992; Leube et al., 1998). ABA activates the vacuolar H^+ATPase (Barkla et al., 1999). Influx of Ca^{2+} across the plasma membrane occurs through ABA-activated channels (Schroeder et al., 1990; Hamilton et al., 2000). Membrane voltage oscillations also initiate [Ca^{2+}] waves and potentate ABA-activated [Ca^{2+}] increases (Grabov et al., 1998). Ca^{2+} has negative effects on the inward-rectifying (Ca^{2+} inhibits) and outward-rectifying (Ca^{2+} activates) K^+ channels that contribute to stomatal opening and closing, respectively. Ca^{2+} regulation of the outward K^+ channel is indirect: the channel is voltage-gated and is thus activated by membrane depolarization, caused largely by the activity of an ABA-activated and Ca^{2+}–activated outward-rectifying slow (S) anion channel that extrudes Cl^- and malate (Grabov et al., 1997; Pei et al., 1997, 1998).

The *abi1* and *abi2* mutants decrease, but do not abolish intracellular [Ca^{2+}] transients and are blocked in the ABA-induced S-anion currents involved in stomatal closure (Allen et al., 1999). Experimental modulation of intracellular free [Ca^{2+}] bypasses the effects of the *abi1* and *abi2* mutations on the S-anion channel and restores stomatal function (Allen et al., 1999). Ca^{2+} is required for ABA-inducible gene expression in transiently transformed protoplasts, embryo and sub-epidermal cells (Rock et al., 1996; Wu et al., 1997; Bustos et al., 1998), and Ca^{2+} perfusion is sufficient to trigger ABA-inducible gene expression in protoplasts and microinjected sub-epidermal cells (Sheen, 1996; Wu et al., 1997).

Recent experiments showed that cADPR (cyclic-ADP ribose) is associated to Ca^{2+} fluxes, ABA-regulated gene expression and stomatal movement. It provides evidence that stomatal and nuclear ABA signalling might share at least some elements. In plant vacuoles, nanomolar cADPR concentrations can activate a Ca^{2+} permeable current (Allen et al., 1995). Microinjection of cADPR into tomato hypocotyl cells shows that cADPR can function in ABA signalling (Wu et al., 1997). In *Commelina* guard cells cADPR causes $[Ca^{2+}]_{cyt}$ increases and elicits stomatal closing (Leckie et al., 1998). However, microinjection of the inactive cADPR analog 8-amino-cADPR or noncyclic ADPR does not elicit $[Ca^{2+}]_{cyt}$ increases or guard cell turgor loss. Ryanodine treatment of guard cells also reduces $[Ca^{2+}]_{cyt}$ increases (Grabov et al., 1998). In addition, ABA-induced stomatal closure is only partly inhibited by either microinjection of 8-amino-cADPR (Leckie et al., 1998) or by nicotinamide, an inhibitor of cADPR production (Jacob et al., 1999; Leckie et al., 1998). This suggests that additional parallel $[Ca^{2+}]_{cyt}$ elevation mechanisms are needed in the ABA signalling cascade.

Various lines of evidence suggest that phospholipase C (PLC) is a component of ABA signal transduction in guard cells. Early experiments showed that release of caged inositol 1, 4, 5-triphosphate (InsP3) into the cytosol of guard cells could cause $[Ca^{2+}]_{cyt}$ increases and stomatal closure (Gilroy et al., 1990) and inhibit K^+_{in} channels (Blatt et al., 1990). Treating

guard cell protoplasts with ABA slightly elevates InsP3 levels (Lee et al., 1996; Parmar et al., 1995). In addition, other inositol-phosphates can also act as second messengers in ABA signal transduction in guard cells. In a recent study, myo-inositol hexakisphosphate (InsP6) was identified as an intermediary of guard cell signal transduction (Lemtiri-Chlieh et al., 2000). ABA stimulates production of InsP6 in guard cells. InsP6 perfused into the cytosol *via* a patch pipette inhibited K^+_{in} channels in potato guard cell protoplasts in a Ca^{2+}-dependent manner. These data suggest that InsP6 production is also an important component of ABA signalling.

Phospholipase D (PLD) has been implicated in ABA signalling in aleurone cells (Ritchie et al., 1998) and in guard cells (Jacob et al., 1999). PLD generates phosphatidic acid (PtdOH), and ABA treatment of *Vicia* guard cells caused PtdOH levels to transiently increase 2.5-fold (Jacob et al., 1999). PtdOH also promotes stomatal closure and inactivates K^+_{in} currents. Guard cell $[Ca^{2+}]_{cyt}$ did not increase following PtdOH treatment, suggesting that PLD acts in a parallel pathway or downstream of Ca^{2+} mobilizing second messenger systems. An inhibitor of PLD activity, 1-butanol, caused only a partial inhibition of ABA-induced stomatal closure whereas near-complete inhibition of stomatal closure resulted from adding 1-butanol together with nicotinamide (Jacob et al., 1999), suggesting a parallel action of PLD to the cADPR-mediated pathway. Figure 5 shows the ABA signal transduction in guard cells (Schroeder et al., 2001).

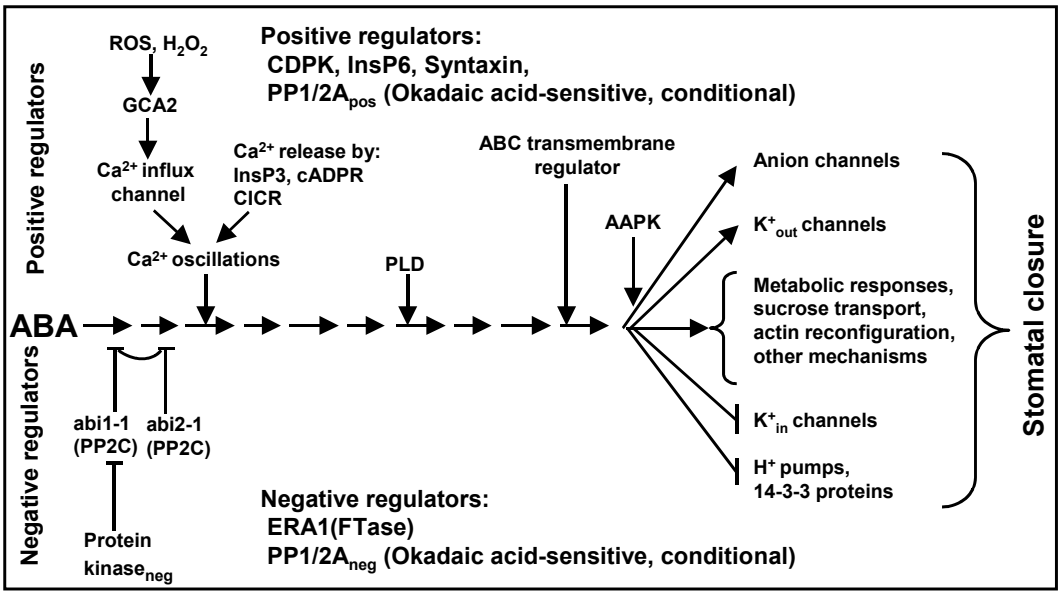


Figure 5. ABA signal transduction in guard cells.

Simplified model for proposed functions of positive (top) and negative regulators (bottom) mediating guard cell ABA signal transduction. [According to Schroeder et al., 2001]

1.2.3. Expression of ABA-regulated genes

About 50% of plant genes are homologous to genes of known function (Somerville et al., 1999). Some of those genes are ABA-inducible or -repressible. Recently, the cDNA microarray analysis showed that the transcripts of 245 genes increased after abscisic acid treatment. Among the ABA-inducible genes identified, 22 transcription factor genes were identified, suggesting that many transcriptional regulatory mechanisms exist in the ABA signal transduction pathways (Seki et al., 2002). Using massively parallel signature sequencing (MPSS) applied to samples from *Arabidopsis thaliana* wild-type and *abi1-1* mutant seedlings to study genome-wide ABA-responsive gene expression, 1354 genes that are either up- or down-regulated following ABA treatment of wild-type seedlings were identified and many of them encode signal transduction components (Hoth et al., 2002). Therefore, the further investigations of their functions will probably reveal a role in ABA physiology or signalling.

Cis-acting elements: Genes which contain ACGT-containing G-boxes and can be recognized by bZIP transcription factors have been shown to function as ABA-responsive elements (ABREs) in promoters (Marcotte et al., 1988; Mundy et al., 1990), such as HVA1 (Hobo, 1999a), OsEm (Shen & Ho, 1997), Dc3 (Siddiqui et al., 1998). The ACGT-core element is required for responsiveness to ABA. A well characterized ABA-inducible and drought-inducible promoter from carrot LEA Dc3 gene (Siddiqui et al., 1998) has been used to visualize gene expression induced by ABA and drought after fusion to the GUS gene and expression in wild-type, *aba1*, *abi1* and *abi2* mutant (Chak et al., 2000). Another *cis*-acting core target sequence CCGAC, designated the C-repeat/dehydration-responsive element (DRE), is found in the promoters of COR genes and imparts cold-regulated and dehydration-regulated gene expression through overlapping ABA-independent pathways (Shinozaki et al., 1997; Thomashow 1999). A novel *cis*-element was identified in HB6 promoter which mediated HB6- and ABA-dependent gene expression (Himmelbach et al., 2002).

Trans-acting factors: Expression of some ABRE-binding bZIPs is induced by ABA (Lu et al., 1996; Kusano et al, 1995; Nakagawa et al., 1996); there is only evidence from *in vitro* studies (Oeda et al., 1991; Izawa et al., 1994; Nantel et al., 1996) that those proteins act as transcription factors in ABA-responsive gene expression. Recently two true functional bZIP genes involved in ABA-inducible expression have been cloned, e.g. TRAB1 from a yeast two-hybrid screen with VP1 as a bait (Hobo et al., 1999b), and ABI5 by map-based cloning of an ABA-insensitive mutant. The DREB2A (dehydration- responsive-element binding factor) has a conserved Ser/Thr-rich region adjacent to the DNA binding domain that might be a target for protein kinases (Liu et al., 1998). It is possible that protein kinases and/or

phosphatases, such as ABI1 and ABI2, might act in environmental or cellular responses that include targets such as DREBs, ABI3/Vp1, ABI4 and ABI5. Consistent with this notion is the fact that *DREB*, *ABI1*, *ABI2* and *ABI5* expression are induced by ABA and dehydration (Leung et al., 1997; Liu et al., 1998; Finkelstein et al., 2000). A variety of cellular functions are activated by CDPKs, e.g. the activation of Cl^- channel in the tonoplast of guard cells involved in stomatal closure (Pei et al., 1996).

Transcriptional regulator HB6 belongs to the plant-specific HD-Zip class. Gene expression of HB6 is upregulated by ABA and during drought stress (Södermann et al., 1999). Reporter expression under control of HB6 promoter is induced by a factor of 2,000 in the presence of ABA and ectopic analysis indicates that ectopic expression of HB6 displays a reduced sensitivity towards ABA during seed germination and stomatal closure (Himmelbach et al., 2002).

In *Solanum* plants, both transcript and protein of chloroplastic drought-induced stress protein (CDSP34) accumulated from early stages of water deficit and in response to ABA. In water-stressed ABA-deficient potato mutants, similar increases in levels of the CDSP34-related transcript amount were observed in comparison with the wild-type, but protein accumulation was reduced, suggesting a post-transcriptional role for ABA in CDSP34 synthesis (Gillet et al., 1998).

The eventual cloning of the genes identified by mutational analyses will reveal their molecular identity and provides access to possible functions based on homologies with known proteins. Genes with unexpected structural features will divulge novel signal transduction mechanisms. The isolation of genes responsive to ABA, coupled with promoter analyses, is necessary to understand how combinatorial and synergistic actions of *cis*-acting elements and *trans*-acting factors are involved in the versatile control of the ABA response. Biochemical purification and those based on interaction cloning will undoubtedly complement the more established technologies in unraveling signalling elements or even signalling complexes (Leung and Giraudat, 1998).

1.3 Yeast hybrid interaction system

In *Arabidopsis*, more than 3,000 genes (13% of *Arabidopsis* genes) are likely to be involved in transcription (Somerville et al., 1999). The development of the yeast two-hybrid system provided a genetic means to identify proteins that physically interact *in vivo*. Since the publication of this technique in the late 1980s (Fields and Song, 1989), the robust nature and far-reaching utility of the yeast two-hybrid systems for functional expression library cloning has led to the identification of many novel proteins in all areas of biological life science research. Additionally, the two-hybrid technique provides a rapid and versatile system for the further characterization of discrete protein-protein interactions (Young, 1998).

The yeast two-hybrid system was a logical extension of studies that demonstrated the modular nature of transcriptional activators (Sadowski et al., 1988). Site-specific transcription factors often have a physically separable, functionally independent DNA-binding domain (BD) and a transcriptional activation domain (AD). In yeast a DNA-binding domain (DNA-BD) binds to a specific enhancer-like sequence, which in yeast is referred to as an upstream activation site (UAS; Heslot and Gaillardin, 1992). One or more activation domains (AD) direct the RNA polymerase II complex to transcribe the gene downstream of the UAS (Keegan et al., 1986; Hope and Struhl, 1986; Ma and Ptashne, 1987). In the two-hybrid system (Figure 6), two putative interacting proteins X and Y are fused to the BD and AD of the yeast transcriptional activator GAL4. If physically separated by recombinant DNA technology and expressed in the same host cell, the DNA-BD and AD peptides do not directly interact with each other and thus can not activate the responsive genes (Ma and Ptashne, 1988; Brent and Ptashne, 1985). However, if the DNA-BD and AD can be brought into close physical proximity in the promoter region, the transcriptional activation function will be restored and gene expression can easily be detected by a suitable reporter system, e.g. cell growth or a quick enzyme assay.

Based on the yeast two-hybrid system, a possible MAPK cascade of *Arabidopsis* was identified (Mizoguchi et al., 2000). Mitogen-activated protein kinase (MAPK) cascades have essential roles in diverse intracellular signalling processes in plants, animals and yeasts (Madhani, 1998). In plants, MAPK and MAPK-like kinase activities are transiently activated in response to environmental stresses and plant hormones (Hirt, 1997).

Using the yeast two-hybrid system biologically relevant interactions between VP1/ABI3 and plant proteins have been demonstrated that might be involved in ABA-regulated transcriptional activity. Proteins that bind to VP1/ABI3 include a bZIP protein that transactivates ABA-inducible promoters (Hobo et al., 1999b), a 14-3-3 chaperone-like

protein that binds a bZIP (Schultz et al., 1998), a novel protein with limited homology to CONSTANS (a gene encoding a nuclear zinc-finger protein involved in flowering that genetically interacts with ABI3) (Kurup et al., 2000), and a zinc-finger transcription factor with homology to *Arabidopsis* COP1 (**CO**nstitutively **P**hotomorphogenic-1) (Jones et al., 2000; Kurup et al., 2000). The homeo-domain protein HB6 was identified by using ABI1 as bait protein to screen the *Arabidopsis* cDNA library with the yeast two-hybrid system (Leube 1996, PhD dissertation). As a regulator of ABA signal transduction pathway, the interaction between HB6 and ABI1 requires an intact protein phosphatase domain of ABI1 and one domain of HB6 containing the DNA-binding site (Himmelbach et al., 2002).

The two-hybrid system is not only frequently used to test for interactions between known proteins, but it also has proved a powerful tool to screen libraries in order to isolate and identify genes that encode interacting partners for a protein of interest (Chien et al., 1991).

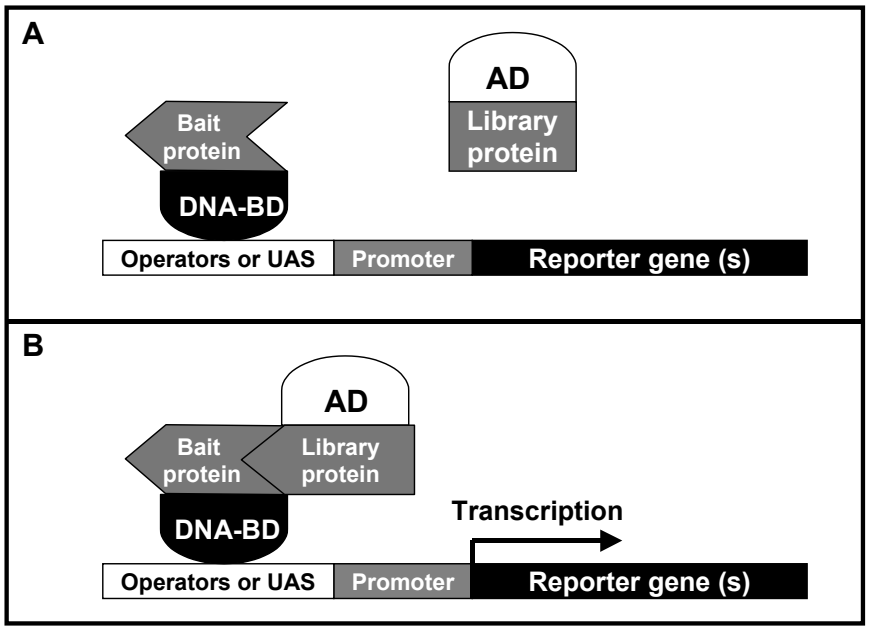


Figure 6. The transcription-based two hybrid system.

A protein of interest is expressed as a fusion to a DNA-BD and another protein is expressed as a fusion to the AD. A: if two proteins do not interact, the BD alone can not activate the transcription of the reporter gene; B: if the two proteins interact, the interaction reconstitutes the activator, leading to transcription of the reporter gene. [modified from Clontech MATCHMAKER SYSTEM]

1.4 The aim of this work

In ABA signalling transduction pathway, the ABA response depends on coordinated interactions between positive and negative regulators. However, it is unclear whether these interactions are direct or indirect in many cases (Finkelstein et al., 2002). Although hundreds of ABA-regulated genes have been identified, many of them as homologs in different species, but there is still abundance of non-characterized ABA-regulated genes in the genome, e.g. at least 2,000 ABA-responsive genes in *Arabidopsis*. Identification and characterization of ABA-response genes in respect to their targets and regulators will certainly elucidate ABA-signalling.

The negative regulators of ABA signalling, ABI1 and ABI2 are class 2C serine/threonine protein phosphatases (PP2C). Both proteins display the same characteristic modular architecture and are central components in abscisic acid (ABA) signal transduction. To study the proteins which interact with ABI1 and ABI2 as potential modulators or components of ABA signal transduction, wild-type ABI2 was used in this work as a bait protein to identify its interacting partners by expression of *Arabidopsis thaliana* cDNA libraries using the yeast two-hybrid system. The interactions between potential candidates and different versions of ABI2, abi2, ΔABI1 and Δabi1 were planned to be characterized by quantification of β-galactosidase reporter activity. Further characterization should include transcription analysis. Consequently, gene expression studies of selected candidates should be performed and in order to understand their role on ABA signal transduction. In addition, functional analysis of those candidates *in vitro* and *in vivo* as well as in transgenic plants was an aim of this work.

2 Materials and Methods

2.1 Materials

2.1.1 Plant materials

Arabidopsis thaliana

Arabidopsis thaliana accessions Reschiev (RLD) and Landsberg *erecta* (La-*er*) were used for generation of the transgenic plants and as a source of wild-type DNA and RNA as well for crossing. All accessions were received from the Arabidopsis Biological Resource Center (ABRC), Ohio, USA.

Mutant plants

ABA-insensitive mutants *abi1*, *abi2* and ABA-deficient mutants *aba1*, *aba2* were used for crossing and obtained from ABRC, Ohio, USA.

Transgenics

The transgenic plants expressing the LUC reporter gene under control of the HB6 promoter (Himmelbach et al., 2002) or Lti65 (Nordin et al., 1993) promoter was generated by Dr. A. Himmelbach.

2.1.2 Micro-organisms

Yeast strains

Yeast strain HF7c (*Saccharomyces cerevisiae*) containing *HIS3* and *lacZ* reporter genes and *trp1* and *leu2* selectable markers (Feilotter et al., 1994) was provided by CLONTECH (Stehelin & Cie AG, Basel, Swiss).

Bacterial strains

The *Agrobacterium tumefacien* stain C58 harboring Ti-plasmid pGV3850 (Zambrisky et al. 1983) or pGV3101 (Koncz and Schell, 1986) were used for plant transformation by vacuum infiltration (Bent et al., 1994). For routine cloning, plasmids were propagated in *E. coli* (*Escherichia coli*) strains DH5α (Stratagene GmbH, Heidelberg) and HB101 (Promega, Swiss).

2.1.3 Libraries

Ohio library

Arabidopsis thaliana Ohio library was provided by the DNA Stock Center, ABRC, Ohio, USA. mRNA was isolated from mature *Arabidopsis* leaves and roots. Adapted cDNA was inserted into the T-filled XhoI site of λACT (Elledge et al. 1991). λACT allows construction of cDNA libraries fused to sequences for GAL4 transcriptional activation domain (aa. 768-881) with subsequent conversion to plasmid from using cre-lox site-specific recombination as with λ YES. pACT10 contains the sequences encoding GAL4 AD and the *LEU2* gene for selection in Leu^- auxotrophic yeast strains.

Clontech library

Arabidopsis thaliana MATCHMAKER cDNA Library generated from 3-week-old green vegetative tissue was obtained from CLONTECH, USA. Cloning vector is pGAD10 (Bartel et al., 1993) with GAL4 AD (aa. 768-881) and the *LEU2* gene for selection in Leu^- auxotrophic yeast strains, and the cDNA is cloned to EcoRI site in the polylinker of pGAD10 vectors.

Cosmid library

Arabidopsis cosmid library in plasmid pBIC20 (Meyer et al., 1994) was constructed by our laboratory (AG Professor E. Grill) and was used to isolate the glutamyl tRNA synthetase gene.

Vector	Reference
pBI121/pBI221	Jefferson, 1987
pSK	Stratagene, Heidelberg
pGEX-2T (GTK)	Phamacia, USA (N-terminal PKA recognition sequence, RRASV, provided by Blanar and Rutter, 1992)
pGBT9	Bartel et al., 1993
pGAD424	Bartel et al., 1993
pMESHI	C. Koncz, MPI, Cologne
pPCV812	C. Koncz, MPI, Cologne

2.1.5 Recombinant vectors

Vector	Insert	Reference
BAC T24H24	Fibrillin gene	ABRC, DNA stock center, Ohio, USA
pGBT9ABI2	full length ABI2	Rodriguez (unpublished results)
pGBT9abi2	full length abi2	Rodriguez (unpublished results)
pGBT9DABI1	N-terminus deleted (aa 121-434)	Leube et al. 1998
pGBT9Dabi1	N-terminus deleted (aa 121-434)	Leube et al. 1998
pGBT9DABI2	N-terminus deleted (aa 95-423)	Rodriguez (unpublished results)
pGBT9Dabi2	N-terminus deleted (aa 95-423)	Rodriguez (unpublished results)
pGBT9NAP	non-active ABI1 (aa 121-434)	Himmelbach et al., 2002
pGBT9NCO	N-terminus of ABI1 (aa 1-180)	Leube et al., 1998
pQE60ABI1	full length ABI1	Leube et al. 1998
pQE60abi1	full length abi1	Leube et al. 1998
pQE70ABI2	full length ABI2	Rodriguez et al., 1998
pQE70abi2	full length abi2	Rodriguez et al., 1998
pVA3	murine $p53_{(72-390)}$ in pGBT9	Iwabuchi et al., 1993
pTD1	SV40 large T $antigen_{(84-708)}$ in pACT2	Li & Fields, 1993

pVA (Iwabuchi et al., 1993) and pTD (Li & Fields, 1993) were used as positive control and pGBT9 and pGAD424 (Bartel et al., 1993) served as negative control. Plasmids were provided by CLONTECH (Stehelin & Cie AG, Basel, Swiss).

2.1.6 Reagents

1 kb and 100bp DNA ladders were purchased from GIBCO/BRL. Protein marker was obtained from New England Biolabs, GmbH, Frankfurt a. M.

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), antibiotic Betabactyl (Timenten) is a mix of Ticarcillin and Clavulan acid from Smith Kline Beecham GmbH & Co KG, Herrenberg. Oligonucleotides were obtained from MBI Fermentas (Vilnius, Lithuania) and MWG-Biotech GmbH (Ebersberg).

Enzyme	Producer
Restriction enzyme	MBI Fermentas GmbH, St. Leon-Rot
T4-ligase / -kinase	MBI Fermentas GmbH, St. Leon-Rot
Alkaline phosphatase	Boehringer Mannheim GmbH
Taq-polymerase	MBI Fermentas GmbH, St. Leon-Rot
Pwo-polymerase	Peqlab Biotechnology GmbH, Erlangen
Vent-polymerase	New England Biolabs GmbH, Frankfurt a. M.
M-MuLV reverse transcriptase	Peqlab Biotechnology GmbH, Erlangen
Klenow-fragment polymerase	MBI Fermentas GmbH, St. Leon-Rot
Ribonuclease A	Serva GmbH, Heidelberg
Lysozym	Fula Sigma-Aldrich GmbH, Deisenhofen
Macerozym R-10	Yakult Honshia Co. Ltd. Japan
Cellulase R-10	Yakult Honshia Co. Ltd. Japan

2.1.7 Primers (5' to 3')

All primers were synthesized by MWG-Biotech GmbH, Ebersberg, Germany.

2.1.7.1 Standard primers

AD forward	TACCACTACAATGGATG
AD reverse	GTTGAAGTGAACTTGCG
35S-core-forward	TCCCCCCGGGGCAAGACCCTTCCTC
Actin forward	TGGGATGACATGGAGAAGAT
Actin reverse	ATACCAATCATAGATGGCTGG
Tubulin forward	AGGTGGAAGATACGTTCC
Tubulin reverse	TTCTCTACAAGCTCATGGAC

2.1.7.2 Gene specific primers

Glutamyl tRNA synthetase :

Glut f1	TGGCCCGGGGATGGATGGGATGAAGC
Glut r2	TCACTTAGCAGATCTTCCATCTGG
Glut f2	TTCAGCTTTACGAATTCAGCC
Glut f3	TGATGCGAATGCCATATCC'
Flut r3	ATCTTTGACCAGTTCC
Glutf720	CCAAGTGGTTATCTTCAC
GTS 129f	GAATTCGCGGCCGCTTCCTGATTTCTATGGC
GTS 129r	GAATTCGCGGCCGCTTCCGGCAAGTCC

Glutamyl tRNA synthetase promoter :

GPRf1	ATTAAGCTTCAACTTTCATGGAAGATTC
GPRr1	TAATCTAGAAACGAGAAATGGATTGTGC
GPRf2	GGTAAGTTTCGTAAGAAACCC
GPRr2	CTCATCACACGAAAGCACCC
GPRf3	AGAGTAGTTGAAGCTTTTAAC
GPRr3	TTTGGATCCAAATCAGAGAAAC
GPRf4	AACTACTCCTTTATCGAGATCC

Fibrillin:

FIB for1	AGATCTGGAATTCGGATCCTC
FIB rev1	TTAAGCTTAGATCTCTCGAGGCCCGAAG
EIB rev2	GATTTCTAGATCTCTCGAGGCC
FIB 129f	GAATTCGCGGCCGCTGGTCCGTTTTCC
FIB 129r	GAATTCGCGGCCGCCTCTCGAGATCC

Fibrillin promoter:

FPRf1	TTAAAGCTTATGAAACATCGTCAGATC
FPRr1	AATGGATCCTGTGTTTGTTCTTCAGAGAAACC

Fibrillin-2:

Fib-2 for	AGAGTTTTCAAAGTCCGAGCC
Fib-2 rev	TGCTAATCGAGTTTGTACC

2.1.8 Media

Media for Yeast

YPD medium:

20g/l	Difco peptone
10g/l	Yeast extract
20g/l	Agar (for plates)

pH 5.8. Glucose was added after autoclavation to 2

SD medium:

Synthetic dropout (SD) is a minimal medium used in yeast transformations to select and test for specific phenotypes.

6.7 g/l	Yeast nitrogen base without amino acids
20 g/l	Agar (for plates)

pH 5.8. After autoclavation, glucose was added to 2% and 10x DO (dropout solution).

Bacteria

LB medium:

10.0 g/l	Bacto Tryptone
5.0 g/l	Yeast extract
10.0 g/l	NaCl
18 g/l	Agar (for plates)

E. coli cultures were grown in LB medium supplemented with appropriate antibiotics. The cultures were grown at 37 C for 12-16 hrs, with shaking at 200 rpm.

Antibiotics:

Ampicillin	50 µg/ml	stock solution 100 mg/ml in H_2O
Kanamycin	50 µg/ml	stock solution 50 mg/ml in H_2O

Plants

MS medium (Murashige and Skoog, 1962):

100ml/l	10 x Macrosalt
2.5ml/l	Vitamin (500x)
2.5ml/l	Microsalt (500x)
1g/l	MES
10g/l	Sucrose
9g/l	Agar (for plates) pH 5.8

2.1.9 Buffers

TE buffer:	
10 mM	Tris-HCl, pH 8.0
1 mM	EDTA

10 x TBE:	
1 M	Tris-HCl, pH 8.0
0.5 M	Boric acid
20 mM	EDTA

20 x SSC:	
3 M	NaCl
0.3 M	Trisodium citrate x $2H_2O$, pH 7.2

10 x PBS:	
0.75 M	NaCl
30 mM	KCl
45 mM	Na_2HPO_4
15 mM	KH_2PO_4

10 x MOPS:	
0.05 M	Na-acetic, pH 7.0
0.1 M	MOPS
0.1 M	EDTA

2.1.10 Antibodies

Polyclonal goat anti-rabbit IgG alkaline phosphatase conjugate antibodies, goat anti-rabbit IgG peroxidase conjugate and goat anti-mouse IgG peroxidase conjugate were purchased from Sigma. Anti-GAL4 AD and anti-GAL4 BD antibodies were obtained from Santa Cruz, Biotechnology. Anti-GST antibody was obtained from Pharmacia Biotech. Anti-GST-ABI1 antibody was provided by Eurogentec (Seraing, Belgien).

2.2 Methods

2.2.1 Standard methods

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

-isolation of plasmid DNA
-electrophoresis of DNA in agarose gels
-restriction of DNA
-dephosphorylation with alkaline phosphatase
-"fill in"-reaction with Klenow polymerase
-ligation with T4 DNA ligase
-preparation of competent *E. coli* cells using $CaCl_2$ method
-polymerase chain reaction (White et al., 1989)
-Northern blotting
-Southern blotting
-Western blot

2.2.2 Yeast two-hybrid system

The yeast two-hybrid screening and subsequent analysis were according to the Clontech protocols (manual PT3204-1, CLONTECH Laboratories, Inc., CA. USA).

2.2.2.1 Preparation of the single strand carrier DNA

According to Schiestl and Gietz (1989), DNA from Salmon sperm (Sigma D1612 type III) was dissolved in 1xTE (10mg/ml). DNA was sonicated twice for 30 sec and then extracted with phenol, phenol plus chloroform (1:1) and chloroform. Subsequently, DNA was precipitated by adding1/10 volume of 3M sodium acetic and 2.5 volume of ethanol, recovered by centrifugation and washed with 70% ethanol. DNA was redissolved (2mg/ml) in 1xTE and boiled for 20 min. DNA was chilled on ice and stored at -20^{o}C.

2.2.2.2 HB101 E. coli electrocompetent cells

In the two-hybrid system, vectors carrying *HIS3*, *LEU2* or *TRP1* markers can be rescued by complementation of the *E. coli hisB, leuB* or *trpC* mutations, respectively. HB101 carrying the *LeuB* mutation (Boliver & Backman, 1979) can be used for selection of yeast plasmids bearing the *LEU2* marker.

One HB101 colony was inoculated in 5 ml of LB liquid culture and incubated at 37^{o}C with vigorous shaking overnight. The cells were transferred to 1 liter of LB liquid culture and incubated for another 3-5 hs. After centrifugation (20 min, 5000 rpm, 4^{o}C), the pellet was washed twice with sterile water and with 10% glycerol. Cells were resuspended in 2-3 ml of 10% glycerol and stored at –80^{o}C.

2.2.2.3 LiAc mediated yeast transformation (small scale)

Preparation of the yeast competent cells

One yeast colony was inoculated in 1 ml of YPD or SD liquid culture. The culture was transferred into a flask containing 50 ml of YPD or appropriate SD medium and incubated at 30^{o}C for 16-18 hs with shaking (200 rpm) until the OD_{600} reached 1.5. 30 ml of overnight culture were transferred into a flask containing 300 ml YPD and incubated for 3 hs. After the OD_{600} of the culture reached 0.4-0.6, cells were harvested (1000g, 5 min), washed with sterile TE and resuspended in 1.5 ml of freshly prepared, sterile 1xTE/1xLiAc solution.

Yeast transformation

0.1µg of plasmid DNA, 0.1 mg of carrier DNA (2mg/ml) and 0.1 ml of yeast competent cells were mixed with 0.6 ml of sterile PEG/LiAc solution and vortexed (10 sec) at high

speed. After incubation at 30°C for 30 min with shaking, the mixture was supplemented with 70µl DMSO. Cells were heat shocked at 42°C for 15 min and chilled on ice. Cells were harvested and resuspended in 0.5 ml of sterile 1xTE buffer. 100 µl of cells were plated on SD selective agar medium and incubated at 30°C until the colonies appeared (2-4 days).

2.2.2.4 β-galactosidase Assays

Colony-lift filter assay

The colony-lift filter assay was used to screen large numbers of cotransformants that survive the HIS3 growth selection in a GAL4 two-hybrid library screening. In addition, the assay was employed to visualize the interaction between two known proteins.

Sterile filter (3mm chr, Whatman®) was soaked in Z-buffer/X-gal solution and placed in a clean petridish. Colonies were replica plated on a dry filter and transferred in to liquid nitrogen. After 10 sec, the filter was removed from the liquid nitrogen and thawed at room temperature. The filter was placed, colony side up, on the presoaked filter and incubated at 30°C until blue colonies appeared.

Quantitative assay

Liquid cultures were assayed for β-galactosidase activity to quantify two-hybrid interactions and to compare the relative strength of protein-protein interactions.

One colony was incubated in 5 ml of SD selection medium at 30°C overnight. The culture was vortexed for 1 min in order to disperse cell clumps. 2 ml of the overnight culture were transferred to 8 ml YPD medium and incubated at 30°C for 5 hs with shaking until the cells were in mid-log phase (OD_{600} =0.5-0.8). 1.5 ml of culture was transferred to a new microcentrifuge tube and centrifuged (14,000 rpm, 30 sec). The cell pellets were washed with Z-buffer and resuspended in 300 µl of Z-buffer. In order to permeabilize the cells, 100 µl suspensions were subjected to 3 freeze/thaw cycles. 700 µl of Z-buffer/β-mercaptoethanol and 160 µl of fresh prepared ONPG (o-nitrophenyl β-D-galactopyranoside) in Z-buffer (4 mg/ml) were added to the reactions and then incubated in 30°C. As a control served a blank with 100 µl Z-buffer. After the yellow color developed, the reaction was stopped by adding 0.4 ml of 1 M Na_2CO_3. The cell debris was removed and the supernatants were transferred to cuvettes. The OD_{420} of the samples was measured in a spectrophotometer (Ultrospec 2000, Pharmacia. Biotech).

1 unit of β-galactosidase is defined as the amount which hydrolyes 1 µmol of ONPG to o-nitrophenol and D-galactose per min per cell (Miller, 1972; 1992):

β-galactosidase units = $1{,}000 \times OD_{420} / (t \times V \times OD_{600})$

"t" is the duration (minute) of appearance of yellow color after adding the ONPG;

"V" is the volume (ml) of cell culture used.

2.2.2.5 GAL4 two-hybrid library screening

Test the DNA-BD/bait protein for transcriptional activation function

The transformants expressing the bait/GAL4 DB fusion and a library fused to the GAL4 AD were plated on appropriate SD selection medium. Transformants were assayed for *LacZ* reporter gene expression by using the colony-lift filter assay for β-galactosidase and *His3* selection.

Elimination of leaky HIS3 expression

3-AT (3-amino-1,2,4-triazole), a competitive inhibitor of the yeast HIS3 protein (His3p), was used to inhibit low levels of His3p expression and to suppress background growth on SD medium lacking HIS (Fields, 1993; Durfee et al., 1993).

Transformants were plated on SD selective medium with different concentrations of 3-AT (0, 2, 4, 8 and 16 mM). After colonies grew up, the medium containing 4 mM 3-AT could eliminate the expression of His3p background.

Preparation of the yeast competent cells

One BD/bait colony was inoculated in 1 ml SD selective medium and transferred into a flask containing 50 ml appropriate SD medium. 30 ml of overnight culture ($OD_{600} \geq 1.5$) were transferred into 300 ml SD medium. After overnight incubation, 200 ml overnight culture were transferred into 2 liter YPD medium and grown until the OD_{600} reached 0.6. Cells were harvested by centrifugation and washed with sterile TE. For transformation, the cells were resuspended in 15 ml of freshly prepared, sterile 1xTE/1xLiAc solution.

Library transformation and screening

200 µg of library plasmid DNA, 40 mg of carrier DNA and 15 ml of yeast competent cells were mixed. 120 ml of sterile PEG/LiAc solution were added with vortexing at high speed for 30 sec and incubated at 30°C for 30 min with shaking (200 rpm). After addition of 14 ml DMSO, the mixture was heat shocked in 42°C for 15 min and subsequently chilled on ice for 2 min. After centrifugation for 5 min at 1000g, cells were resuspended in 10 ml of sterile 1xTE buffer and plated on SD selective medium with 8mM 3-AT. The plates were incubated at 30°C until the appearance of colonies.

2.2.2.6 Analysis and verification of putative positive colonies

Elimination of duplicates

His$^+$ colonies were streaked on SD selective medium containing histidine to test the *LacZ*$^+$ reporter gene expression. Then the *His*$^+$ and *LacZ*$^+$ positive clones were restreaked and tested for their *His* and *LacZ* phenotypes again. This process was repeated twice to ensure the positive to both reporter genes. It is important because some of the initial library cotransformants contain more than one AD/library plasmid.

Recovery of library plasmids from yeast

A His$^+$ and LacZ$^+$ positive clone was inoculated to 3 ml SD selective medium and incubated for 2 days at 30^{o}C with shaking. The culture was centrifuged shortly at high speed. Cell pellets were resuspended by adding 200 µl of breaking buffer. One volume of acid-washed glass beads (0.45-0.55 mm, Sigma) and 200 µl phenol, chloroform and isoamylalcohol (25:24:1) were added and vortexed for 2 min at high speed. After centrifugation, the DNA was precipitated from the supernatant by adding 1/10 volume of 3 M NaAc (pH6.0) and 2.5 volume of ethanol. DNA was recovered by centrifugation, washed with 70% ethanol and dissolved in sterile water.

Analysis of cDNAs in AD/prey plasmids

The plasmid DNA from His$^+$, LacZ$^+$ yeast transformant colonies was used to amplify the inserts in AD/preys by PCR with specific primers (AD forward and AD reverse). PCR products were analysed by digestion with different restriction enzymes.

Transformation of mapped physically positive library plasmids in E. coli HB101

1-2 µl of plasmid DNA isolated from yeast was added into 40 µl of HB101 electrocompetent cells and transferred into a prechilled cuvette (0.2 cm gap). Electroporation was performed according to the manufacturer's instructions (V, 2500; T, 0.005; Ω, 201; EasyjecT, EquiBio). After 1 ml of SOC medium was added immediately after the pulse into cuvette. Cells were transferred into a new tube and incubated at 37^{o}C for 1 h with shaking. After centrifugation, the pellets were washed with sterile water and dissolved in 100 µl of 1 x TE. The suspension was plated on supplemented M9 minimal medium (lacking the specific nutrient that will allow selection of the desired plasmid) and incubated in 37^{o}C for 2 days.

Sequence analysis

The inserts in the positive AD/preys were sequenced (ABI PRISM, MWG) using the GAL4 AD forward or reverse primer. The sequences were compared to those of other cDNAs for known or unknown proteins in GenBank.

2.2.2.7 Solutions

10xDropout (DO) supplements:

L-Isoleucin	300 mg/l
L-Valine	1.5 g/l
L-Adenine hemisulfate salt	200 mg/l
L-Arginine HCl	200 mg/l
L-Lysine HCl	1 g/l
L-Methionine	300 mg/l
L-Phenylalanine	500 mg/l
L-Threonine	2 g/l
L-Tyrosine	300 mg/l
Uracil	200 mg/l

100x Histidine HCl monohydrate	1 g/l
100 x Leucine:	10 g/l
100 x Tryptophan:	2 g/l

X-gal: 20 mg/ml

Dissolve 20 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) in 1 ml of N,N-dimethylformamide. Store in dark at -20ºC.

10 x TE buffer:

0.1 M	Tris-HCl,
10 mM	EDTA, pH7.5

Z-buffer:

$Na_2HPO_47H_2O$	60 mM
$NaH_2PO_4H_2O$	40 mM
KCl	10 mM

Breaking buffer:

2%	Triton X-100
1%	SDS
0.1M	NaCl
10mM	Tris-Cl (pH8.0)
1 mM	EDTA (pH8.0)

1 M 3-AT (3-amino-1,2,4-triazole):

Prepare in deionized H_2O and filter sterilize. Store at 4ºC

M9 minimal medium:

M9 (Sambrook et al., 2001)

$MgSO_4$	2 mM
Glucose	4 % (W/V)
$CaCl_2$	0.1 mM
Proline	40 mg/L
Thiamine-HCl	1mM
Dropout	1x
Ampicillin	50 µg/ml

PEG/LiAc solution:

PEG 4000	40%
TE buffer	1 x
LiAc	1 x

50% PEG 3350:

prepare with sterile deionized H_2O

10 X LiAc:

1 M lithium acetic, adjust to pH 7.5 with dilute acetic acid

Z-buffer/X-gal solution:

Z-buffer	100 ml
β-mercaptoethanol	0.27 ml
X-gal stock solution	1.67 ml

ONPG (o-nitrophenyl β-D-galactopyranoside):

4 mg/ml in Z-buffer. pH 7.0

2.2.3 Cosmid screening

For the isolation of the glutamyl tRNA synthetase gene, the target fragment was amplified by PCR with specific primers for glutamyl tRNA synthetase in cosmid pools. After the positive cosmid pool was detected, the nylon-membrane of selected cosmid pool was hybridized with digoxigenin-11-dUTP labelled probe for glutamyl tRNA synthetase. After single positive cosmid was identified, the cosmid DNA which contained the target gene was prepared and digested with appropriate restriction enzyme in order to get the desired fragment.

2.2.4 Methods for DNA analysis

Mini-preparation of plasmid DNA and maxi-preparation of plasmid DNA with PEG solution were according to the protocols described by Sambrook and Russell, 2001.

2.2.4.1 Maxi-preparation of plasmid DNA (modified by Myashev et al.)

E. coli cells were lysed according to the alkali lyses protocol (Sambrook et al., 2001). After reinoval of cell debris, the supernatant was filtered and 50 ml of isopropanol were added. The nuclei acids were recovered by centrifugation (10,000g, 30 min, 4^{o}C) and dissolved in 2 ml of 0.1 x TE. RNA was precipitated by addition of 8 ml of 5M LiCl. After centrifugation, the supernatant was supplemented with 10 ml of Bind mix. After centrifugation (2,000g, 3 min), the Silica was resuspended in 10 ml of 3M GuSCN (guandinc thiocyanate) and 4%(v/v) of Triton-X 100. After centrifugation, Silica was washed twice in 50% ethanol. The Silica resin was dried for 15 min. DNA was eluted by 2 ml TE. After centrifugation (5 min, 10,000g), the DNA was transferred in a new tube.

Bind mix: 10 ml 3M GuSCN and 4% Triton X-100, 100mg Silica (Sigma) for 100 ml overnight culture of high copy plasmids and 20 mg Silica for 100 ml of low copy plasmids.

2.2.4.2 Genomic DNA Isolation from plants

2-3 *Arabidopsis* leaves were collected and added 100 µl micro isolation buffers (Rogers and Bendich, 1994). After the leaves were homogenized, 550 µl of micro isolation buffer was added. The sample was vigorously mixed and incubated at 65^{o}C for 1 h. 750 µl of chloroform were added. After phase separation, DNA was precipitated by addition of 1 volume isopropanol. The DNA was recovered by centrifugation and dissolved in 50 µl of 1 x TE (with RNAse, 20 ng/µl). DNA was precipitated by addition of 2 volume ethanol and 1/10 volume 3 M Na-acetic (pH5.2). After centrifugation, the DNA was washed with 70% ethanol and dissolved in 20 µl of 0.1 x TE.

Extraction buffer:	
0.1 M	Tris-HCl (pH8.0)
5 mM	EDTA (pH8.0)
0.35 M	Sorbitol
Nucleus lysis buffer:	
200 mM	Tris-HCl (pH8.0)
50 mM	EDTA (pH8.0)
2 M	NaCl
2% (w/v)	CTAB (cetyltriethylammoniumbromid)
Micro isolation buffer:	
1 V	Extraction buffer
1 V	Nucleus lysis buffer
0.4 V	5% Sarcosyl
0.38g/100ml	Sodium disulfite
pH 7.5	

2.2.4.3 Digoxigenin-11-dUTP labeling of the probes

Digoxigenin-11-dUTP was incorporated into probes by using PCR and Taq DNA polymerase. Incorporation of digoxigenin allows the synthesis of labeled DNA probe. In this work, the digoxigenin-11-dUTP was purchased from Roche Diagnostics GmbH, Germany. For a standard PCR reaction, the following nucleotide concentrations were used: 70 µM Dig-11- dUTP, 130 µM dTTP and 200 µM dATP, dGTP, dCTP each. Prior to hybridization, the DNA probe was purified through Gel Elute Kit (QIAgen).

2.2.5 Methods for RNA analysis

2.2.5.1 RNA isolation

Total RNAs were isolated from *Arabidopsis* leaves or plant grown for 10 days in MS liquid culture as described (Himmelbach et al., 2002). The seedlings were treated with 30 µM ABA before RNA isolation. The isolation of RNAs was according to the protocol described by Sambrook and Russell, 2001.

2.2.5.2 RT-PCR

Synthesis of first strand cDNA

First strand cDNA synthesis Kit (#K1611, MBI Fermentas) was used in this work. 1–2.5 µg of total RNA isolated from *Arabidopsis* materials were used as template. For a typical synthesis of first strand cDNA, 0.5 µg of oligo$(dT)_{18}$ primer and distilled DEPC water were added and incubated at 70°C for 5 min. The mixture was supplemented with 4 µl of 5x reaction buffer, 20U of ribonuclease inhibitor and 2 µl of 10mM dNTP. After incubation at 37°C (5 min) 40U of M-MuLV reverse transcriptase (MBI Fermentas, Lithuania) were added. The reaction was incubated at 37°C for 1 h and stopped by heating at 70°C (10 min).

PCR

0.15--0.5 µl of first strand cDNA synthesized above was used as template DNA for PCR. The PCR was according to the standard protocol described by Sambrook and Russell 2001.

Quantitative analysis of RT-PCR

To quantify the amount of target gene transcript, endogenous standards such as housekeeping genes, β-actin and tubulin, were employed as internal standard controls. Quantification of the PCR products was achieved by comparing the amount of amplified product generated by the endogenous standard (β-actin and tubulin) and the target sequence (Chelly et al. 1990). The products were separated by gel electrophoresis and quantified by computer analysis (BioAnalyst, Biorad) of gels stained with ethidium bromide.

2.2.5.3 Quantitative real time PCR

Real time PCR system is dependent on the detection and quantification of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. In this work, the reporter is the double-strand DNA specific dye SYBR Green (Master SYBR Green I, Cat. No. 2239264, Roth, Germany). SYBR Green binds double-stranded DNA, and upon excitation emits light, as a PCR product accumulates, fluorescence increases. LightCycler (Roche, Germany) was used to detect the intensity of fluorescence. In order to eliminate the variation of PCR reaction, an invariant endogenous control (β-actin) was used to normalize the value. The PCR cycles were designed as following: denaturation (95°C, 10 min); amplification (95°C, 15 sec; 50°C, 9 sec; 72°C, 15 sec; 45 cycles); melting at 65°C (15 sec); cooling at 40°C (35 sec).

2.2.5.4 Northern blotting, hybridization and immunodetection

Northern blot analysis, hybridization and immunodetection with digoxigenin-11-dUTP labeled probe were according to the standard protocols described (Sambrook and Russell, 2001).

2.2.6 Methods for protein analysis

2.2.6.1 Protein extracts from yeast

Yeast cell lyses:

Extraction of yeast proteins was performed according to the protocol from Clontech (protocol PT3204-1). Yeast cells were resuspended in ice-cold water. After centrifugation, the cell pellets were frozen in liquid nitrogen and thawed in pre-warmed cracking buffer (100 µl of cracking buffer per 7.5 OD_{600} units of cells). The cell suspension was transferred into a new tube containing glass beads (425-600 µM; Sigma # G-8772; 80 µl of glass beads per 7.5 OD_{600} units of cells). The sample was heated (70°C, 10 min) and vortexed vigorously. After centrifugation at high speed (14,000 rpm, 5 min), the protein concentration was determined spectrophotometrically from the supernatant (1 OD_{580}=26 µg)

PMSF (phenylmethl-sulfonylfluoride) stock solution (100X):
174 mg PMSF (Sigma # 7626) in 10 ml isopropanol

Protease inhibitor:

Trypsin	4mg / ml
Pepstatin A	0.1 mg / ml
Leupeptin	1mM
E64	1 mg / ml

Cracking buffer stock solution:

Urea	8 M
SDS	5 % (w/v)
Tris-HCl (pH 6.8)	40 mM
EDTA	0.1 mM
Bromophenol blue	0.4 mg / ml

Cracking buffer:

Cracking buffer stock solution	1 ml
β-Mercaptoethanol	10 µl
Protease inhibitor	10 µl
PMSF	50 µl of 100 X stock

Yeast sonication:

Yeast cells were resuspended in 1 x buffer and sonicated (15 impulses, 30% output, 3 times; Branson Sonifier 250) on ice. The extracts was cleared by centrifugation (14,000 rpm, 10 min, 4°C) and stored at –80°C.

1 x buffer:		TTBS:	
5 ml	TTBS	20 mM	TrisHCl, pH 8.0
250 µl	PMSF 100X stock solution	150 mM	NaCl
50 µl	Protease inhibitor	0.05%	Tween 20
50 µl	0.5 M EDTA		
50 µl	β-Mercaptoethanol		
500 µl	80 % glycerol		
50 ul	1 M ascorbic acid		

Immunoprecipitation

20 µg of yeast protein extracts were added to 500 µl of NET-gel buffer and 1:5000 diluted anti-GAL4 AD or BD antibody (Santa Cruz Biotechnology). The mixture was incubated on ice for 1 h on a rocking platform. Protein A-sepharose (P-3391, Sigma) was added, and mixture was incubated on ice (1 h) and centrifuged (14,000 rpm, 4^{o}C, 20 min). Pellets were washed with NET-gel buffer and with 10 mM Tris, 0.1 % NP-40. For analysis on SDS-PAGE, pellets were supplemented with 30 µl 1 x SDS gel loading buffer.

NET-gel buffer:		1 x SDS gel loading buffer:	
50 mM	Tris-HCl (pH 7.5)	50 mM	Tris-HCl (pH 6.8)
150 mM	NaCl	100 mM	DTT
0.1 %	Nonidet P-40 (NP-40)	2 %	SDS
1 mM	EDTA	0.1 %	Bromophenol blue
0.25 %	Gelatin	10 %	Glycerol
0.02 %	Sodium azide		

2.2.6.2 Expression and purification of GST-fusion proteins in E. coli

pGEX vectors (Pharmacia Biotech) were used to express polypeptides as fusions with glutathione-S-transferase (GST) in bacteria. Fusion proteins were purified from lysed cells under non-denaturing conditions by absorption to glutathione-sepharose beads, followed by elution in the presence of free glutathione (GST gene fusion system, Pharmacia, Biotech).

Induction of expression of GST-fusion proteins

A pGEX vector carrying DH5α colony was inoculated into 100 ml of LB/ampicillin medium and incubated at 37°C overnight with shaking. The culture was diluted 1:10 into 1 liter of fresh 2 x YTA medium and incubated at 30°C with shaking until the OD_{600} reached 0.5. Expression of fusion proteins was induced by adding IPTG (isopropyl-β-D-thiogalactoside) to a final concentration of 0.1 to 1mM. The culture was incubated at 30°C for an additional 2-3 hs and centrifuged (7700 g, 10 min, 4^{o}C). Pellets were resuspended in ice cold 1 x PBS (50 µl per ml culture) and lysed by sonicator (15 impulses, 30% output, 3

cycles, Branson Sonifier 250). The crude extracts was cleared by centrifugation (12,000 g, 10 min, 4°C) and used for affinity purification.

Affinity purification

The clear extract was incubated with glutathione-Sepharose 4B beads (Pharmacia) at 4°C for 30 min. The charged resin was transferred into a mini-column and washed with 1 x PBS. GST-fusion proteins were eluted by using the buffer E. The purified proteins were stored in aliquots containing 10% glycerol at –80°C.

1 x PBS:		2 x YTA:	
140 mM	NaCl	Tryptone	16 g / l
2.7 mM	KCl	Yeast extract	10 g / l
10 mM	Na_2HPO_4	NaCl	5 g / l
1.8 mM	KH_2PO_4 (pH 7.3)	Ampicillin	100 µg / ml

Buffer E:		IPTG stock solution:
10 mM	Reduced gluthatione	500 mg of isopropyl-β-D-thiogalactoside
50 mM	Tris-HCl (pH 8.0)	(IPTG) was dissolved in 20 ml of distilled water and sterilized.

2.2.6.3 Expression and native purification of 6xHIS-tagged protein in E. coli

pQE60ABI1/abi1 (Leube et al., 1998) and pQE70ABI2/abi2 (Rodriguez et al., 1998) were used to express 6xHis-tagged recombinant ABI1, ABI2 and their mutant forms in *E. coli*. Purification of His-tagged protein was according to the protocol described by QIAexpress protein purification system (QIAGEN).

2.2.6.4 Determination of protein concentration by Bradford assay

The measurement of protein content was according to a modified Bradford test (Ramagli and Rodriguez, 1985). 1 µl of protein extracts was added with 10 mM HCl and 200 µl of 1:3 diluted Bradford-reagents (Bio-Rad). Serial diluted BSA with the same extraction buffer was served as standard for the purified proteins. The measurement carried in micro plate (Nunc GmbH & Co. KG) in HTS 7000 Plus Bioassay Reader from Perkin Elmer by 590 nm. To normalize the luciferase activity in transgenic plants, the protein content was usually used as standard control.

Bradford reagent:	
0.01%	Coomasie Brilliant Blue G 250
8.5%	Phosphoric acid
5%	Methanol

2.2.6.5 Detection of proteins

Silver staining

According to the protocol (Sambrook and Russell, 2001), the SDS-PAGE was incubated for 1 h in fixation solution, washed three times for 20 min in 50 % ethanol, soaked in thiosulfate solution for 1 min, flushed three times for 20 sec in water and incubated in silver solution for 20 min. Subsequently, the staining reaction was initiated in developing solution. The reaction was terminated by addition of stop solution. Gels were dried under vacuum at 80°C.

Fixation solution:		Developing solution:	
50 %	Methanol	6 %	Na_2CO_3
12 %	Acetic acid	4 mg/ml	$Na_2S_2O_3$
Thiosulfate solution:		**Stop solution:**	
0.2 g/l	$Na_2S_2O_3$	20 %	Methanol
		3 %	Glycerol
Silver solution:			
2 g/l	$AgNO_3$		

Ponceau S

Ponceau S {3-hydroxy-4-[2-sulfo-4-(sulfo-phenylazo)phenylazo]-2,7-naphtalene disulfonic acid} staining was used to monitor the protein transfer and to locate the molecular weight markers. The membrane was shortly incubated in Ponceau S solution and rinsed in water until protein bands appeared.

Ponceau S solution:	
0.2 %	Ponceau S
1.0 %	Acetic acid

Western blot

Western blots can be used to separate 0.0005-25 µg of complex mixtures of proteins. As described by Sambrook and Russell, 2001, the gel was blotted onto a nylon membrane using Miniprotein II (Biorad) at 59 V for 30 min. The membrane was washed in distilled water and incubated in TTBS buffer containing 5 % milk for 1 h with gentle shaking to block unspecific binding of antibody. The blot was decorated with the primary antibody and incubated for 1 h with gentle shaking. Depending on the antibody's specificity and affinity, the antibody was diluted to 1:100 to 1:5000 in TTBS containing 5 % of milk. The membrane was washed with TTBS and the secondary antibody, peroxidase conjugated

anti-IgG (mouse, rabbit, peroxidase-conjugated, Sigma) diluted to 1:5000-1:10000 in TTBS containing 5 % of milk was added. After 1 h incubation the blot was washed with TTBS and bands were revealed by addition of the BCIP/NBT substrate.

Alkaline phosphatase buffer:		NBT stock solution:
100 mM	Tris-HCl, pH 9.5	0.5 % NBT in 70 % DMF
100 mM	NaCl	
5 mM	$MgCl_2$	BCIP stock solution:
		5 % BCIP in 70 % DMF

2.2.6.6 In vitro protein-protein interaction

Affinity chromatography

Ni-NTA resin slurry (QIAGEN GmbH) was equilibrated in binding buffer. His-tagged proteins (ABI1, ABI2, abi1 or abi2) were tethered to Ni-NTA resin and kept on ice for 30 min. the charged resin was applied to a mini-column and washed with binding buffer. Then the test proteins were added into mini-column. Fractions were collected after washing with binding buffer. Subsequently, proteins bound to the Ni-NTA were eluted with elution buffer. Protein samples were stored at –80^{o}C.

Binding buffer:		Elution buffer:	
30 mM	NaCl	50 mM	Na-phosphate buffer (pH 7.0)
10 mM	Mg-acetic	300 mM	NaCl
10 mM	Na-phosphate buffer (pH 7.0)	250 mM	Imidazol
5 mM	β-Mercaptoethanol		

Pull-down assay

The fibrillin was incubated with 5 µl of [γ-^{33}P] ATP (10 µCi/µl, Amersham, UK), 9 µl of protein kinase (1U/µl) and 1 x kinase buffer at 30^{o}C for 1 h. Unincorporated label was removed by gel filtration in a mini-column with Sepharose G-50 resin which was equilibrated in Z'-KCl. Proteins were eluted by addition of Z'-KCl. Fractions containing proteins were analyzed for radioactivity by using the Liquid Scintillation System (LS 5800 Series, Beckman, USA) and scintillator Quicksafe A (Diisopylnaphthalene & surfactant, Zinsser Analytic, Germany).

Z'-KCl:		5 x kinase buffer:	
25 mM	Tris-HCl pH 7.5	100 mM	Tris-HCl pH 6.5
17 %	Glycerol (w/v)	5 mM	DTT
50 mM	KCl	500 mM	NaCl
0.1 %	BSA (w/v)	60 mM	$MgCl_2$
1 mM	DTT		

Protein kinase:
1 U / µl (70U plus 70 µl of 40 mM DTT)

2.2.6.7 ABA/protein binding

For ABA/protein binding assays, ^{14}C-ABA [2,000 cpm/µl, DL-*cis*-*trans*-(2-^{14}C)-abscisic acid, Amersham, UK] was used. The experiment was performed in one container which was separated by a membrane. ^{14}C-ABA diffused freely through this membrane; however, proteins did not allow crossing it.

^{14}C-ABA was added into one side of the container and the potential binding proteins of ABA were added into another side. The buffer in both sides contained 30 mM $MgCl_2$, 10 mM ATP and 1 mM DTT. After incubation, the samples were collected from both sides and the radioactivity was determined by Liquid Scintillation System as described in 2.2.6.6.

2.2.6.8 PP2C enzyme assay

Assay with methylumbelliferyl phosphate (MUP) as substrate:

MUP (Methylumbelliferyl phosphate), as described for difluoro-MUP (Fjeld and Denu 1999), was used as substrate to determine the activity of ABI1 in the presence of Mn^{2+}. The assay was carried out in 100 mM Tris-HCl, 15 mM $MgCl_2$ and 5 mM DTT. Catalysis was monitored by the formation of the fluorescent product methylumbeliferone in a microplate reader (Perkin-Elmer HTS 7000 Plus). The excitation and emission wavelength were 360nm and 465 nm, respectively.

Incubation buffer:		Assay buffer:	
100 mM	Tris-HCl (pH8.0)	100 mM	Tris-HCl (pH8.0)
10%	Glycerol	1 mM	MUP
5 mM	DTT	1 mM	$MnCl_2$
15 mM	$MgCl_2$	5 mM	DTT

Assay of PP2C activity with casein as substrate:

Labeling of casein with [γ-^{33}P] ATP:

PP2C activity can be assayed by its ability to dephosphorylate casein. Γ-33P casein was prepared by incubating casein (10 mg) with cAMP-dependent protein kinase (70 Sigma units) for 8 hs at 30^{o}C in solution A containing 10 mM magnesium acetic and 100 µCi of γ-^{33}P ATP (50 pmol). The reaction was terminated by the addition of 0.1 ml of 100 mM EDTA and 100 mM sodium pyrophosphate (pH7.0), and subjected to gel filtration on Sephadex G-50 equilibrated with solution A plus 5% (v/v) glycerol to remove unincorporated γ-^{33}P ATP.

PP2C assay using casein:

Protein phosphatase activity was assayed as described by Leube et al. (1998). ABI1 (PP2C) was diluted in solution A and pre-incubated at 30°C with 0.01 ml of solution A containing magnesium acetic by pH8.0. This reaction initiated with phosphocasein. After the mixture was incubated at 30°C for 10 min, the reaction was terminated by addition of 0.1 ml of 20% (w/v) TCA to precipitate the proteins. After centrifugation (13,000 g, 2 min), the supernatant containing free phosphate was mixed with 2 ml of scintillator Quicksafe A and the radioactivity was measured by Liquid Scintillation System as described in 2.2.6.6.

10 x solution A:	
0.5 M	Tris-HCl (pH 7.5)
1 mM	EGTA

Solution A:	
50 mM	Tris-HCl pH7.5)
0.1 mM	EGTA
0.1% (v/v)	β-mercaptoethanol

Termination solution:	
100 mM	EDTA
100 mM	sodium pyrophosphate (pH7.0)

Labeling of casein:	
20 µl	5% casein
100 µl	10 x solution A
1 µl	β-mercaptoethanol
100 µl	100 mM Mg(AC)2
10 µl	0.6 mM cAMP
1 µl	[γ-^{33}P]ATP (100 µCi=50 pmol)
5 µl	0.5 mg protein kinase (Sigma P-8289)
add distilled water to 1 ml	

2.2.7 Methods for plant analysis

2.2.7.1 Plant transformation of Arabidopsis thaliana

Successful and stable transformation of *Arabidopsis thaliana* was according to vacuum-mediated method with *Agrobacterium tumefaciens* (Bent et al., 1994). Agrobacteria strains carrying vector (pBI121 derivation) were grown in LB medium with kanamycin (50 µg/ml) and rifampicin (10 µg/ml) at 30°C overnight. The overnight culture was transferred to a fresh LB medium without antibiotic and incubated at 30°C overnight. Cells were washed with LB medium and resuspended in infiltration medium. For transformation, the 4-5 week old *Arabidopsis* plants were selected. The inflorescences of plants were infiltrated with *Agrobacterium* cells for 5 min in vacuum (100 mbar). The infiltrated plants were kept under standard condition (22°C, 16 hs with light, 200 lux, 8 hs in dark, 60 % humidity) to grow until the seeds were harvested.

Transgenic seeds of *Arabidopsis* T_0-generation were screened for *kan* resistance. Seeds were sterilized and plated on MS medium containing kanamycin (50 µg/ml) and betabactyl (30 µg/ml). Putative transformants were analyzed by PCR.

Infiltration medium:	
0.5 x	MS medium
50 g / l	Saccharose
10 µg / l	BAP
50 µl / l	Silvet L-77

2.2.7.2 Physiological analysis of transgenic plants

Germination assay

Seeds were sterilized with 80% ethanol, 4 % NaOCl, washed with H_2O and plated on MS medium with different ABA concentrations. Then the plates were incubated at standard conditions and the rate of germinated seeds was determined from 1-7 days.

Root growth assay

3 day old seedlings were transferred to MS medium containing different ABA concentrations. The root growth was determined after 3 days.

Water loss assay

6 leaves were excised from 4-5 week old plants raised in phytochambers and exposed to ambient conditions. The loss of weight was determined during 1 h.

2.2.7.3 Assays for GUS activity

Histochemical GUS staining

GUS-proteins expressed in transgenic plants were detected histochemically by incubating plant tissues with the substrate X-gluc (5-brom-4-chlor-3-indolyl-β-glucuronid) (Jefferson et al., 1987). X-gluc is separated into 5-brom-4-chlor-indoxyl and glucoronate in the presence of GUS-protein. 5-brom-4-chlor-indoxyl converts to blue stain 5, 5'-dibrom -4, 4'-dichlor-indigo by oxidation.

The transgenic seedlings were fixed in 90% acetone and rinsed with the rinse solution. The tissue was incubated with X-gluc staining solution at 37^{o}C for several hs. Chlorophyll was removed by addition of 70% ethanol at 65^{o}C prior to microscopic examination (Zeiss Axioskop, Zeiss Stemi SV 11, Nikon Coolpix 990).

Rinse solution:		staining solution:	
50 mM	Na-phosphate buffer (pH7.2)	50 mM	Na- phosphate buffer (pH7.2)
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

Fluorometric GUS activity

Quantification of GUS activity was according to Fütterer (1990). Transgenic seeds were incubated in MS liquid culture for 8 days and then placed immediately in liquid nitrogen. After the plant materials were ground, GUS extract buffer was added (50 mg plant materials pro 200 µl extraction buffer) and the mixture was centrifuged (14,000 rpm, 5 min). GUS activity was measured in the supernatant after adding MUG. The substrate MUG (4-methyl-umbelliferyl-β-D-glucuronid) was hydrolyzed by GUS-protein into 4-MU (4-methyl-umbelliferon or 7-hydroxy-4-methyl-cumarin) and glucoronid. GUS activity was determined in a dark micro-plate (Nunc GmbH & Co. KG) using the HTS 7000 plus Bioassay Reader (emission, 460 nm; excitation, 365nm) from Perkin Elmer.

GUS extraction buffer:		Assay buffer:	
50 mM	Na-phosphate buffer (pH 7.0)	1 mM	4-Methylumbelliferyl- β-D-glucuronid
10 mM	β-Mercaptoethanol	50 mM	Na-phosphate buffer (pH 7.0)
10 mM	EDTA	10 mM	β-Mercaptoethanol
0.1 %	Sodium lauryl sarcosin	10 mM	EDTA
0.1 %	Triton X-100	0.1 %	Sodium lauryl sarcosin

2.2.8 Protoplast expression in *Arabidopsis thaliana*

The isolation of protoplasts was according to modified protocol from Abel and Theologis (1994).

2.2.8.1 Isolation of protoplasts from plant leaves

3-4 g leaves from 4-week old *Arabidopsis* plants were washed in distilled water and incubated with 20 ml enzyme solution for about 3-4 hs at 23°C with shaking (50 rpm/min). The protoplast suspension was filtered through a net with 150 µm pore diameter. After centrifugation (60 g, 2 min), the supernatant was discarded and the cells were resuspended in washing solution 1. Subsequently, the protoplasts were washed with in washing buffer 2 and twice with Magma solution, respectively. The concentration of protoplast was determined. The viability of the cells was verified by staining with FDA (fluoresce in diacetat) and subsequently analysis with the fluorescent microscopy (Zeiss Axioskop) (Nunberg and Thomas, 1993). The protoplast concentration was adjusted to 10^6/ml.

Enzyme solution:

1%	Cellulase R-10
0.25%	Macerozyme R-10
400 mM	Mannitol
8 mM	$CaCl_2$
1 %	BSA
5 mM	MES-KOH (pH 5.6)

FDA solution:

1 mg/ml in Aceton

Washing solution 1:

167 mM	Mannitol
133 mM	$CaCl_2$

Washing solution 2:

333 mM	Mannitol
67 mM	$CaCl_2$

MaMg solution:

400 mM	Mannitol
15 mM	$MgCl_2$
5 mM	MES-KOH (pH 5.6)

2.2.8.2 Protoplast transfection

Plasmid DNA solutions were adjusted to 0.5 M mannitol and mixed with 5 x 10^5 protoplasts. After addition of equal volume PEG solution, the suspension was incubated at room temperature for 10 min and washed with WI. The pellets were resuspended in WI after centrifugation. The transfections were incubated in the dark (23°C, 30 rpm, overnight). For ABA induction, the suspension was divided into different tubes, treated with ABA and incubated for another 16 hs.

PEG solution:

300 mM	$CaCl_2$
40%	PEG-4000
0.5 %	MES-KOH (pH 5.8)

WI:

0.5 M	Mannitol
20 mM	KCl
1 %	BSA
4 mM	MES-KOH (pH 5.8)

2.2.8.3 Protein extraction

The suspension was centrifuged (60 g, 2 min) and washed with 0.5 M mannitol. Pellets were dissolved in 105 µl of extraction buffer which is different according to the reporter gene. Extracts were vortexed and kept in -80°C for more than 1 h or in liquid nitrogen for several min.

2.2.8.4 Assays for activity of the reporter genes

GUS

100 µl GUS extraction buffer or CCLR (Luehrsen et al., 1992) were added to the 50 µl protein extracts. The measurement was described in 2.2.7.3.

CCLR:

25 mM	Tris-phosphate buffer (pH 7.8)
2 mM	EDTA
2 mM	1,2-diaminocyclonhexan-N,N,N',N'-tetraessig acid
10 %	Glycerol
1 %	Triton-X-100

Photinus pyralis luciferase

Assay for luciferase activity in protein extracts was according to Luehrsen et al. (1992). 105 µl of CCLR was added to 50 µl protein extracts from protoplasts. After centrifugation (14,000 rpm, 5 min), luciferase activity was assayed in the supernatant. Luciferase activity was measured in a luminometer (flask'n glow, Berthold) with a 90 sec integration period of light emission.

Substrate buffer:

20 mM	Tricine (pH 7.8)
1.07 mM	$(MgCO_3)4Mg(OH)_2 \times 5H_2O$
2.67 mM	$MgSO_4$
0.1 mM	EDTA
33.3 mM	DTT
0.27 mM	Coenzyme A
0.47 mM	D-luciferin (PJK GmbH)
0.53 mM	ATP

Aequorin

Aequorin is used in transient expression for standardization of transfections. Protoplasts were suspended in CCLR without Triton X-100. Aequorin activity was measured in a luminometer as described for LUC. For the assay of aequorin activity, 50 µl of substrate solution was used.

Substrate solution:

200 mM	$CaCl_2$
2 µM	Coelenterazin (PJK GmbH)

2.2.9 Cellular localization of fusion proteins

2.2.9.1 Agroinfection of Arabidopsis cell cultures

Agrobacterium GV3101 pMP90RK carrying plasmid pPCV812 derivates was plated on YEB plate containing rifampicin (100 µg/ml), kanamycin (50 µg/ml) and carbenicillin (100 µg/ml) and incubated at 28°C. A single colony was inoculated in 5 ml of liquid YEB with appropriate antibiotics at 28°C with shaking for 2 days. Subsequently, the culture was transferred into 50 ml of selective YEB for 24 hs. Cells were harvested, washed in cell culture medium and used for Agroinfection. 25 ml of fresh cell culture from root or leave tissue were inoculated with 500 µl Agrobacteria suspension (10^{10} cells) and incubated for 3 days at 22°C with shaking (50 rpm). Claforan (500 µg/ml) was added for 2 days to prevent *Agrobacteria* growth.

2.2.9.2 GUS staining of cell suspension

Cells were suspended in fixing solution and washed in rinse solution. Alternative, cells were suspended and washed with 0.1 M of Na-phosphate buffer (pH=7.0) Expression of GUS- fusion protein was monitored in plant cells after addition of staining solution and incubation at 37°C. Nuclei were counterstained with DAPI (4', 6-diamino-2-phenylindol dihydrochloride) and visualized by fluorescence microscopy (Zeiss Axioskop).

YEB Medium:

Beef extract	5 g
Yeast extract	1 g
Peptone/Tryptone	5 g
Saccharose	5 g

pH 7.2 (NaOH) and autoclave, $MgCl_2$ added to 70 mM

100 x B5 Vitamine:

Nicotinic acid	1 mg/ml
Pyriodixin HCl	1 mg/ml
Thiamine HCl	10 mg/ml
Myo-Inositol	100 mg/ml

Filter sterilize and store at 4°C

Medium for cell culture:

MS basal salt	4.3 g
100 x B5 Vitamine	4 ml
87 mM Saccharose	30 g

pH 5.8 (KOH), 1 µg/ml 2,4-D added after sterilization

Fixing solution:

Formaldehyde	1 %
Triton X-100	0.1 %
Sodium phosphate	150 mM (pH 7.0)

X-Gluc solution:

10 mg	X-Gluc
20 µl	DMSO (Dimethylsulfoxide)
10 µl	Triton X-100
10 ml	0.1 M Na-phosphate buffer (pH 7.0)

2.2.10 Computer analysis of DNA sequences, data and pictures

Programs:

Comparison of DNA sequences and protein sequences, analysis in databank, analysis of restriction enzyme sites: BLAST (Altschul et al., 1990; 1997), Genescan (DKFZ-Heidelberg), BioEdit (Hall, 1999), AtDB (*Arabidopsis thaliana* Databank, Stanford, USA), TAIR (The *Arabidopsis* Information Resource at the Carnegie Institution of Washington, Stanford, USA), SignalP (CBS), Multialin (Corpet, 1988), Align (Pearson et al., 1997).

Images and graphics: Micrografx Picture Publisher, Microsoft Excel and Powerpoint

Computer: PC-Compaq with Windows® 98

Digital camera: NIKON Coolpix 990

Gel documentation: CAMAG Reprostar 3, Molecular Analyst™ , BioRad Lab., USA

3 Results

3.1 Identification of interaction partners of ABI2

To identify proteins which may interact with the protein phosphatase ABI2 as potential modulators or components of ABA signal transduction, the yeast two-hybrid system was used to screen *Arabidopsis thaliana* cDNA libraries in this work.

3.1.1 Library screening with the yeast two-hybrid system

3.1.1.1 Quality control of the plasmids for transformation

Prior screening for ABA interacting proteins, the efficiency of yeast transformation was tested (up to 10^6 transformant/µg plasmid). The transformation rates (Table 2) were in accordance with an efficient yeast transformation of bait and prey plasmids. Binding domain (BD) plasmids with or without bait sequence were plated onto selective medium without the amino-acid tryptophan since the BD plasmid has the *TRP1* selection marker. Active domain (AD) plasmids with or without library insert were plated on selective medium without leucine since the AD plasmid has the *LEU2* selective marker. In order to check the plasmids which will be applied for library screening, different plasmids were introduced into yeast HF7c competent cells, auxotrophic for tryptophan and leucine, and then plated on selective media.

Table 2. Transformation of the yeast with plasmids used for interaction screening

plasmid	DNA(ng)	colonies	cfu/µg	selection
pGBTABI2fl	100	1000	10^6	-Trp
pGBTabi2fl	100	1600	1.6×10^6	-Trp
pTD+pVA	100	10	10^3	-Trp, -Leu
pGBT9	100	1000	10^6	-Trp
pGAD424	100	400	4×10^4	-Leu
pGBT9+pGAD424	100+100	21	2.1×10^3	-Trp, -Leu
Ohio library	10	720	7.2×10^6	-Leu

For selection of transformants, selective medium without tryptophan (Trp) and leucine (Leu) or without both were used. High transformation ratios were obtained with the yeast strain HF7c cells. pGBT harbouring full length ABI2 and mutant form abi2, empty BD (pGBT9) and AD (pGAD424) as well as positive controls were used for transformation. Ohio library was transformed into HF7c. cfu: colony forming units.

For selection of co-transformants, SD selective medium without tryptophan and leucine were used. High transformation ratios were obtained with the yeast strain HF7c cells. The Ohio library also showed high transformation efficiency ($7.2x10^6$ cfu/µg). It was digested with BglII to detect the different inserts. Inserts in the Ohio library ranged from 350 to 1200 bp length. The Clontech library gave the same fragment patterns as the Ohio library after digestion with BglII.

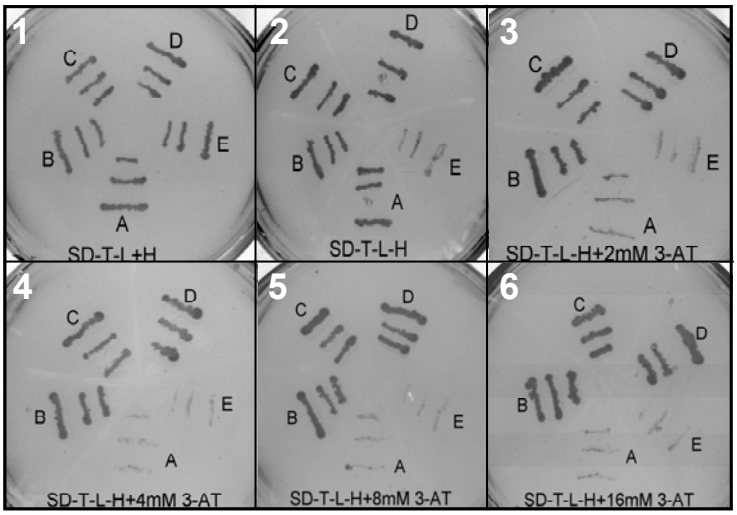


Figure 7. Optimal 3-amino-1,2,4-triazole (3-AT) test to eliminate the background.

Growth assay of co-transformants in selective medium without Trp and Leu.
1: with His (histidine); 2-6: without His; 3-6: with different 3-AT concentrations,
2mM, 4mM, 8mM and 16mM, respectively. Cotransformants: A: pGBTABI2fl+pGAD;
B: pGBTABI2fl+pGADHB6fl; C: pGBTΔABI1+pGADHB6fl; D: pVA+pTD; E: pGBT+pGAD

3.1.1.2 Optimal 3-amino-1,2,4-triazole (3-AT) concentration to eliminate the background

As a competitive inhibitor of the yeast HIS3 protein (His3p), 3-AT (3-amino-1,2,4-triazole) is used to inhibit low levels of His3p expressed in a leaky manner and to suppress background growth on selective medium lacking HIS (Fields, 1993; Durfee et al., 1993). To determine which 3-AT concentration was suitable to eliminate the background expression of His3p, different concentrations of 3-AT were added to SD selective medium as follows: 0, 2, 4, 8 and 16 mM. The results are presented in Table 3 and Figure 7. The BD/bait used in this test was pGBT9 harboring full length ABI2 cDNA sequence (pGBTABI2fl) which was used for library screening, the second was pGBT9 harboring an N-terminal-deleted ABI1 cDNA sequence (pGBTΔABI1). The AD fusion was pGAD424 harboring a full length HB6 cDNA sequence (pGADHB6fl). HB6 has already been shown to be an interaction partner of ABI1 (Himmelbach et al., 2002). The empty plasmids pGBT9 and

pGAD424 were used as negative controls and pVA and pTD from Clontech as positive controls. After co-transformation, the growth of colonies on different selective media with different 3-AT concentrations was determined. Growth of the negative controls was completely suppressed in the presence of 4 mM, 8 mM and 16 mM 3-AT, but there was no effect on positive controls, so 8 mM 3-AT concentration was used in further experiments for eliminating background *His* expression.

Table 3. 3-AT test

plasmid	-T-L+H	-T-L-H	-T-L-H 3-AT (2mM)	-T-L-H 3-AT (4mM)	-T-L-H 3-AT (8mM)	-T-L-H 3-AT (16mM)
pGBTABI2 +pGAD	+	+	- +	-	-	-
pGBTABI2 +pGADHB6	+	+	+	+	+	+
pGBTdABI1 +pGADHB6	+	+	+	+	+	+
pVA+pTD	+	+	+	+	+	+
pGBT+pGAD	+	-	-	-	-	-

Qualifying the growth of cotransformants on selective medium with or without 3-AT.
+: the colonies grew well; -+: the colonies grew poorly; -: no growth on selective medium.

3.1.2 Results of library screenings

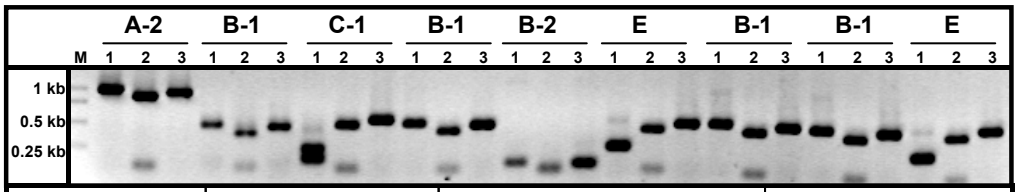
To identify the interacting proteins of ABI2, two different available libraries were transformed into yeast strain HF7c harboring the GAL4-ABI2fl bait. The Ohio and Clontech *Arabidopsis* cDNA libraries yielded 10^6 and 5 x 10^6 transformants, respectively. For each library the His3 positive transformants (indication of protein-protein interaction) were estimated to be above $2x10^3$. The His3 positive colonies, 143 single colonies from the Ohio library and 70 single colonies from the Clontech library, were further randomly selected for LacZ filter assay. Filter assay demonstrated that 85 out of 143 from Ohio library and 63 out of 70 single colonies from Clontech library were both His3 and LacZ positive (Table 4).

In order to selectively rescue AD/preys, plasmids of selected cotransformants were transferred into *E. coli* HB101 which carries the *LeuB* mutation and can be used to select for yeast plasmids bearing the *Leu2* marker (Boliver and Backman, 1979). The plasmids bearing *Leu2* marker (AD/preys) were isolated and 79 AD/preys from Ohio library and 16 AD/preys from Clontech library were retransformed into yeast to eliminate false positive transformants. Of those, 46 from the Ohio library and 15 from the Clontech library were

again both His3 and LacZ positive, indicating true positive interacting partners of ABI2. Subsequently, PCR and restriction enzyme analysis revealed that the positive AD/preys can be grouped to 9 classes (Figure 8). According to their different lengths of inserted fragments and different digestion patterns with restriction enzymes, 20 AD/preys were finally selected for DNA sequence analysis.

Table 4. Summary of *Arabidopsis* cDNA library screenings with yeast two-hybrid system

Arabidopsis cDNA library	Ohio library	Clontech library
Transformants	10^6	5×10^6
His positive colonies	≥ 2000	≥ 2000
His and lacZ positive colonies after single colony test	85 out of 143	63 out of 70
PCR and restriction enzyme analysis	9 groups	9 groups
retransformation to yeast	79	16
His and lacZ positive colonies	46	15
sequencing	12	8



Type	length of PCR product	BamHI	HindIII
A-1	1.1 kb	0.55 kb+0.45 kb	1.1 kb
A-2	1.1 kb	1 kb+0.1 kb	1.1 kb
A-3	1.1 kb	0.7 kb+0.25 kb+0.15 kb	0.75 kb+0.4 kb
B	0.7 kb	0.5 kb+0.2 kb	0.7 kb
B-1	0.55 kb	0.5 kb	0.55 kb
B-2	0.25 kb	0.2 kb	0.25 kb
C	1 kb	0.95 kb	0.7 kb+0.2 kb
C-1	0.55 kb	0.48 kb	0.3 kb+0.25 kb
E	0.55 kb	0.5 kb	0.27 kb

Figure 8. Analysis of AD/preys by PCR and digestion patterns of restiction enzymes.

All positive clones containing AD/preys were amplified by PCR with specific primers for the inserts in AD/preys. The primers used here are AD forward and AD reverse (see 2.1.7). After amplificatior the PCR products were digested with BamHI and HindIII to analyse their digestion patterns and then the AD/preys were grouped according to the length and digestion pattern of their inserts. Upper panel: one example of digestion pattern by restriction enzymes. M: DNA marker; 1: digestec with HindIII; 2: digested with BamHI; 3: undigested sample. Lower table: different types of the inserts in AD/preys according to the analysis by PCR and restriction enzymes.

Table 5. Identified ABI2 interaction partners: DNA sequence analysis

Clone ID	Description	Assigned functions	AC	location
Ic9	Catalase *	Decompose H_2O_2 to H_2O and O_2	ATU43340	Chr. I
Ic1	Fibrillin	Structural stabilization of cell, response to stresses	AF075598	Chr. IV
IIc3	Erd15	Response to dehydration	ATHERD15	Chr. II
IIc6	RibA	Involved in biosynthesis of riboflavin	ATHJ0053	Chr. V
Ic35	Glutamyl tRNA synthetase	Transfer of glutamic acid to $tRNA^{Glu}$, involved in cellular activity	AF067773	Chr. V
CT134	unknown protein	unknown	AB025622	Chr. V
CT137	HB6 *	Transcriptional factor	ATH5J17	Chr. IV
CT226	Unknown protein	unknown	ATAC006587	Chr. II
IV17	hypothetical protein	unknown	AC007123	Chr. V
VII45	Ath Cab binding protein	Involved in photosystem I (PSI)	ATCAB1	Chr. III
VII48	Ath mitochondrial genome	unknown	MIATGENB	Mitochondrial
XIII6	Peroxiredoxin	Reducation of hydrogen peroxide (H_2O_2) and alkyl hydroperoxides	AB006700	Chr. V
CT179	unknown protein	unknown	AC023628	Chr. I

AC: accession number in gene bank; Chr. I-V: indicating Arabidopsis chromosome I-V; Clone ID: original number of clone in the yeast two-hybrid screening; *: not in the reading frame of GAL4.

3.1.3 Sequence comparison in database

3.1.3.1 Comparison of DNA sequence

DNA sequence analysis revealed that the primary interacting partners of ABI2 included different known and unknown proteins of *Arabidopsis thaliana* (Table 5 and sequencing results see Appendix 5.2). Of them, *Arabidopsis* glutamyl tRNA synthetase (AtGluRS) and unknown protein (Ic35 and CT176, Genbank accession numbers AF067773 and AC023628, respectively) were identified in both libraries. Ic9 and CT137, cDNA fragments of catalase (ATU43340) and HB6 genes (ATH5J17) of *Arabidopsis*, were not in the open reading frame of GAL4. The other positive clones encode fibrillin (Ic1, AF075598), Erd15 (IIc3, ATHERD15), RibA (IIc6, ATHJ0053), *Arabidopsis* Chlorophyll A/B binding protein gene (VIIc45, CAA39534), peroxiredoxin (XIII6, CAA71503), and one sequence with homology to part of the *Arabidopsis* mitochondrial genome (VII48, MIATGENB). Proteins with unknown function were CT134, CT226 and IV17 (AB025622, ATAC006587 and AF007271, respectively). There were also some sequences identified which did not yielded any homology to sequences in the Genbank database because their sequences were too short to find significant similarity. All the identified genes are located on different

chromosomes of the *Arabidopsis* genome (Table 5).

3.1.3.2 Comparison of protein sequence

BLAST analysis (Altschul et al., 1990) revealed that all of the cDNA encoding putative interacting proteins identified have high similarity compared to those in the database (Table 6, except for the sequences which are not in the open reading frame of GAL4).

The cDNA of fibrillin (AF075598) and Erd15 (ATHERD15) were almost completely present in the AD-domains comprising 315 out of 318 amino acids and 162 out of 163 amino acids identical with full length sequences in the database, respectively. The cDNA of unknown protein (AC023628) encodes 170 out of 188 amino acids with 100% amino acid identity. Thus, the three candidates encode almost complete protein sequences. cDNA of AtGluRS (AF067773) and CT134 (unknown protein, AB025622) encode only N-terminal protein domains (1-262 out of 719 and 1-157 out of 188 aa, respectively).

Table 6. Identified ABI2 interaction partners: protein sequence analysis

Clone ID	Gene in *A. thaliana* databank	a. a. in library	a. a. in databank	Similarity
Ic1	Fibrillin	1--315	318	100%
IIc3	Erd15	1--162	163	100%
IIc6	RibA	48--387	543	100%
Ic35	Glutamyl tRNA synthetase	1--262	719	100%
CT134	Unknown protein	1--172	188	100%
CT226	Unknown protein	Not found	Not found	
IV17	Hypothetical protein	81--198	212	100%
VII45	Cab binding protein	13--142	241	100%
VII48	Mitochondrial genomic sequence	Not found	Not found	
XIII6	Peroxiredoxin	62--183	265	95%
CT179	Unknown protein	2--186	186	100%

Other candidates also encode the N-terminal sequences: i.e. cDNA of RibA (ATHJ0053) encodes 340 amino acids (48-387 out of 543 full length protein; cDNA of Chlorophyll A/B-binding protein gene (CAA39534) encodes 130 amino acids (13-142 out of 241 full length amino acids).

One candidate, the cDNA of hypothetical protein (AF007271) encodes 117 amino acids (81-198 of 212 amino acids of the protein). cDNA of another candidate, XIII6, encodes 188 amino acids but only C-terminus was identity to peroxiredoxin. The amino acids from 80-188 is similar to 2-Cys peroxiredoxin (62-183 out of 265 full length amino acids) in

Arabidopsis with 93% amino acid identity, however, no homology is found to the first 80 amino acids in *Arabidopsis* and possibly represent a cloning artifact.

Two candidates, ATAC006587 and mitochondrial genome (MIATGENB), encode cDNA of unknown proteins but the encoded amino acids have only a short open reading frame encoding a peptide of 29 and 100 amino acids, respectively. No homology was found in the protein database.

3.1.3.3 Analysis of protein sequences of potential interacting candidates

The potential interacting partners of ABI2 include known and unknown proteins. Of the known proteins, fibrillin (AF075598) is a 32-kD protein that forms fibrils in which carotenoids are stored (Deruére et al., 1994b). Fibrillin is expressed during fruit ripening in *Capsicum annuum*, leading to its accumulation in chromoplasts. Fibrillin-related proteins constitute a well-conserved group expressed in plants. It is a chloroplastic drought-induced protein (CDSP) in *Solanum tuberosum* plants (Gillet et al., 1998) and a chloroplast protein associated with the thylakoid membranes in potato (Monte et al., 1999). *ERD15* is a member of dehydration-stress induced genes in *Arabidopsis thaliana*. The deduced polypeptide is hydrophilic, with 49 charged residues out of 163, and lacks a Cys residue (Kiyosue et al., 1994). Its cellular function is unknown.

RibA is involved in the biosynthesis of riboflavin in plants and specifies a bifunctional GTP cyclohydrolase II/3, 4-dihydroxy-2-butanone-4-phosphate synthase. By comparison with the bacterial *ribA* gene, the *Arabidopsis* gene contains an additional 5' element encoding about 120 amino acid residues. This segment contains numerous serine and threonine residues and does not show similarity with other known sequences. The N-terminal segment is not required for catalytic activity and is likely to serve as signal sequence for import into chloroplasts (Herz et al., 2000).

Glutamyl tRNA synthetase (GluRS) belongs to the class I aminoacyl-tRNA. The most important function of GluRS is to transfer glutamic acid to $tRNA^{Glu}$ (Deutscher, 1967a, b; Lapoinate and Söll, 1972). Acylation of $tRNA^{Glu}$ with glutamic acid is also the first step in the biosynthesis of porphyrins (Kumar et al., 1996). Most reports focus on GluRS from bacteria, yeast or animals and little is known about plant GluRS except for the cloning of the GluRS in wheat, barley, tobacco and *Arabidopsis thaliana* (Freist et al., 1997).

Another protein (CAA39534) identified as a likely target of ABI2 interaction is involved in photosystem I as chlorophyll a/b-binding protein (Jensen et al., 1992). Peroxiredoxin of *Arabidopsis* (2-Cys peroxiredoxin, CAA71503) is highly homologous to that of *Spinacia oleracea* (X94219), *Hordeum vulgare* (CAA84396), *Triticum aestivum* (AB000405) and

Secale cereale (AF076920) according to the *in silico* analyses.

One predicted sequence has no homology to identified sequences in the protein database. The DNA fragment (MIATGENB) of 456 bp is similar to part of the *Arabidopsis* mitochondrial genome with 99% nucleotides identity and also 99% and 98% to chloroplast 23S ribosomal RNA gene of *Alnus incana*, tobacco, rice, and maize. It appears to encode part of 23S ribsomal RNA.

The two unknown proteins, CT134 (AB025622) and CT176 (AC023628) show 77% amino acid identity as shown in Figure 9. They are similar to pathogenesis-related proteins in a central peptide domain of about 60 amino acids with more than 50% similarity. A predicted 117 amino acids long protein IV17 is a hypothetical protein (AF007271) without homology to any known protein in the database.

```
CT134  MEA.NGIENL TNPNQEREFI RRHHKHELVD NQCSSTLVKH INAPVHIVWS 49
CT176  MDGVEGGTAM YGGLETVQYV RTHHQHLCRE NQCTSALVKH IKAPLHLVWS 50
 Cons  M#a.#Ggea$ tngn#er#%! RrHHqHecr# NQCsSaLVKH InAPlHiVWS

CT134  LVRRFDQPQK YKPFISRCVV KGNMEIGTVR EVDVKSGLPA TRSTERLELL 99
CT176  LVRRFDQPQK YKPFVSRCTV IGDPEIGSLR EVNVKSGLPA TTSTERLELL 100
 Cons  LVRRFDQPQK YKPF!SRCtV iG#mEIGslR EV#VKSGLPA TrSTERLELL

CT134  DDNEHILSIR IVGGDHRLKN YSSIISLHPE TIEGRIGTLV IESFVVDVPE 149
CT176  DDEEHILGIK IIGGDHRLKN YSSILTVHPE IIEGRAGTMV IESFVVDVPQ 150
 Cons  DD#EHILgIr I!GGDHRLKN YSSIislHPE iIEGRaGT$V IESFVVDVP#

CT134  GNTKDETCYF VEALIKCNLK SLADISERLA VQDTTESRV 188
CT176  GNTKDETCYF VEALIRCNLK SLADVSERLA SQDITQ... 186
 Cons  GNTKDETCYF VEALIrCNLK SLAD!SERLA sQDiT#...
```

Consensus symbols:
! is anyone of IV; **$** is anyone of LM; **%** is anyone of FY; **#** is anyone of NDQEBZ; identical amino acids are in dark; different amino acids are in grey.
Underlined: potential domain of intracellular pathogenesis-related protein

Figure 9. Alignment of protein sequences of two proteins--CT134 and CT176 identified in the yeast two-hybrid with unknown cellular function.

3.1.4 Quantification of protein-protein interaction

The yeast two-hybrid system was also used in this work to further characterize the interactions between the AD/preys identified from *Arabidopsis* cDNA libraries and different BD/bait constructs. Enzymatic assays for expressed β-galactosidase activity in yeast extracts were employed to quantify the strength of interactions between AD/preys and BD/baits.

3.1.4.1 DNA-binding domain fusions

In order to verify and test the specificity of protein-protein interactions, different BD/bait constructs were used for quantitative β-galactosidase assay. The baits used in this work were ABI2fl (a.a. 1-423, Rodriguez et al., 1998), which represents a full-length ABI2 fused to the GAL4 transcription factor, N-terminal deleted ABI2 (ΔABI2, a.a. 95-423), both point-mutated forms abi2 full-length and Δabi2 cDNA ($ABI2^{Gly168Asp}$) with a deficient functional catalytic domain of PP2C, that resulted in greatly reduced PP2C activity. In addition, the N-terminal truncated ABI1 (ΔABI1, aa. 123-434, Himmelbach et al., 2002) was used. Preliminary experiments revealed that the ABI1 cDNA fused to GAL4 DNA-binding domain (BD) resulted in auto-activation of the LacZ reporter expression. The N-terminal truncated ABI1 reduced transcription activity to background levels (Himmelbach et al., 2002). Furthermore, the point-mutated form of Δabi1 ($\Delta ABI1^{Gly180Asp}$, its PP2C activity is reduced more than ten times), NAP, a non-active ABI1 ($\Delta ABI1^{Asp177Ala}$; Himmelbach et al., 2002), and NCO, C-terminally truncated from the NcoI site in the *ABI1* gene (aa. 1-180; Leube et al., 1998) were used. All of the baits were fused to the GAL4 DNA-binding domain (BD). pGBT9 and pGAD424 (Bartel et al., 1993) empty vectors were used as negative control and pVA3 (Iwabuchi et al., 1993) and pTD1 (Li & Fields, 1993) as a positive control in this work.

3.1.4.2 Quantitative β-galactosidase assay

The colony-lift filter assay, see 2.2.2, showed that all selected AD/preys have very strong or relatively strong LacZ activity. The liquid quantitative β-galactosidase assay (described in 2.2.2) was used to quantify and compare the relative strength of the protein-protein interactions observed in selected transformants between BD fusions and AD/preys. The assay showed that some of the genes encoding interacting protein of interest strongly interacted with both ABI2 and ABI1, some of them have a clear preference with one PP2C form and others have only a weak interaction.

As shown in Figure 10, there is a strong interaction between fibrillin and ABI2 (3.35 β-gal units, 1 unit of β-galactosidase is defined as the amount which hydrolyses 1 μmol of ONPG to o-nitrophenol and D-galactose per minute per cell according to Miller, 1972) but very low activity with its mutated form, abi2. Protein-protein interaction is very strong between ΔAtGluRS (amino acids from 1-262 out of full length 719 a.a.) and ABI1 and its mutated version abi1 (over 30 β-gal units) but relatively low with ABI2 (only 1 β-gal units). No detectable activity was observed with the abi2 mutated version. In control cotransformations with empty pGAD, the β-galactosidase activities were all under 0.2 units.

In order to test the protein-protein interaction between full length protein of AtGluRS and BD versions, the full length AtGluRS cDNA (2.16 kb, see Appendix 5.3) was amplified by RT-PCR and cloned into an AD fusion plasmid as shown in Appendix (Figure 78-C). The liquid quantitative assay for two hybrid interaction showed that there were no indications for binding of AtGluRS and ABI2, N-terminal deleted ABI1 and their mutated versions (β-gal units measured were like those of the control).

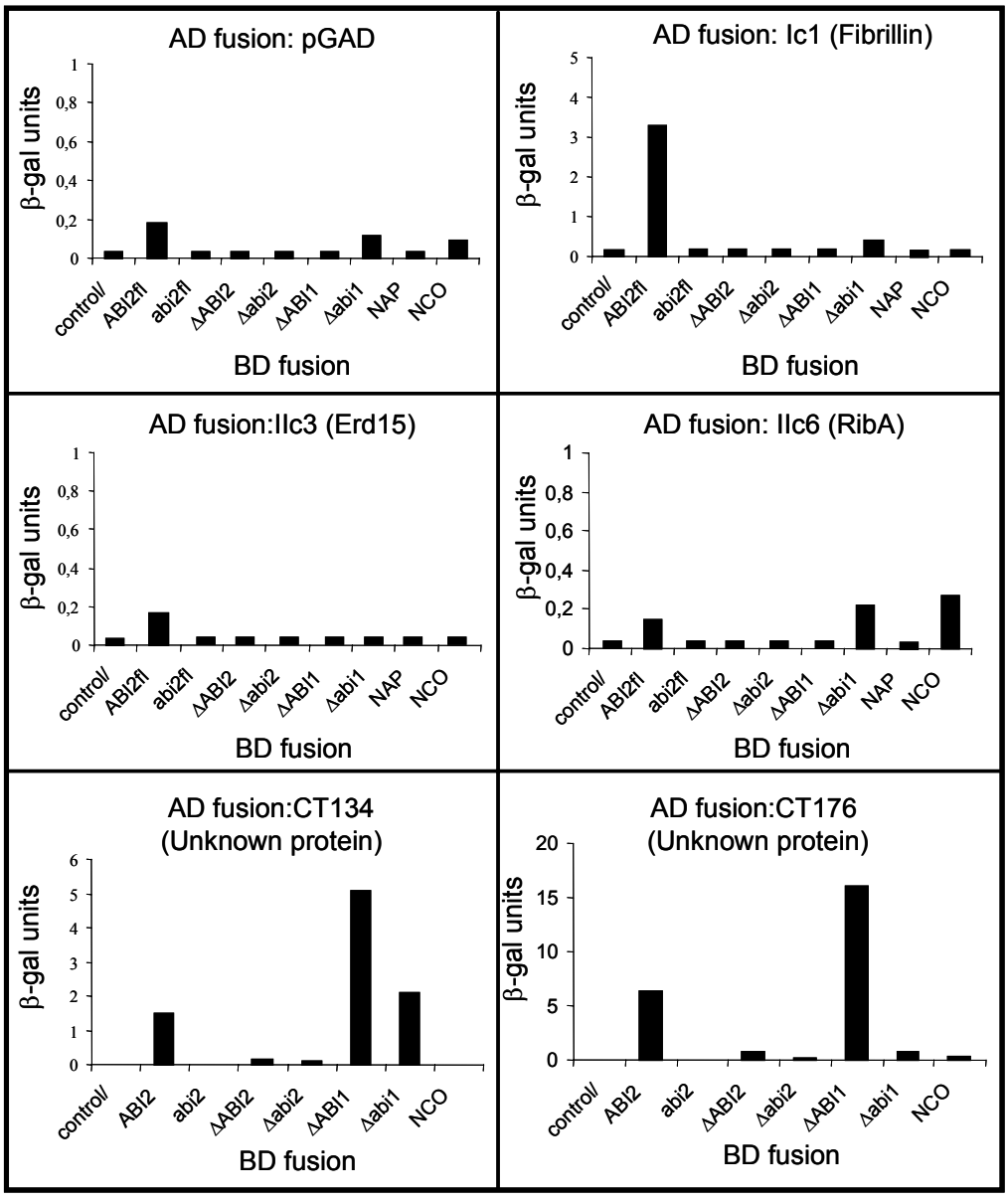


Figure 10. Quantitative β-galactosidase assay

Various ABI1 and ABI2 versions were fused to the GAL4 DNA binding domain (BD-fusions) and tested for interaction with the potential interacting candidates fused to the GAL4 activation domain (AD) in the yeast two-hybrid system. The different ABI1 and ABI2 versions are as described in in text and under 3.1.4.1. Cells expressing non-fused AD and BD domains were used as control. Interaction is indicated by the level of transactivation of β-galactosidase expression. The reporter activity is given in Miller units.

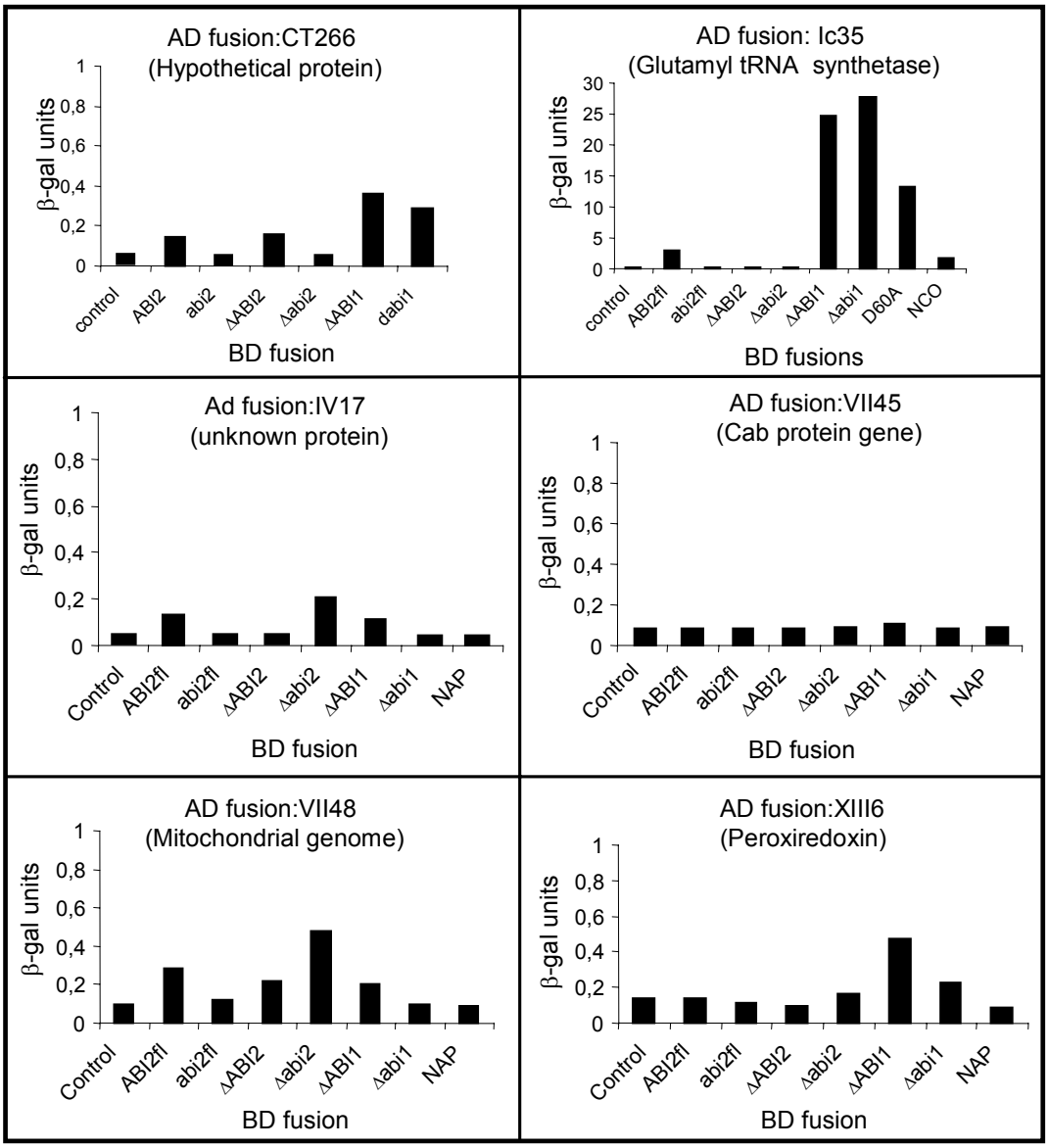


Figure 10. Quantitative β-galactosidase assay (continued)

The identified unknown protein CT134 (AB025622) showed in these assays a strong interaction (more than 5 β-gal units) with N-terminal deleted ABI1, and a relatively strong interaction with deleted abi1 and ABI2 (2 and 1.5, respectively), but no with the other BD versions (Figure 10); Another unknown but structurally related protein CT176 (AC023628) revealed an even stronger interaction (more than 15 β-gal units) with N-terminal deleted ABI1, strong activity with ABI2 (above 6), and very low activity with N-terminal deleted ABI2 and abi1. These two unknown proteins have similar interaction pattern with ABI1 and ABI2 as well as their deleted and mutated versions. According to their protein sequence (Figure 9), they have strong amino acid identity (77%), providing an explanation for their

similarity in the interactions. However, ABI2 and ΔABI1 interact clearly stronger with CT176 than with CT134. This difference is perhaps due to the somewhat different N-terminal domains of these two unknown proteins.

The other identified proteins encoded by IIc3 (Erd15, ATHERD15), IIc6 (RibA, ATHJ0053); VIIc45 (Cab binding protein gene, CAA39534); XIII6 (peroxiredoxin, CAA71503) and VII48 (mitochondrial genome, MIATGENB), CT226 (ATAC006587) and IV17 (AF007271) have very weak protein-protein interaction with either ABI1 or ABI2, and their mutated versions. The quantitative β-gal assay supports a detectable degree of protein-protein interaction.

3.2 Fibrillin and glutamyl tRNA synthetase (AtGluRS)---potential cellular target proteins of ABI1 and ABI2

From the yeast two-hybrid screen for ABI2 interacting *Arabidopsis* proteins, several candidate proteins were identified which clearly indicate *bona fide* interaction due to their specificity to ABI1/ABI2 or their discrimination of the PP2C-deficient mutant versions thereof. In this work fibrillin and AtGluRS were selected for further analysis.

3.2.1 Fibrillin

Fibrillin was first identified in bell pepper (Deruére et al., 1994a). It is a 32-kD protein that forms fibrils in which carotenoids are stored. The gene encoding fibrillin is specifically expressed during fruit ripening in *Capsicum annuum*, leading to its accumulation in chromoplasts. Proteins belonging to the fibrillin family are present in other chromoplastic structures from different species (Deruére et al., 1994b). Chloroplastic drought-induced protein (CDSP) in *Solanum tuberosum* plants displays a high homology (91%) with pepper fibrillin (Gillet et al., 1998) and is preferentially associated with stromal lamellae (Pruvot et al., 1996). In potato a cDNA clone 40.4 has high homology to fibrillin in bell pepper and the protein is located in the chloroplast, associated with the thylakoid membranes (Monte et al., 1999). *Citrus unshiu* (Moriguchi et al., 1998), *Arabidopsis thaliana* (Genbank accession number AF075598 and AL021712), *Zea mays* (Genbank accession number AA979828) and *Brassica* (Hernandez-Pinzon, et al., 1999) also have genes homologous to fibrillin. It appears that fibrillin-related proteins constitute a well-conserved family of proteins expressed in both di- and monocotyledonous plants.

The fibrillin homologue of *Arabidopsis* (AF075598) [which its coding sequence is predicted in BAC T24H24 according to computer analysis using Genefinder (Washington University Genome sequences Center, 1998)] was isolated from an Arabidopsis cDNA library using ABI2 as bait protein. The identified cDNA encodes a nearly the entire protein

(315 out of 318 amino acids) and is fused in frame of GAL4. From the quantitative assay, fibrillin has strong β-galactosidase activity with the ABI2 full-length protein but no activity with the other BD fusions such as N-terminal-deleted and mutated forms of both ABI1 and ABI2 and also N-terminal form of ABI1 (Figure 11-A). This suggests that both the N-terminal part of ABI2 and the PP2C activity are crucial for protein-protein interaction between fibrillin and ABI2.

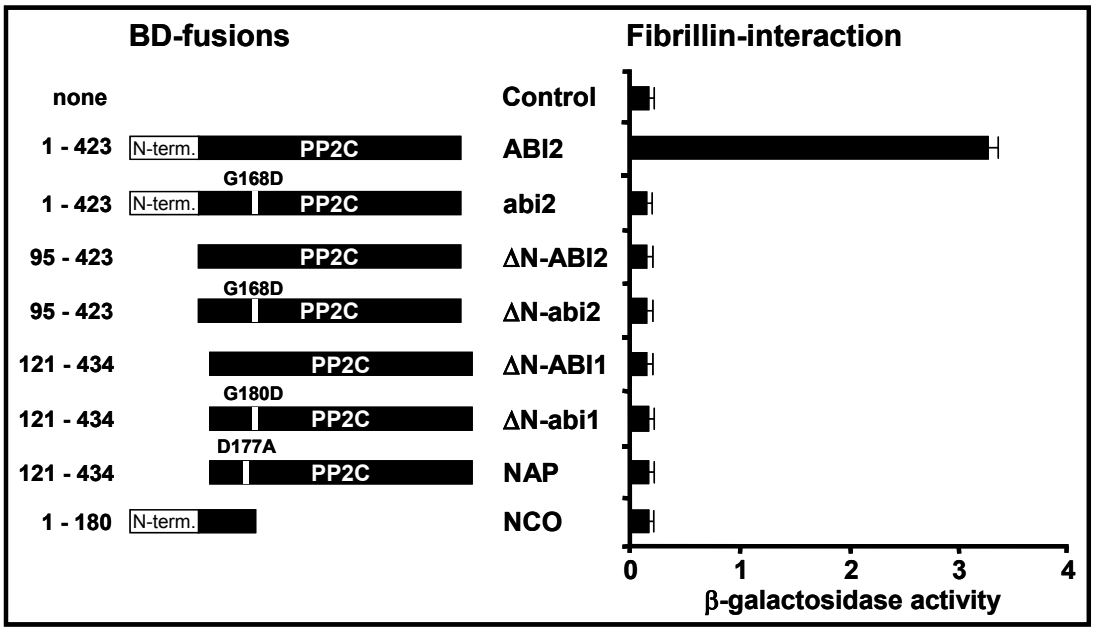


Figure 11-A. Two-hybrid interaction of fibrillin with ABI1 and ABI2 and their mutated versions.

Various ABI1 and ABI2 versions were fused to the GAL4 DNA binding domain (BD-fusions) and tested for interaction with the fibrillin protein fused to GAL4 activation domain (AD) in the yeast two-hybrid system. The amino acid residues of the protein phosphatase versions are given and are schematically presented on the left. The catalytic domain is signified by PP2C and the aminoterminal part is labelled N-term. The amino acid exchange in mutant abi1 and abi2 are presented as well as the mutation in the catalytically non-active PP2C (NAP). Cells expressing non-fused AD and BD domains were used as control. Interaction is indicated by the level of transactivation of β-galactosidase expression. The reporter activity is given in Miller units and represents the mean value of three independent experiments (± standard deviation).

3.2.2 Glutamyl tRNA synthetase (AtGluRS)

GluRS belongs to the class I aminoacyl-tRNA synthetases and shows several similarities with glutaminyl-tRNA synthetase concerning structure and catalytic properties (Freist et al., 1997). GluRS esterifies glutamic acid, which is used in nature as a constituent of proteins and in the nervous system as neurotransmitter (Seeburg, 1993). The most important function of GluRS is to transfer glutamic acid to $tRNA^{Glu}$ in a two-step process in which there is first a formation of an aminoacyl adenylate and then an ATP/PPi exchange reaction (Deutscher, 1967a, b; Lapointe and Söll, 1972). In addition, acylation of $tRNA^{Glu}$ with

glutamic acid is also the first step in biosynthesis of porphyrins, because the universal precursor of porphyrins, δ-aminolevulinic acid, is synthesized from Glu-tRNA^{Glu} in plant (Kumar et al., 1996).

In plants, different GluRS were isolated as dimeric forms and found in chloroplasts, mitochondria and the cytoplasm (Ratinaud et al., 1988; Thomas et al., 1983). In *Arabidopsis thaliana*, cytoplasmic GluRS has been cloned, and the deduced protein from the cDNA contained 719 amino acids which were confirmed following expression in *E. coli* (Day et al., 1998). Except for these reports, little is known about plant GluRS.

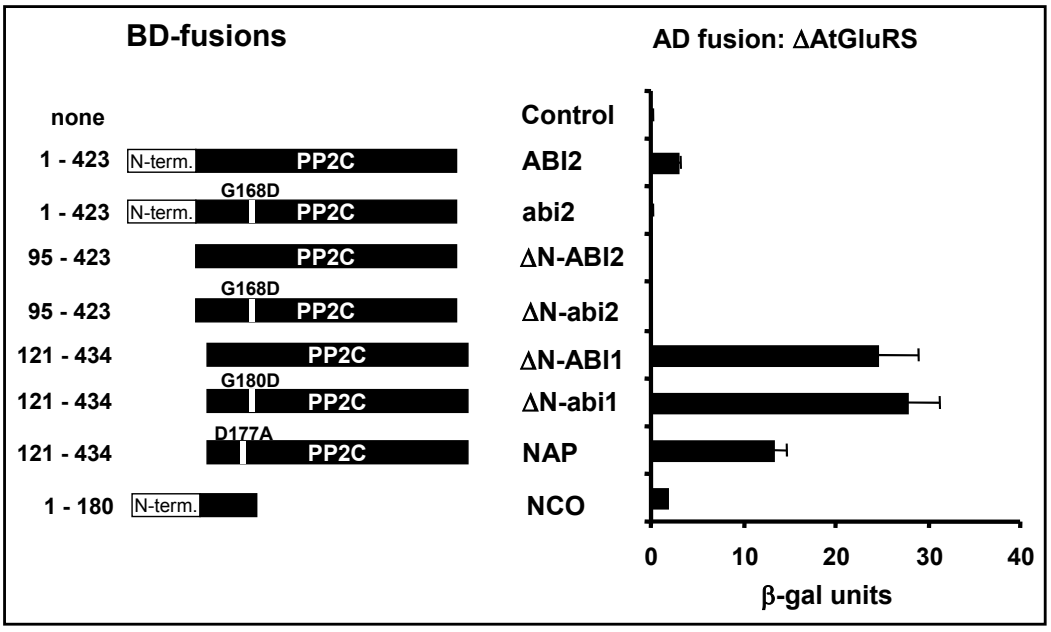


Figure 11-B. Two-hybrid interaction of ΔAtGluRS with ABI1 and ABI2 and their mutated versions.

Various ABI1 and ABI2 versions were fused to the GAL4 DNA binding domain (BD-fusions) and tested for interaction with the ΔAtGluRS protein fused to GAL4 activation domain (AD) in the yeast two-hybrid system. The amino acid residues of the protein phosphatase versions are given and are schematically presented on the left. The catalytic domain is signified by PP2C and the aminoterminal part is labelled N-term. The amino acid exchange in mutant abi1 and abi2 are presented as well as the mutation in the catalytically non-active PP2C (NAP). Cells expressing non-fused AD and BD domains were used as control. Interaction is indicated by the level of transactivation of β-galactosidase expression. The reporter activity is given in Miller units and represents the mean value of three independent experiments (± SD).

In this work AtGluRS (Genbank accession number AF067773) isolated from an *Arabidopsis* cDNA library using ABI2 as bait protein encoded only the N-terminal domain (ΔAtGluRS), with 262 out of the 719 amino acids of full-length protein.

The quantitative assay demonstrated that the identified domain of ΔAtGluRS very strongly interacted with N-terminal deleted and mutated versions of ABI1 (around 25 β-gal units). It

has relatively weak activity with the ABI2 wild type protein (2 β-gal units) and N-terminal version of ABI1 (1.8 β-gal units) (Figure 11-B). Interestingly, the full length AtGluRS did not interact with any BD versions tested. All β-galactosidase activities measured with full-length AtGluRS and different ABI1 and ABI2 versions were lower than 0.2, and were comparable to the control. It seems that the protein phosphatase activity is not required for protein-protein interaction between ΔAtGluRS and ABI1 since the prey also interacted strongly but somewhat reduced with catalytically non-active ABI1 (NAP). Thus, both proteins could form a stable complex. It was tempting to determine the function of ΔAtGluRS and elucidate whether it has a role in ABA signal transduction.

3.2.3 Immunoprecipitation in yeast

To analyse the expression of GAL4-fused proteins in yeast system, the AD/prey proteins, GAL4-fibrillin and GAL4-AtGluRS were extracted from expressing yeasts and detected using anti-GAL4 AD antibodies according to methods described in 2.2.2. Figure 12 illustrates that the GAL4-fibrillin fusion protein had a Mr of 54 kD Mr and GAL4-AtGluRS approximately 50 kD. The masses are similar to those predicted (GAL4, 19 kD and fibrillin 35 kD, ΔAtGluRS 29 kD in AD/preys). So these two selected cDNAs were expressed in the correct reading frame of GAL4 in yeast.

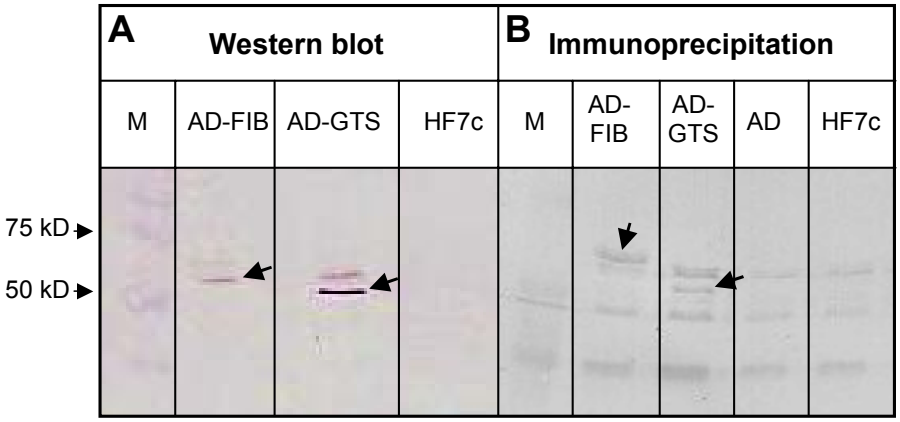


Figure 12. Detection of AD fusion proteins by western blot and immunoprecipitation.

A: Western blot. Yeast protein extracts with AD/fibrillin, AD/AtGluRS and empty AD were directly loaded on SDS-PAGE. AD-fusion proteins were detected by anti-GAL4 AD antibody after separating and blotting; **B: Immunoprecipitation.** AD-fusion proteins were purified by incubation of yeast protein extracts with Protein-A Sepharose and then loaded on SDS-PAGE. AD-fusion proteins were detected by anti-GAL4 AD antibody. Arrows indicate the positive AD-fibrillin and AD-AtGluRS, respectively; M: protein marker.

3.3 Characterization of fibrillin's role in ABA signal transduction

3.3.1 Expression analysis of fibrillin regulation by ABA at the transcriptional level

Gene expression is controlled at different stages, such as transcription, processing and translation. Fibrillin is known as a drought- and wound-induced protein. The promoter of the pepper fibrillin gene is upregulated after wounding, drought, and oxidative stress and during cold and salt stress (Manac'h and Kuntz, 1999). Little is known about the role of ABA in these regulations. In this study, the regulation of fibrillin gene by ABA was investigated at the transcriptional level. ABA-mediated increase in fibrillin mRNA abundance occurred rapid and within 3 hours of hormonal exposure (30 µM) in *Arabidopsis* seedling before isolation of RNA with maximal induction levels of 2.5 to 3 fold (Fig. 13-1-A). The moderate increase remained constant for at least two days. The dose-response dependence of the process illustrates that maximal induction levels are reached already at 3 µM ABA (Figure 13-1-B). Treatment of seedling with 0.3 µM ABA for 24 hours increased the expression of fibrillin mRNA 50% approximately over the control. Expression of fibrillin mRNA increased with increasing ABA concentration. From the results, it is clear that mRNA abundance of fibrillin is moderately but consistently up-regulated by ABA.

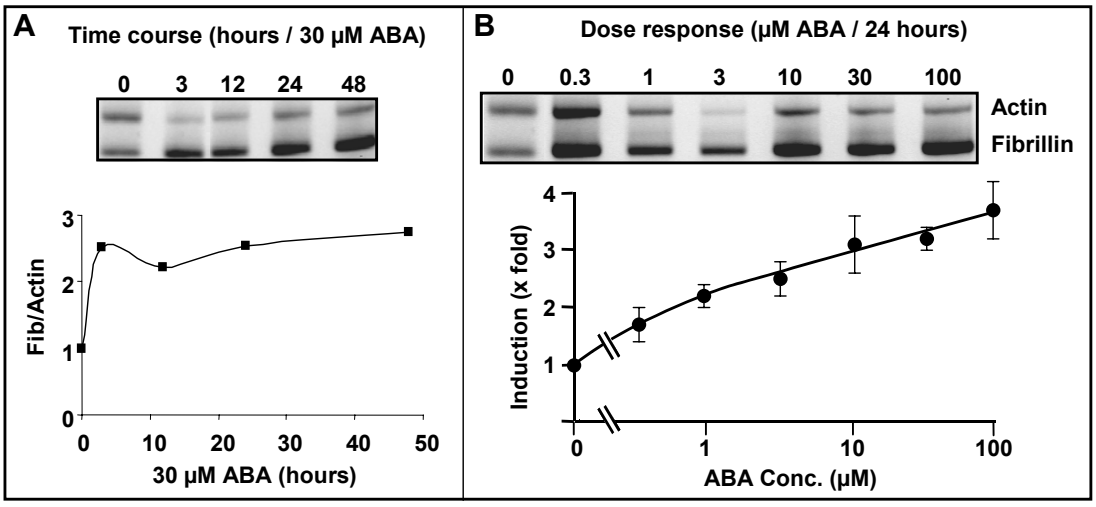


Figure 13-1. Level of fibrillin transcripts influenced by ABA.

Total RNAs were isolated from sterile seedlings of *Arabidopsis* ecotype RLD cultivated in liquid medium and analysed by RT-PCR. **A: Time course.** Seedlings germinated for 10 days were treated with 30 µM ABA for 0, 3, 12, 24 and 48 hours; **B: Dose-response.** Seedlings were treated with 0, 0.3, 1, 3, 10, 30 and 100 µM ABA for 24 hours prior RNA extraction. PCR was performed with fibrillin cDNA-specific primers and normalized for actin transcripts. Quantification was performed with imaging software Molecular Analyst (Biorad).
Upper panels: amplified DNA fragment stained by ethidium bromide in agarose gel;
lower panels: Quantification analysis.

Northern analysis using RNA isolated from *abi1* and *abi2* mutant lines as well as wild-type (La-*er*) also demonstrated that fibrillin mRNA was up-regulated by ABA in wild-type plants. The hybridization signal with fibrillin-specific probe was much stronger in the treatment with 30 or 100 µM ABA than without ABA (Figure 13-2). Interestingly, the ABA-induced alteration of fibrillin gene expression is strictly dependent on ABI1 (Figure 13-2). The signal was hardly detected in *abi1* after treatment with 30 µM ABA, but a weak signal after treatment with 100 µM ABA. In the *abi1* mutant background the mRNA abundance of fibrillin is lowered by at least a factor of 10 irrespective of the presence of ABA in comparison to wild-type and *abi2*. At 100 µM ABA, which is about 30-fold excess over the ABA concentration required for maximal induction in wild-type, a basic level of fibrillin transcripts is detected in the *abi1* mutant. There was almost no hybridization signal without ABA in both mutant lines. After the treatment with 30 or 100 µM ABA, the hybridization signals of the fibrillin were much stronger in *abi2* than in *abi1*.

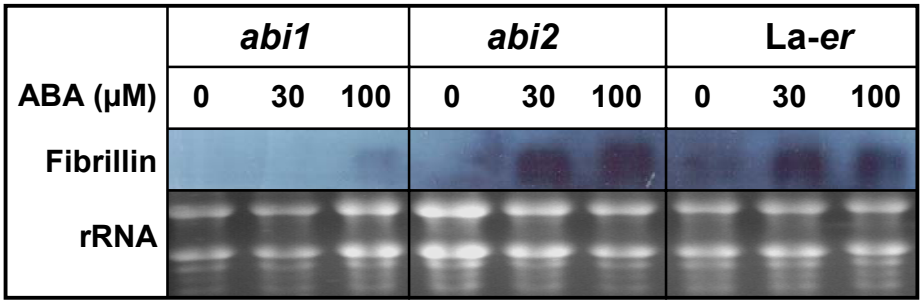


Figure 13-2. Dependence of fibrillin expression on ABI1.

Total RNAs were isolated from 10-day seedlings of *Arabidopsis* wild-type Landsberg and *abi1*, *abi2* plants. Prior RNA isolation seedlings were treated with ABA (0, 30, 100 µM) for 24 hours. Approximately 20 µg of RNA was loaded per lane and rRNAs were visualised by ethidium bromide staining (lower panel). Digoxigenin-labelled fibrillin cDNA was employed for Northern analysis of fibrillin transcripts (upper panel).

3.3.2 Interaction analysis --- *in vitro*

3.3.2.1 Expression, purification and detection of GST-fibrillin fusion protein

A fusion protein between fibrillin plus glutathione S-transferase (GST) was heterologously expressed in *E. coli* by using the plasmid construct shown in Appendix (Figure 78-A). To induce the expression of GST-fusion proteins, 0.1 mM of IPTG was added into the bacterial culture after the OD_{600} reached 0.5. The fusion protein was purified from bacterial extracts by glutathione sepharose 4B–affinity chromatography using 10 mM reduced glutathione as

elution buffer. The successful induction of GST-fibrillin and its purification is documented in Figure 14. Silver staining and Western blotting of extracts and purified proteins using mouse anti-GST antibody and anti-mouse IgG alkaline phosphatase conjugate showed that the GST-fibrillin fusion protein was induced after adding IPTG treatment. Subsequently, the fusion protein and GST as a control were purified to homogeneity.

To determine the concentration of GST fusion proteins, silver staining serial dilutions of BSA served as standard. Figure 15 shows that purified GST-fibrillin fusion protein was recovered at a concentration of about 200 ng /µl and a total of 200 µg from 200 ml of bacterial cultures.

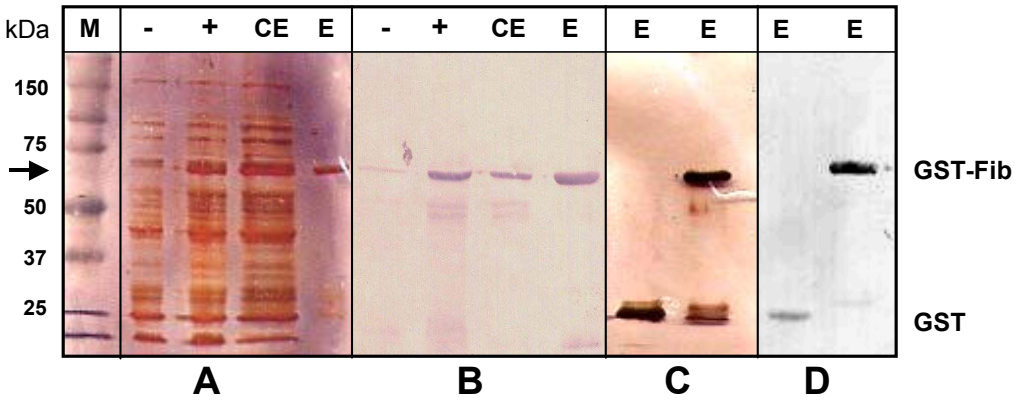


Figure 14. Purification of GST-fibrillin fusion proteins.

Silver staining (**A**) and western blot (**B**) showed that the GST-fibrillin fusion protein was induced by IPTG in *E. coli* extracts. Purified GST-fibrillin and the control GST proteins by Glutathione Sepharose 4B were detected by silver staining (**C**) and western blot (**D**). GST-fibrillin and GST were detected by anti-GST antibody after western blotting. M: protein marker; -: without IPTG; +: with IPTG; CE: crude extracts; E: eluate after purification; arrow: GST-fibrillin (GST-Fib).

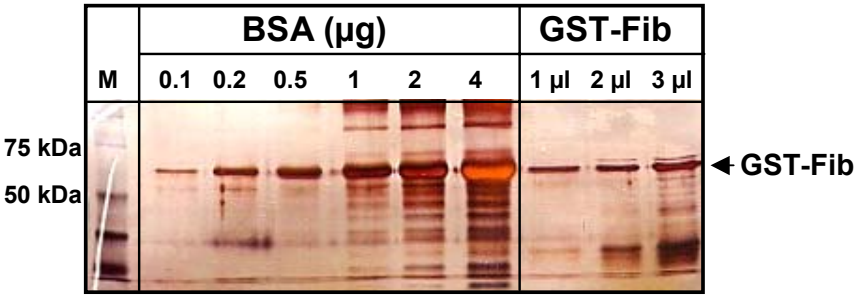


Figure 15. Determination of GST-fibrillin fusion protein concentration by silver staining.

Purified GST-fibrillin fusion protein (right panel) was analysed by SDS-PAGE together with a serial dilutions of BSA to determine its protein concentration (left panel). Arrow indicates the GST-fibrillin fusion protein. M: protein marker.

3.3.2.2 Expression and purification of 6 x His tagged ABI1, ABI2 and their mutated forms

E. coli plasmids of pQE70 expressing full-length ABI1, ABI2 and their mutated forms were constructed by Meyer et al. (1994) and Rodriguez et al. (1998). To induce expression of these proteins, IPTG was added to the growth medium. However, the induction with IPTG was not a crucial factor for expression in *E. coli*. Recombinant proteins were purified by nickel-affinity interaction according to the methods in 2.2.6.4. Western blotting and silver staining showed that the native purified 6xHis-tagged recombinant proteins (Figure 16) were approximately 50 kD. The molecular weights of ABI1 and abi1 are near 49 kD (ABI1/abi1: 47.7kD; 6xHis: 1kD) and of ABI2 and abi2 are 47 kD (ABI2/abi2: 46.5kD; 6xHis: 1kD), so the determined molecular weight of purified proteins were in agreement with prediction. Later, an enzymatic assay for PP2C activity confirmed functionality (see Figure 47-B).

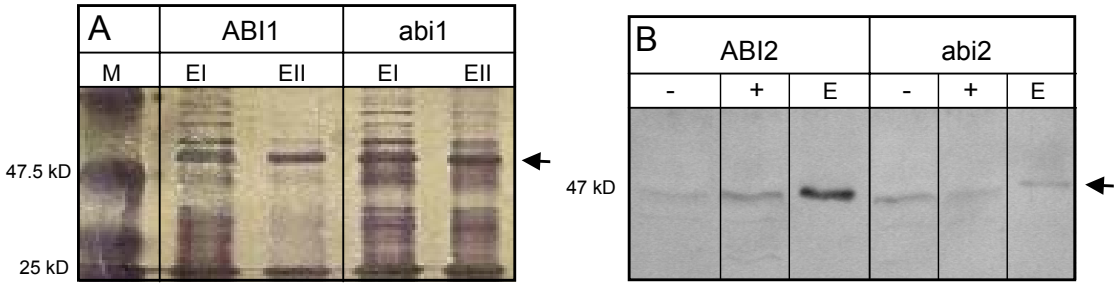


Figure 16. Purification of 6xHis-tagged recombinant proteins expressed in *E. coli*.

6xHis tagged recombinant proteins were expressed in *E. coli* and purified by using Ni-NTA resin. **A:** Silver staining of 6xHis-tagged ABI1 and abi1 recombinant proteins; **B:** 6xHis-tagged ABI2 and abi2 recombinant proteins immuno-detected by anti-ABI1 antibody *via* Western blot. Arrow indicates recombinant proteins, ABI1 and abi1 (**A**) as well as ABI2 and abi2 (**B**); M: protein marker; EI and EII indicate different eluates; -: without IPTG; +: induced by IPTG; E: eluate.

3.3.2.3 Assay of protein-protein interaction in vitro

Based on the data of the yeast two hybrid assay, it became clear that the protein-protein interaction is specific for fibrillin and ABI2 protein. In order to compare the protein-protein interaction *in vivo* and *in vitro*, affinity chromatography and pull down assays were used to confirm the protein-protein interaction between fibrillin and ABI2 *in vitro*.

Affinity chromatography assay

Affinity chromatography is a procedure that takes advantage of the biological important binding interactions that occur on protein surfaces. If a substrate molecule is covalently coupled to an inert matrix such as nickel-NTA bead, for example, the enzyme that operates

on that substrate will often be specifically retained by the matrix and can then be eluted. If one protein interacts with the protein which is covalently coupled to the matrix, it generally will be also retained.

In this study, 1 µg of recombinant protein GST-fibrillin (0.016 nM) and 0.4 µg of GST protein (0.016 nM) were incubated with 0.5 µg of ABI2 proteins (0.01 nM) tagged with 6 x His affinity and then were purified using nickel-NTA resin. The antibody used in this work to detect the proteins was anti-GST-ABI1 antibody which could detect GST and also ABI1 and ABI2 proteins. Western blotting showed that 83% of recombinant protein was found primarily in the ABI2 eluates (Figure 17). Very little fusion protein was detected in washing fraction. In contrast, in the control with GST protein, most of GST protein was found in washing fractions (about 76 %) (Figure 17). Silver staining and western blot immunodetected by another antibody, anti-flag antibody, also proved this result (data not shown).

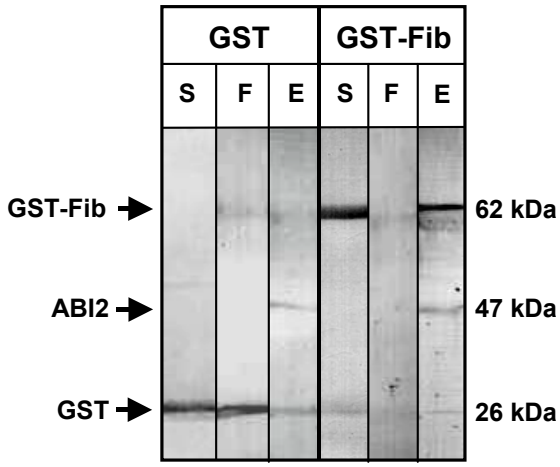


Figure 17. Binding of ABI2 and fibrillin revealed by affinity chromatography and immunodetection.

Purified GST and GST-Fib (GST-fibrillin) fusion protein were subjected to chromatography over ABI2-immobilised resin. The ABI2 protein had been tethered onto Ni-NTA resin *via* a His-tag. Aliquots of applied sample (S), flow through (F) and the eluate of ABI2 (E) were examined after SDS-PAGE and immunodetection using polyclonal antibodies raised against ABI2-GST. The position of visualized protein bands are indicated by arrows and the predicted Mr is given. Interaction of GST-Fib with ABI2 is reflected by a strong decrease of the signal for GST-Fib in the F fraction compared to the sample and by a high recovery rate in the eluate (83%) in contrast to control (GST; 24%). GST-Fib did not significantly interact with undecorated resin. Quantification was performed with imaging software Molecular Analyst (Biorad).

3.3.2.4 Pull down assay

The pull down assay is based on the same principle of specific binding as affinity chromatography. Due to prior radio-labeling of one protein, the interaction between two proteins can be quantified by autoradiography.

Autoradiography of the labeled GST-fibrillin protein indicates efficient ^{33}P-phosphorylation at the introduced PKA phosphorylation site (Figure 18). In the protein-protein interaction analysis between GST-fibrillin and ABI2 *in vitro*, most of the radioactivity was found in eluates of GST-fibrillin and ABI2 (around 80%), and 20% in unbound washing fractions. In the interactions between GST and ABI2 as a control, recombinant protein GST:fibrillin and abi2, or GST and abi2 less than 18 % of radioactivity were found in eluates of bound fractions. Most radioactivity was recovered in the washing fractions. This result is similar to the results obtained *in vivo* (in yeast two hybrid β-galactosidase assay) and support the conclusion that fibrillin interacts with ABI2 protein but does not or not detectable interact with its mutated version abi2.

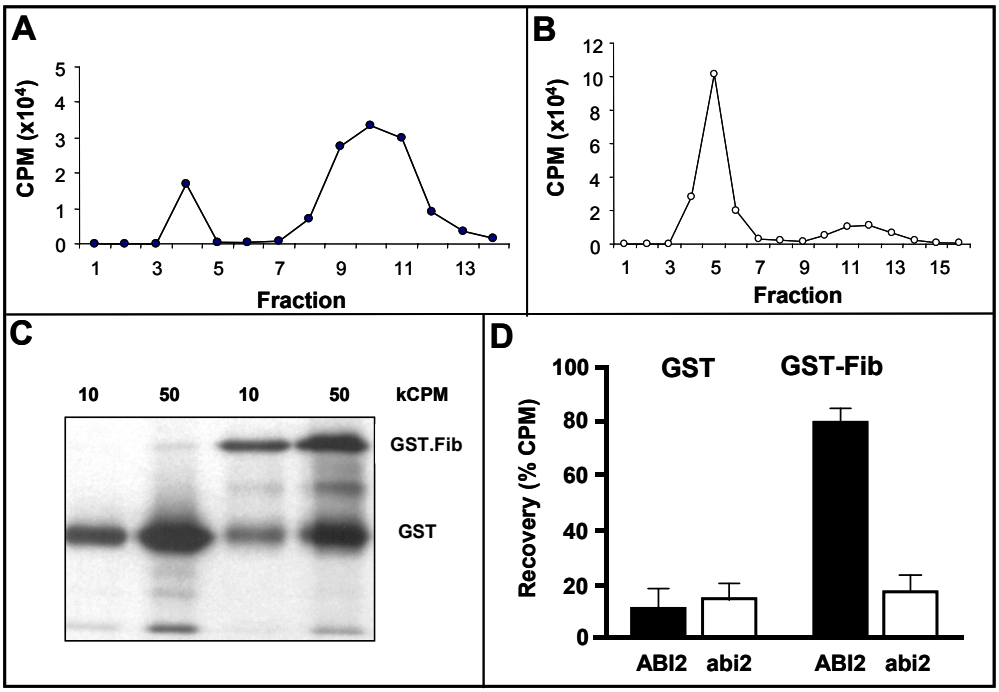


Figure 18. Pull down assay----*in vitro* protein interaction between fibrillin and ABI2/abi2.

Recombinant GST-Fib protein and GST protein both containing a protein kinase A target site were labeled with ^{33}P by phosphorylation. ABI2 and mutant abi2 proteins immobilised on Ni-NTA resin were incubated with purified and labelled GST-Fib or GST as a control. The distribution of radioactivity was examined after separation of the incubation mixture into supernatant and resin fraction by a single centrifugation step. **A, B:** labelling efficiency of GST-Fib fusion (**A**) and GST proteins (**B**) with γ-P^{33} ATP examined by gel filtration. **C:** autoradiography shows the radioactivity of labeled proteins. **D:** pull down assay shows the interaction between fibrillin and ABI2. The values are presented as recovery of the initial label present in the sediment of GST-Fib and GST analysis from two independent experiments (± SD).

3.3.3 Functional characterization by transient analysis

3.3.3.1 Protoplast expression system in Arabidopsis thaliana

The protoplast expression system is a sensitive and fast cellular system to analyze the ABA signal transduction mechanism through ABA-regulated reporter gene constructs (Sheen, 1996; 1998). The different ABA-dependent promoters such as RD29B (Yamaguchi-Shinozaki and Shinozaki,1994), Lti65 (Nordin et al, 1993), RAB18 (Lang and Palva, 1992), HB6 (Sönderman, 1999; Himmelbach, 2002) are strongly induced by ABA. Co-transfection of ABA-regulated reporter gene constructs together with the effector proteins that are expressed under the control of 35S promoter is an efficient tool to analyze the ABA signal transduction. In our laboratory, the transient expression system of *Arabidopsis* protoplasts was used to characterize HB6 (Himmelbach et al., 2002). The ABA signalling cascade was studied in this system by using the ABA-regulated reporter gene constructs (RD29BLUC and RAB18LUC) (Hoffmann, 2001).

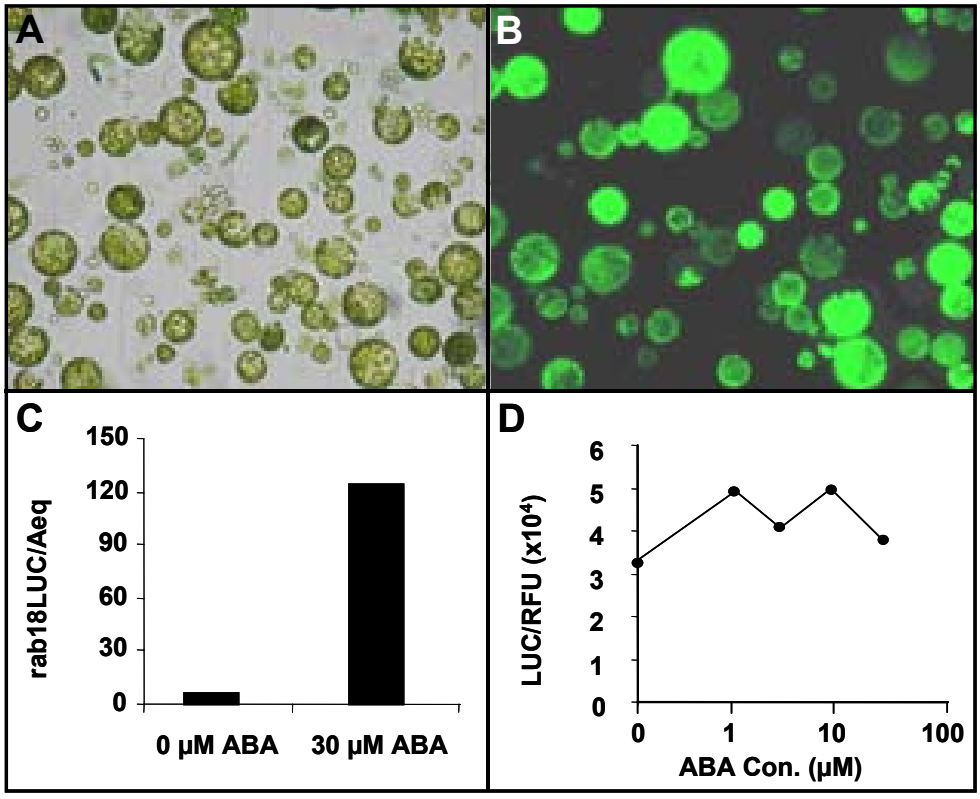


Figure 19. Protoplast expression system.

Protoplast expression system is a sensitive and fast system to study gene regulation, signal transduction pathway and localization of fusion proteins (Hoffmann, 2001).
A: protoplasts of *Arabidopsis* in light microscope. **B:** the same picture as in (**A**) showing living protoplasts coloured with the flouresent dye FDA (fluoresein diacetat).
C: expression of the luciferase reporter gene fused to ABA-regulated RAB18 promoter.
D: ABA-independent expression of luciferase by the constitutive 35S promoter.

Photinus pyralis luciferase (LUC) (Luehrsen et al., 1992) is usually used as reporter gene in the protoplast expression system. Luciferase oxidizes luciferin to oxyluciferin and this process will release photons which can be sensitivity measured by a luminometer. Figure 19 illustrates the expression of the luciferase reporter gene under the control of the ABA-regulated promoter RAB18 and the constitutive 35S promoter in the protoplast system.

3.3.3.2 Regulation of fibrillin promoter by ABA in the protoplast expression system

To determine whether the fibrillin promoter is regulated by ABA, plant material was treated with ABA before or after protoplast isolation and subsequently co-transfected with genes to be tested. Plasmid constructs used are shown in detail in the Appendix (Figure 81). 20 µg of fibrillin promoter-LUC plasmid was co-transfected together with 50 µg of 35S-GUS into protoplasts and then the protoplasts were treated with different ABA concentrations, i.e. 0, 1, 3, 10 and 30 µM. The amount of protoplast per independent transfection was 5 x 10^5 protoplasts per ml. Figure 20 illustrates the relative LUC expression driven by the fibrillin promoter of transfected protoplasts. GUS activity under the control of the 35S promoter was not affected by ABA in cotransfected protoplasts and was used as an internal control to normalize independent transfection for expression efficiency. The results shown in Figure 20 represent data of three individual cotransfections and indicate that the fibrillin promoter is approximately 2-4 fold up-regulated by ABA. Either protoplasts (Figure 20-A) or 4-week old plants prior to the isolation of protoplasts (Figure 20-B) were treated with ABA. Several repeats of this experiment gave similar results. The results showed that the induction is about 3 fold at 1 µM ABA and 3 to 4 fold at 30 µM ABA (Figure 20-A).

However, ectopic expression of abi1 and abi2 abrogated the induction by ABA. No induction of fibrillin-promoter controlled reporter expression was observed in the presence of ABA (Figure 20-C). Thus, the activity of luciferase reporter gene was not induced by ABA if the abi1 and abi2 were overexpressed as effectors in the protoplasts. Compared to the results of the Northern analysis (Figure 13), where the expression of fibrillin occurred in an ABI1-dependent manner, in this system besides abi1 also abi2 blocked the activation of the fibrillin promoter by ABA.

The pepper fibrillin promoter is up-regulated by high light intensities but not in darkness (Manac'h et al., 1999). The fibrillin-like protein of potato C40.4 is involved in photosynthesis by modulating photosynthetic efficiency and in dissipation of excess absorbed light energy within the antenna complex (Monte et al., 1999). To study the relationship between ABA and light on the *Arabidopsis* fibrillin promoter, protoplasts were co-transfected with 20 µg plasmids having the fibrillin promoter fused to the LUC reporter

gene and 50 µg 35S-GUS plasmids and then treated with light, 30 µM ABA or light plus 30 µM ABA overnight. The results shown in Figure 21 document that both ABA and light induce the expression of the reporter gene driven by the fibrillin promoter. Compared with the control cotransfection without ABA and light, the expression of the reporter gene was induced nearly 2 fold by light, more than 3 fold by ABA and more than 6 fold by ABA and light together.

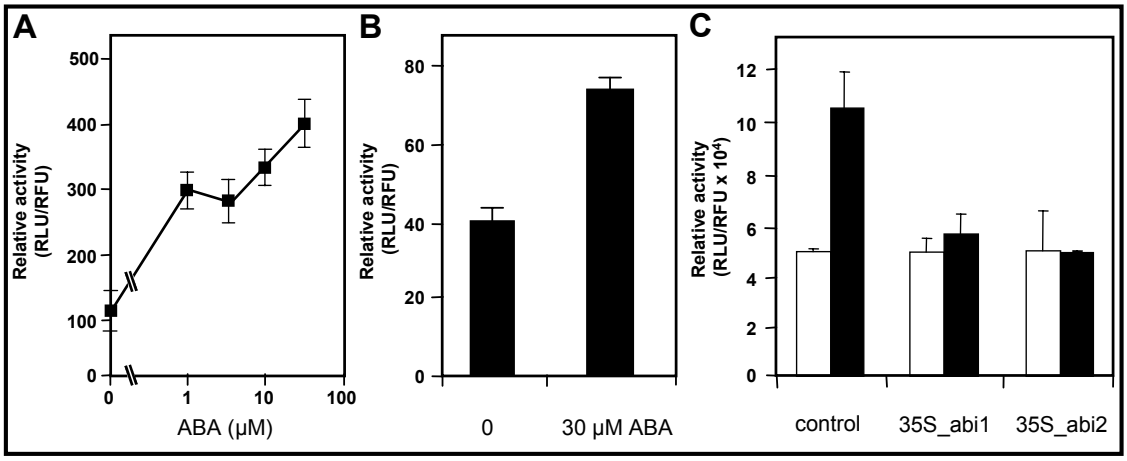


Figure 20. Regulation of fibrillin promoter activity by ABA in protoplasts.

A.B: ABA-dependent activation of the fibrillin promoter driving luciferase expression. *Arabidopsis* protoplasts were transfected with the reporter construct and a constitutively expressed β-glucuronidase gene. The protoplasts were either treated with different ABA concentrations for 24 h after transfection **(A)** or the 4-week old plants had been treated by applying 30 µM ABA (24 hs) prior protoplast isolation **(B)**. The normalized promoter activity shows that fibrillin promoter is up-regulated by ABA. **C:** In the presence of cotransfected abi1 and abi2, fibrillin promoter activation was inhibited even when the protoplasts were treatet with 100 µM ABA. Open columns: without ABA; dark columns: treatment with 30 µM ABA. 20 µg of reporter construct and 50 µg of 35S-GUS construct were used for cotransfection. The luciferase activity was normalized to GUS expression. The data represent three different cotransfections (± SD).

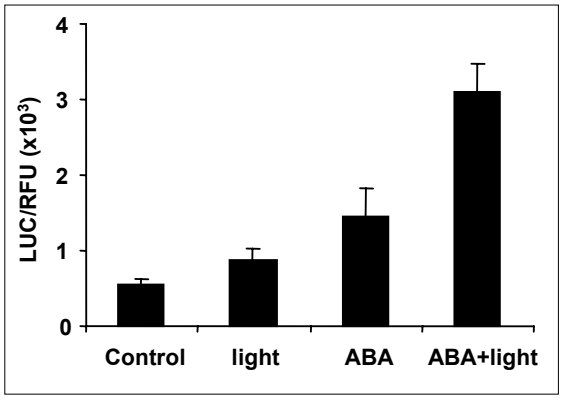


Figure 21. Regulation of fibrillin promoter activity by ABA and light in protoplasts.

Arabidopsis protoplasts were transfected with the reporter construct mentioned in Fig. 20 and a constitutively expressed β-glucuronidase gene. The protoplasts were either treated with light or 30 µM ABA or light and 30 µM ABA together for 24 h after transfection. 20 µg of reporter construct and 50 µg of the constitutive 35S-GUS construct were used per transfection. The luciferase activity was normalized to GUS expression. The data represent three different cotransfections (± SD). Light intensity is 100 µE photon/m^2 per sec.

3.3.3.3 *Ectopic expression of effector: Influence on ABA signal transduction*

Overexpression of proteins and subsequent analysis of the effect is also an efficient method to study the signal transduction pathway (Downward, 2001). In transient studies, overexpression of ABI1 and ABI2 inhibited the induction of ABA-dependent reporters by ABA (Hoffmann, 2001). In the yeast system, there was a strong protein-protein interaction between ABI2 and fibrillin. To examine the role of fibrillin on ABA signal transduction, fibrillin was overexpressed in protoplasts under the control of the CaMV 35S promoter. The construct used is illustrated in the Appendix (Figure 85).

The results indicated that fibrillin might be a negative regulators of the ABA signal transduction pathway. As shown in Figure 22, the expression of two reporter genes, rab18LUC normalized for aequorin expression and Lti65-LUC normalized for GUS expression, were reduced in the presence of the effector fibrillin. The expression of the reporter gene rab18LUC was inhibited by a factor of 6 at 0.3 µM ABA and by a factor of 4 at 3 µM ABA by fibrillin expression compared to the cotransfection without the effector (Figure 22-A). Similarly, reduction of Lti65-LUC reporter expression was by a factor of 2 at 3 µM and by a factor of 3 at 10 µM ABA compared to transfection without the effector (Figure 22-B).

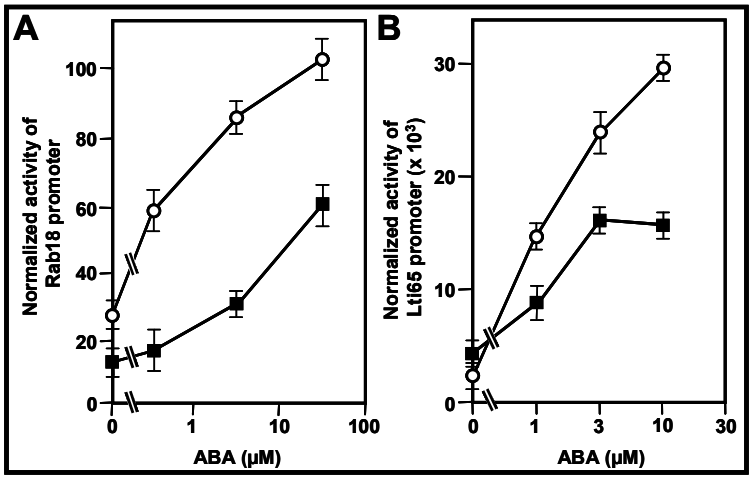


Figure 22. Transient fibrillin expression results in reduced responses of ABA-regulated promoters.

Ectopically expressed fibrillin was tested for interference with ABA signal transduction in Arabidopsis protoplasts. Protoplasts transfected with or without the 35S-fibrillin effecter in addition to an ABA-responsive luciferase construct containing either the Rab18 **(A)** or Lti65 promoter **(B)** were exposed to increasing ABA levels after a 16 hs period of phenotypic expression. Luciferase expression was normalized to the 35S-promoter driven aequorin (**A**) and β-glucuronidase expression (**B**). The data are presented in the presence and absence of fibrillin effector (closed and open symbols ± SE, respectively). Several independent transfections gave comparable results.

3.3.4 Functional characterization by analysis of stable transgenic plants

3.3.4.1 Promoter regulation-----Histochemical GUS staining

In order to understand the expression pattern of fibrillin gene in *Arabidopsis*, transgenics stably transformed with a fibrillin promoter-glucuronidase construct were used to monitor reporter expression in this study. The plasmid construct of GUS reporter gene under the control of the fibrillin promoter which was used for generation of transgenic plants is shown in the Appendix (Figure 81). In order to analyse the activity of the promoter-reporter construct in plants, six independent transgenic lines, which were demonstrated to be stably transformed by analysis of genomic DNA isolated from those six lines, were used to detect GUS activity. Histochemical GUS staining showed that the fibrillin promoter was most active in leaves, such as the leaf vascular system and mesophyll cells (Figure 23-A, -B, -C, -F, -J), in the hypocotyl region (Figure 23-A, -C), the shoot meristem region (Figure 23-A, -D) and was also active in trichomes, being strongest in the bottom of the trichomes (Figure 23-G). The fibrillin promoter was sometimes active in lateral roots (Figure 23-H, -I), but it was rarely active in the root. In 2 out of the six transgenic lines the fibrillin promoter driven GUS activity was strongly expressed in stipula of seedlings (Figure 23-D, -E).

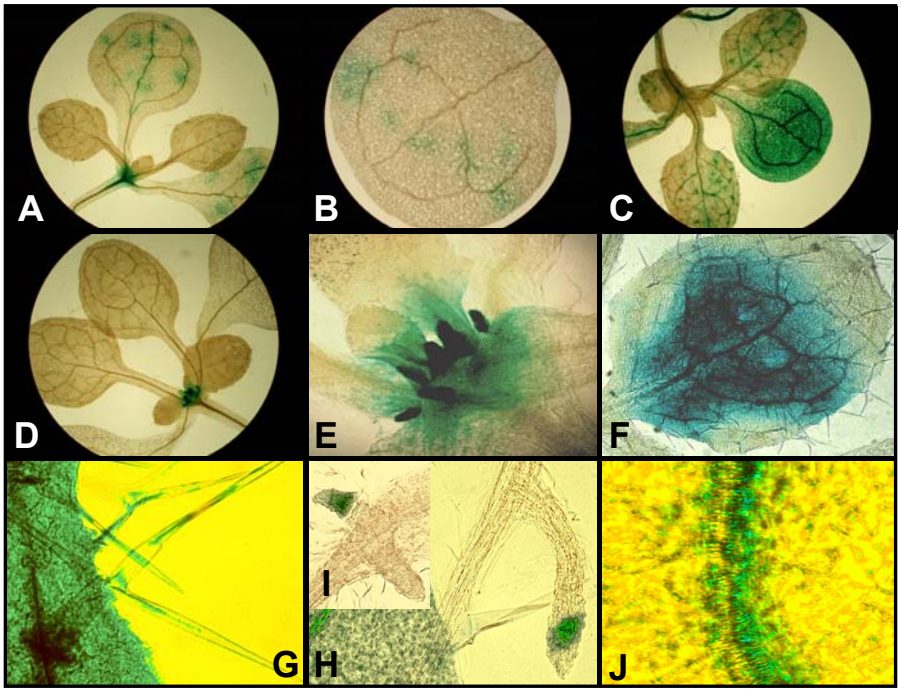


Figure 23. Histochemical localization of fibrillin promoter activity in *Arabidopsis.*

Arabidopsis transgenics for a fibrillin promoter-GUS chimeric construct were analysed for organ-specific and developmentally regulated GUS expression. The reporter activity was preferentially detected in different areas of seedlings of transgenic lines, in plant leaves (A,B,C), vascular tissues (C,J), mesophyll and hypocotyl (A, B, C, F), stipula (D, E), trichomes (G), and lateral roots (H, I).

To study the activity of the fibrillin promoter in mature tissues and organs, whole plants grown on soil were fixed for histochemical GUS staining. In all six lines, the expression of the fibrillin promoter::GUS construct was found in reproductive organs (Figure 24). GUS activity was very strong in flowers, especially in the corolla, anthers and stigma (Figure 24-A, B). GUS activity was also seen in siliques, and the younger the silique, the stronger the GUS staining was (Figure 24-C). GUS activity was also found in the upper region of stems (Figure 24-C).

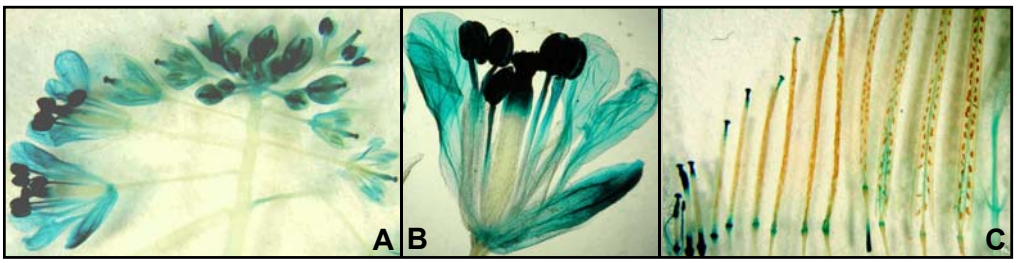


Figure 24. Histochemical localization of fibrillin promoter activity in *Arabidopsis.*

Arabidopsis transgenic for fibrillin promoter::GUS chimeric construct were analysed for organ-specific and developmentally regulated GUS expression. The reporter activity was preferentially detected in different areas of reproductive organs of the transgenic lines. **A, B:** in flowers; **C:** in different developmental stages of siliques.

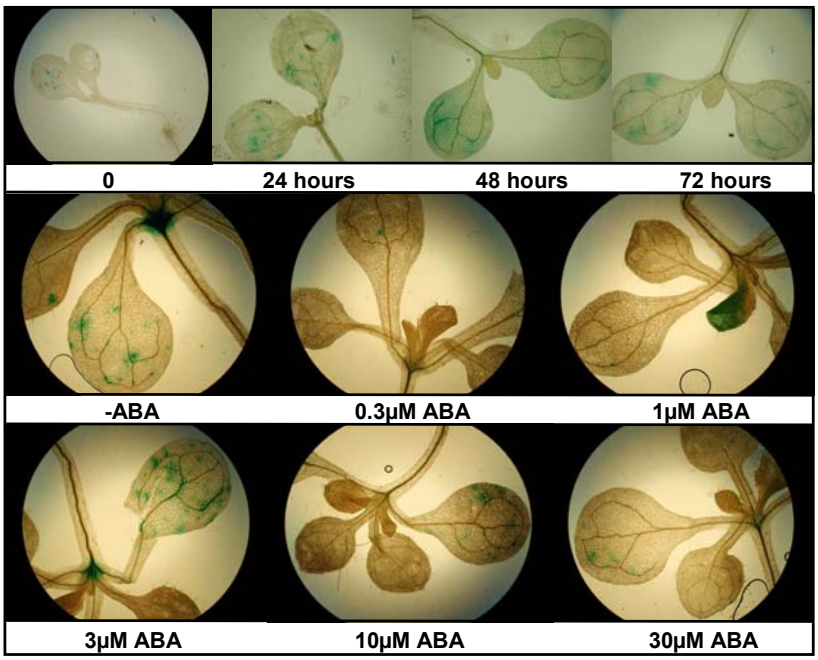


Figure 25. The effect of ABA on fibrillin promoter activity in transgenic *Arabidopsis* plants.

Arabidopsis plants transgenic for fibrillin promoter::GUS chimeric construct were analysed for organ-specific and developmentally regulated GUS expression and for the effect of ABA. Seedlings of transgenic plants were grown on MS plates and then transfered to MS plates with 30 μM ABA for 0, 24, 48 and 72 hours (upper panels), and to plates with different ABA concentration, 0, 0.3, 1, 3, 10 and 30 μM ABA for 24 hours (middle and lower panels).

To investigate the influence of ABA on the activity of the fibrillin promoter, the seedlings were treated in a time-course experiment with 30 µM ABA. In addition, seedlings were exposed to different ABA concentrations for 24 hours (Figure 25) after the seedlings had been grown on MS plate for two weeks. Treatment with 30 µM ABA for 0, 24, 48 and 72 hours, and with 0, 0.3, 1, 3, 10 and 30 µM for 24 hours, failed to reveal any obvious differences. Histochemical GUS staining did not allow the detection of differences in the activity of the fibrillin promoter due to ABA.

3.3.4.2 Expression of fibrillin under ABA and diverse stress conditions ---fluorometric GUS assay

To determine whether the activity of the fibrillin promoter is affected by ABA and by different stress conditions, a fluorometric GUS assay, more accurate and sensitive to detect differences than histochemical GUS staining, was used. After treatment with 30 µM ABA for 4 and 24 hours, five of the six transgenic lines (T1) which had a 3 to 1 segregation ratio on selective kanamycin medium showed GUS activity induced by ABA. GUS activity in PRF1 (fibrillin promoter-GUS transgenic line-1) and PRF3 were induced by a factor of 3 and 2 after seedlings were treated with 30 µM ABA for 4 hours compared to the treatment without ABA (Figure 26-A). Before treatment with ABA, the GUS activity was 0.028 and 0.007 (RFU/s per 100 µg protein) for PRF1 and PRF3, respectively. After treatment with ABA for 4 hours, GUS activity was 0.08 and 0.014. After treatment for 24 hours, the induction of GUS activity by ABA was increased by a factor of more than 4 and 8 in PRF1 and PRF3, respectively. GUS activity was 0.14 and 0.05 in PRF1 and PRF3, respectively. In the control, 35S-GUS line also treated with ABA there was no induction of GUS activity detected. GUS activity were 0.39, 0.31 and 0.34 (RFU/s per 100 µg protein) after treatment with ABA for 0, 4 and 24 hours, respectively. The results in Figure 26-A were derived from three independent experiments.

Other experiments to investigate the expression of the fibrillin promoter-GUS fusion under stress conditions showed that salt and oxidized glutathione could also induce the activity of the fibrillin promoter. After cultivation of the seedlings in liquid medium for two weeks and then adding 100 mM NaCl or 10 mM oxidized glutathione (GSSG) for 24 hours, the latter induced the expression of fibrillin promoter-GUS fusion 6.5 fold in PRF1 and 2.4 fold in PRF3 compared to the control without treatment. Under salt stress (100 mM NaCl) expression of GUS driven by the fibrillin promoter was induced 2.5 fold in PRF1 and 6 fold in PRF3. Treatment with 0.1 mM methyl viologen, no changes in GUS activity were detected (Figure 26-B).The 35S-GUS line as a control showed no induction under those different stresses. All results in Figure 26-B are derived from 3 independent experiments. Other transgenic lines tested also had similarly induced GUS activity under ABA or stress conditions (data not shown).

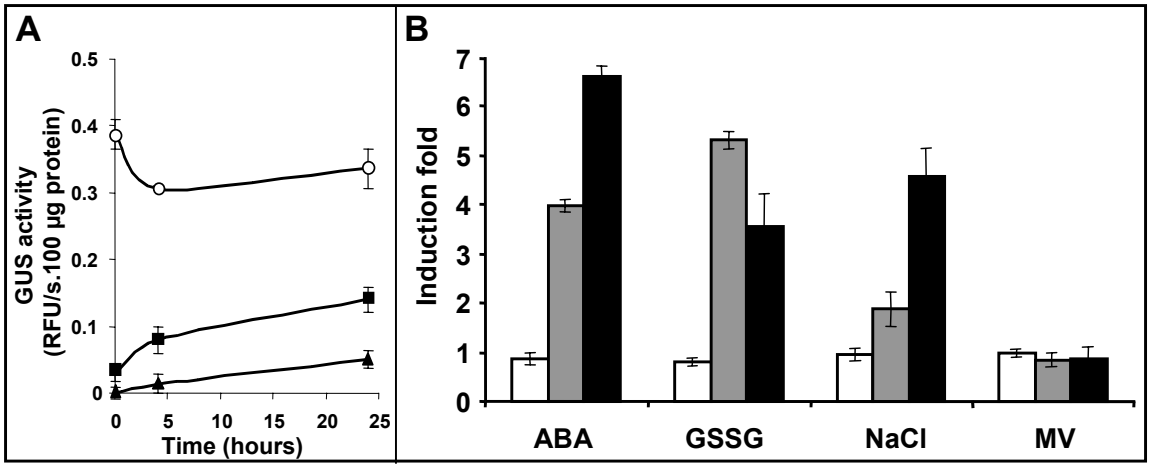


Figure 26. Fluorometric GUS assay to analyse the regulation of the fibrillin promoter in *Arabidopsis*.

A: Effect of ABA on the expression of fibrillin.
Transgenic seedlings were incubated in MS liquid culture with 30 µM ABA for 0, 4 and 24 hours and then GUS activity was measured and normalized to the protein concentration. The results of three independent values are shown. Transgenic line of PRF:GUS (line 1, closed squares and line 3, closed triangles), 35S-GUS as control (opened circles).
B: Induction of fibrillin promoter by verious stresses.
Two transgenic lines of fibrillin promoter-GUS (line 1, grey columns and line 3, dark column) and control 35S-GUS (open columns) were treated 10 mM oxidized glutathione (GSSG), 100 mM NaCl, 0.1 mM methyl viologen and water for 24 hours after the seeds were allowed to germinate and the seedlings to develop in MS liquid culture for 8 days. The protein extracts were used to measure the GUS activity and were normalized to the protein content.

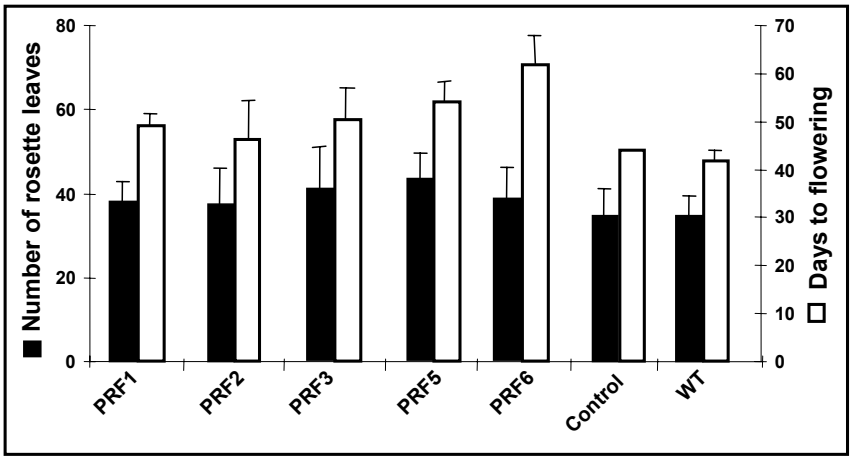


Figure 27. Change in flowering time of fibrillin-GUS transgenic plants.

Arabidopsis plant transgenic for the fibrillin promoter-GUS chimeric construct were analysed for phenotypical alterations. Closed circles: days to flowering; closed columns: number of rosette leaves at flowering initiation. PRF1 to PRF6 indicate independent transgenic lines; control: 35S-GUS transformant plant; WT: wild-type (RLD). n=20 3

3.3.4.3 *Phenotype of fibrillin promoter-GUS transgenic plants*

The analysis of five independent fibrillin promoter transgenic lines for phenotypic variation showed that their growth characteristics were altered. Increased numbers of rosette leaves and increased numbers of days till the onset of flowering was observed (Figure 27). Thus the vegetative growth phase of the transgenic plants was prolonged. Compared with 35S-GUS transformants and wild-type plants which had 35 rosette leaves on average prior onset of flowering, transgenic lines had from 38 to 44 leaves. Initiation of flowering was also delayed significantly from 44 days in 35S-GUS control and 42 days in wild-type lines to around 50 days in fibrillin promoter-GUS transgenic lines.

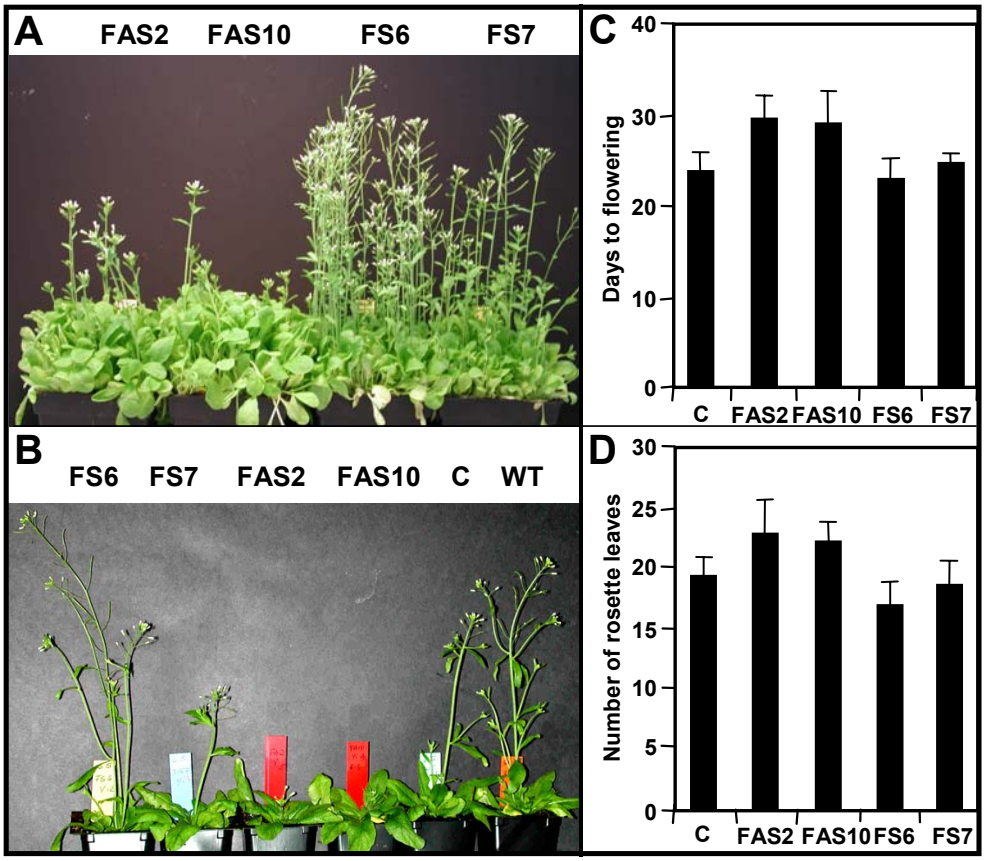


Figure 28. Alteration of flowering time of fibrillin transgenic plants.

A, B: Phenotypic appearance of fibrillin sense and anti-sense transgenic plants 4 weeks after sowing; **C:** days to flowering; **D:** number of rosette leaves at flowering initiation. FS6, FS7: fibrillin sense lines; FAS2, FAS10: fibrillin anti-sense lines; WT: wild type (RLD); C: 35S-GUS transgenic plants as the control.

3.3.4.4 Ectopic expression of fibrillin in plants

To analyse the effect of ectopic expression of fibrillin in plants, transgenic plants were generated. The fibrillin gene was cloned into the binary vector pBI121 in sense and anti-sense orientations under the control of the 35S promoter (Figure 85) and the T-DNA constructs were introduced into *Arabidopsis* plants (RLD) *via Agrobacterium*-mediated transformation. The T_0 transgenic plants were identified according to the introduced resistance marker and by subsequent PCR analysis of genomic DNA isolated from kanamycin resistant plants. T_1 transgenic plants were selected in the presence of kanamycin and the segregation ratios determined. Heterozygous and homozygous T_2 plants were selected according to the segregation ratio under the selective pressure of kanamycin.

3.3.4.4.1 Phenotype of fibrillin expressing transgenic plants

Seven independent primary sense lines and nine independent primary anti-sense lines of fibrillin were analyzed and (several) primary transgenic lines were selected for further analysis.

The obvious phenotypic alterations observed in fibrillin sense and anti-sense transgenic lines were similar to the ones observed with the fibrillin promoter lines namely changes in flowering time and in the number of rosette leaves. The appearance of several plants in pots as well as individual fibrillin sense and anti-sense transgenic plants were documented (Figure 28-A and B). As shown in Figure 28-C, the onset of reproductive development under standard conditions (16 hs light/ 8 hs dark at 100 μE photon/m^2.s) was delayed in fibrillin anti-sense lines. The anti-sense plants flowered at this light regime after 30 (± 3.1) days compared to 35S-GUS transgenic control plants which flowered after 23.5 (± 2.7) days. In contrast, there were no differences observed between fibrillin sense lines and control plants. The number of rosette leaves was increased in fibrillin anti-sense lines, with an average of 22.5 (± 2.2) rosette leaves per plants compared to 17.5 (± 1.8) per plants in control lines and fibrillin sense lines, as shown in Figure 28-D.

The population and individual plants from fibrillin sense and anti-sense lines are presented in Figure 28-A and 28-B, respectively, clearly showing the difference in plant growth behavior between these transgenic lines. There were no differences in lateral and secondary inflorescences, but main stems were shorter in transgenic sense plants than in control and anti-sense transgenic plants after days of development indicating delayed induction of flowering time. The transgenic fibrillin sense plants also displayed suppression of apical dominance and sometimes fascinated inflorescences that is the fusion of several inflorescences (Figure 29).

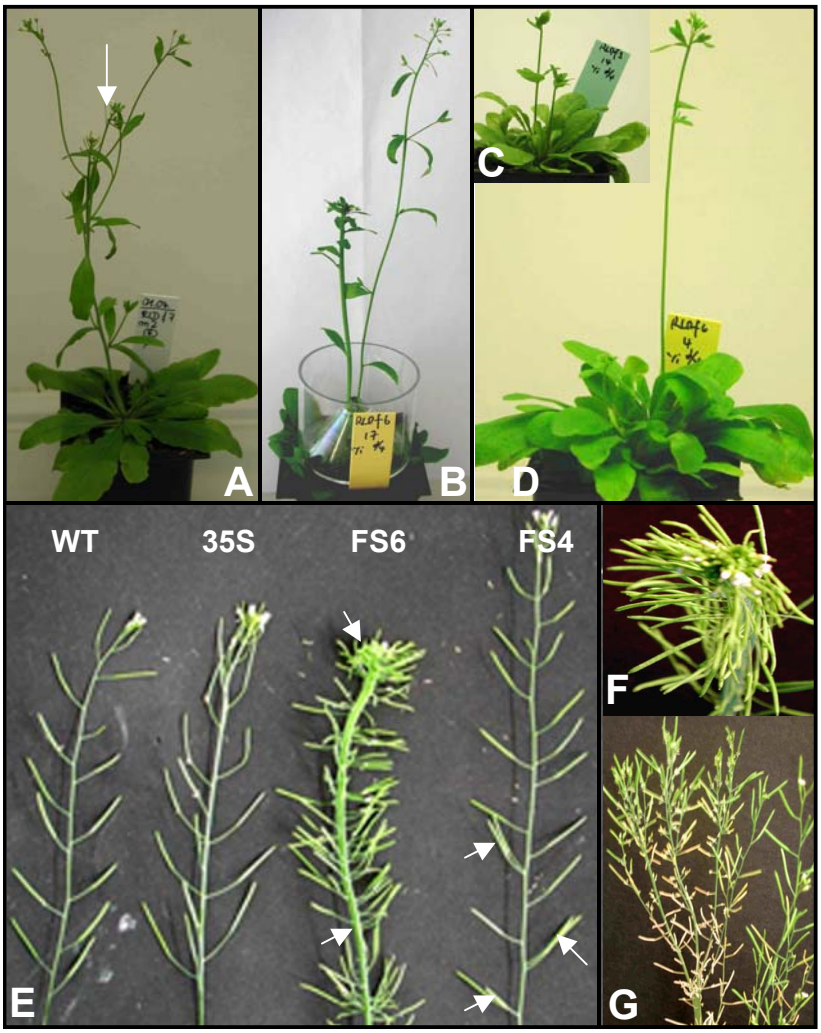


Figure 29. Phenotypical alteration of fibrillin transgenic plants.

The alterations of ectopic fibrillin expression in transgenic plants during different developmental stages. **A,C:** suppression of apical dominance; **B:** fusion of inflorescences; **D:** elongation of the main stem; **E:** siliques comparison of wild-type (RLD), 35SGUS transformant plant (35S), two fibrillin sense transgenic plants FS4 and FS6; **F, G:** bushy appearance of fibrillin sense lines FS6 (**F**) and FS7 (**G**), respectively. Arrows indicate the bushy appearance of FS6 as well as fasciated stems of FS6 and FS4.

3.3.4.4.2 Physiological analysis of ABA responses

To analyse the physiological response of ABA-mediated inhibition of germination and vegetative growth as well as control of stomatal aperture, two fibrillin sense lines and two anti-sense lines were randomly selected. Five sense and five anti-sense lines were also used to analyse root growth. The analyses of ABA-inhibited seed germination revealed that fibrillin anti-sense lines were more sensitive to ABA than fibrillin overexpression lines.

Compared to the control plants, the anti-sense lines was more sensitive by a factor of more than 3 reflected in an IC_{50} value of half-maximal inhibition by exogenous ABA of 0.25 µM (Figure 30-A). The IC_{50} of control was 0.75 µM. After 6 days there was only 10% germination of the fibrillin anti-sense seeds but 80% of the control seeds and around 60% of seeds from fibrillin overexpression in the presence of 1 µM ABA (Figure 30-B).

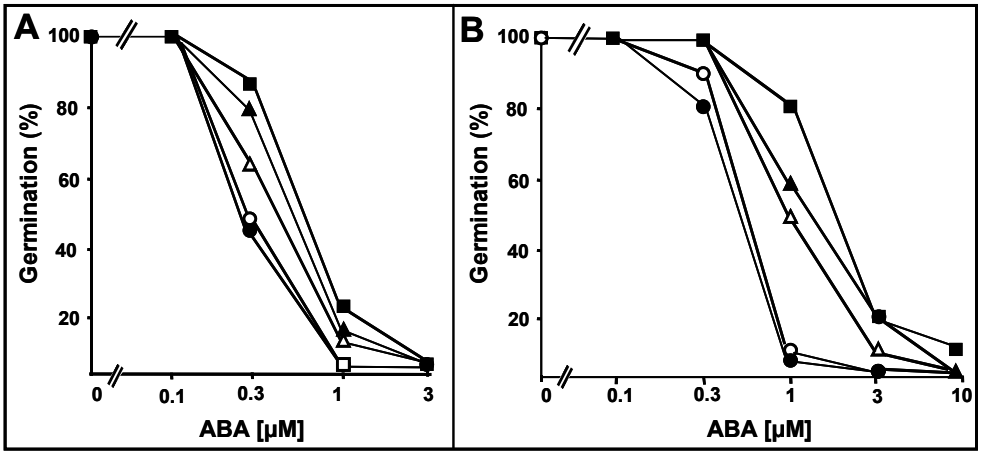


Figure 30. Germination assay of fibrillin transgenic lines in the presence of ABA.

Transgenic seedlings of fibrillin sense and anti-sense lines (n=50 ± 5) were grown on MS plates with different ABA concentrations. The germination rate was determined after 4 days (**A**) and 6 days (**B**) of incubation. Closed columns: 35S-GUS control; open and closed triangles: fibrillin sense lines (FS6 and 7); open and closed circles: fibrillin anti-sense lines (FAS2 and 10).

The germination assay provided evidence that the fibrillin anti-sense transgenic plants were hypersensitive to ABA, but there was no significant enhanced inhibition by ABA of seeds from fibrillin overexpression lines. Interestingly, inhibition of vegetative growth by ABA, as determined by root growth, was identical in all lines tested (Figure 31). However, the analysis of stomatal regulation supported a role of fibrillin influencing also vegetative ABA responses. Transpiration is controlled by stomatal aperture, which is reduced by ABA, and altered stomatal responses of ABA-insensitive or –hypersensitive mutants to ABA are mirrored in enhanced or reduced water loss by detached leaves, respectively (Meyer et al., 1994; Pei et al., 1998). Leaves of the same age and size from fibrillin overexpression plants, anti-sense plants and control plants were tested (Figure 32). The rate of water loss was increased by 17% in fibrillin overexpression lines and reduced by 20% in fibrillin anti-sense lines as compared to the control 35S-GUS transformant line. The difference in water loss between fibrillin sense and anti-sense lines was more than 30% after one hour of leaf excision from the plants. This result also supports the notion that fibrillin anti-sense lines were hypersensitive to ABA.

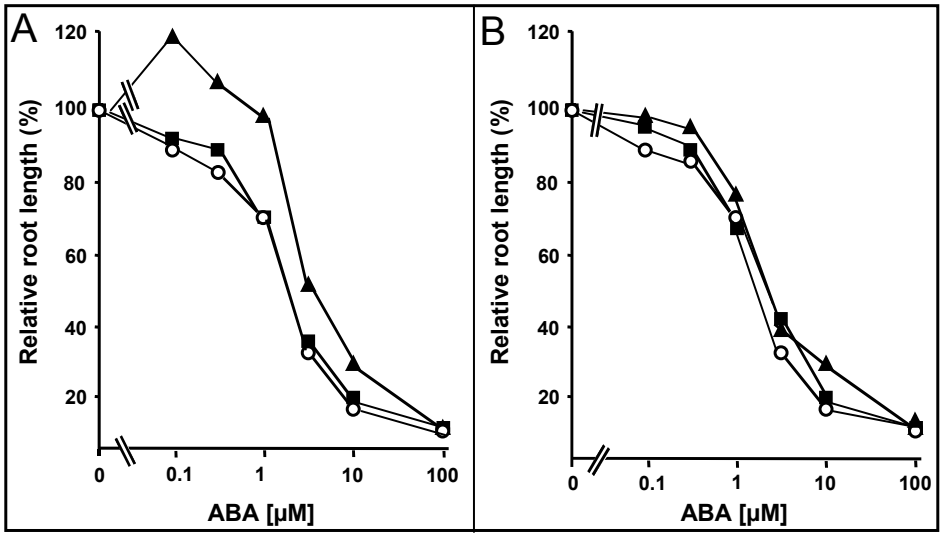


Figure 31. Root growth assay of fibrillin modulated lines.

Four-day-old seedlings grown under sterile condition were transferred on solid medium with various ABA concentrations. Root growth within 4 days after transfer was determined (n=20, SD 15%). In the absence of ABA, root growth equalled 13.7 1.8, 13.5 1.9, 9.3 2, 11.6 2.2 and 11.2 1 mm for the control, fibrillin sense 6, sense 7, anti-sense 2 and anti-sense 10 lines, respectively. The values are indicted for the control (open circles), fibrillin sense lines (closed triangles and squares in **A**) and fibrillin anti-sense lines (closed triangles and squares in **B**).

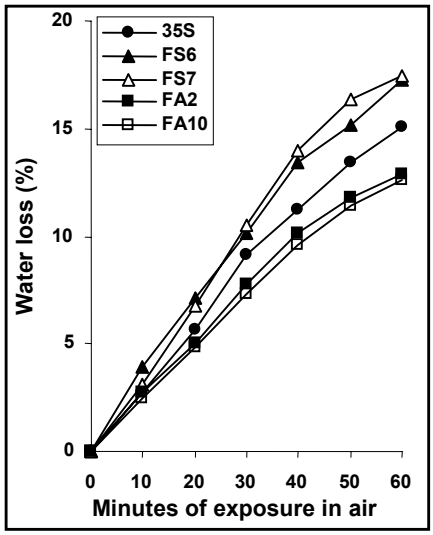


Figure 32. Water loss of leaves from fibrillin transgenic plants.

Stomatal aperture is revealed by water loss of excised leaves. Comparably developed leaves (n=6) from several 4-week-old plants were excised and the loss of the fresh weight was measured at ambient conditions. 35S: the control 35SGUS transformant; FS6, FS7: fibrillin sense lines; FA2, FA10: fibrillin anti-sense lines.

3.3.4.5 Over-expression of fibrillin in abi1, abi2 and reporter lines

Crosses between fibrillin transgenic lines and *abi1* and *abi2* mutant

Two fibrillin stable transgenic lines (FS6 and FS7) were selected for crosses to the *abi1* and *abi2* mutant. After selection of the resulting F1 generation for kanamycin resistance, there were no obvious phenotypic alterations in the F1 generation compared to the control crosses between wild-type and mutant lines.

Crossing fibrillin over-expression lines with HB6LUC reporter line

Again, the two fibrillin stable transgenic lines (FS6 and FS7) were selected for crosses to the HB6LUC reporter line. The luciferase reporter gene is under the control of the HB6 promoter which was shown to be up-regulated by ABA (Himmelbach et al., 2002). After selection of the resulting F1 generation for kanamycin resistance and later by specific PCR identification, the seeds of the F1 generation were used to assay luciferase activity. The heterozygous F_2 seeds were incubated in MS liquid culture to allow germination for 10 days. Before extracting the protein, the seedlings were treated with 30 µM ABA for 0, 4 and 24 hours or 100 mM NaCl for 0 and 24 hours. Luciferase activity was subsequently determined in protein extracts and normalized to protein content.

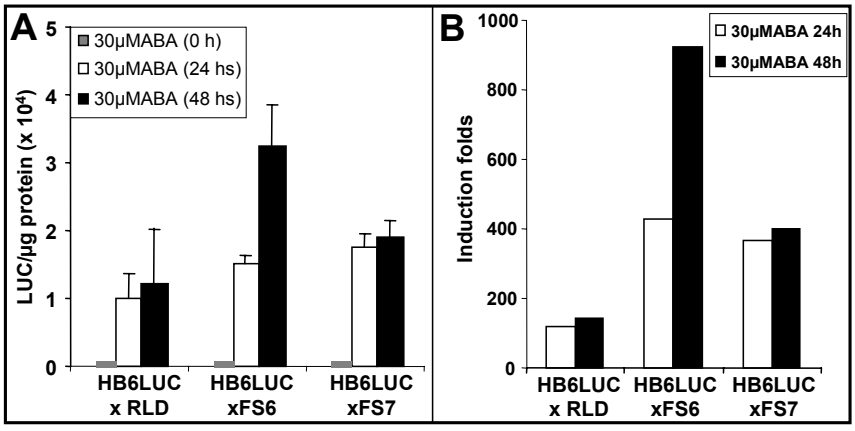


Figure 33. Effect of ectopic fibrillin expression on the ABA- and ABI1-dependent HB6LUC reporter.

Transgenic plants ectopically expressing fibrillin were crossed with the ABA- and ABI1-dependent HB6LUC reporter line. The specific LUC activity of 10-day old seedlings (n=50) in MS liquid culture was determined in protein extracts 0, 24 and 48 hours after ABA administration to the seedlings. Values are expressed as (**A**) relative luciferase activity (luciferase activity per µg of protein) or (**B**) fold induction of specific LUC activity relative to the untreated control. The standard deviation among three independent experiments is indicated. RLD: wild-type; FS6, FS7: fibrillin sense lines; HB6LUC: transgenic plants with luciferase reporter gene under the control of HB6 promoter.

Effect of the expression of reporter gene by ABA

The specific luciferase activities (Figure 33-A) show that the expression level of the HB6 reporter gene was higher in crosses with fibrillin overexpression lines than with wild-type after treatment with ABA. The induction of LUC activity by ABA was increased by a factor of 4 in fibrillin over-expression lines (HB6LUC x 35SFS6, HB6LUC x 35SFS7) after incubation with 30 µM ABA for 24 hours. After incubation with 30 µM ABA for 48 hours induction was increased by a factor of 6 in HB6LUC x 35SFS6, and by a factor of 4 in HB6LUC x 35SFS7 compared to the control line (HB6LUC x RLD) (Figure 33-B).

Effect of the expression of reporter gene by stress

As shown in Figure 34, after over-expression of fibrillin in the reporter line, the expression of the luciferase reporter gene was increased when exposed to100 mM NaCl by factors of 4 and 3 in heterozygous HB6LUC x 35SFS6 and HB6LUC x 35SFS7 plants, respectively, compared with control plants HB6LUC x RLD wild-type (Figure 34-B). In the control, the relative luciferase activity was increased from 80 to 400 (LUC/µg protein) after treatment with 100 mM NaCl. However, the relative luciferase activities in hybrids of fibrillin over-expression lines and reporter lines were increased from 35 to 600 and from 45 to 600 (LUC/µg protein) in HB6LUC x 35SFS6 and HB6LUC x 35SFS7, respectively (Figure 34-A). This difference was observed consistently in 3 independent experiments.

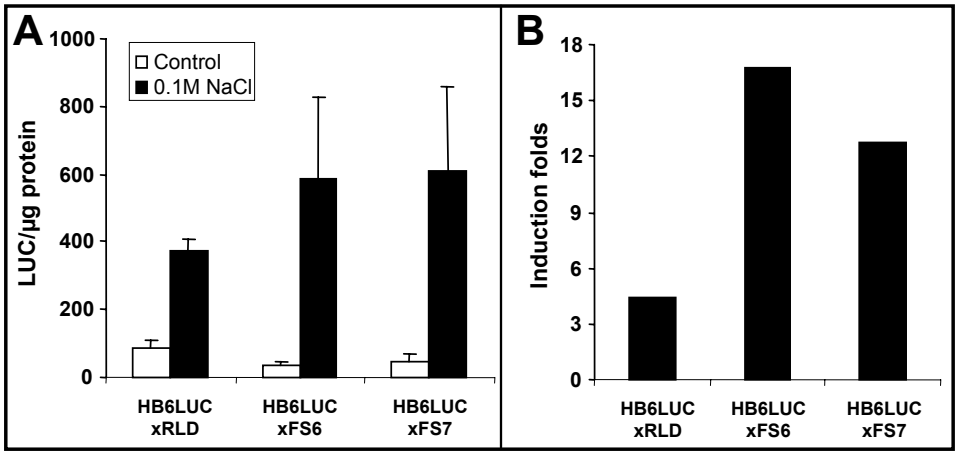


Figure 34. Expression of ABA- and ABI1-dependent HB6LUC reporter during salt stress and ectopic expression of fibrillin.

Fibrillin ectopically expressing plants were crossed with the ABA- and ABI1-dependent HB6LUC reporter lines. The specific LUC activity of 10-day old seedlings in MS liquid culture was determined from extracts 0 and 24 h after 0.1 M NaCl treatment. Values are expressed as (**A**) relative luciferase activity (luciferase activity per µg of protein) or (**B**) fold induction of specific LUC activity relative to the untreated control. The standard deviation among three independent experiments is indicated. RLD: wild-type; FS6, FS7: fibrillin sense lines; HB6LUC: transgenic plants with luciferase reporter gene driven by HB6 promoter.

3.3.4.6 Transcriptional analysis of fibrillin transgenic lines

Transcription is the first stage in gene expression and is controlled by different factors. To clarify whether over-expression and knock-out of fibrillin gene activity in transgenic plants affect their expression at the transcriptional level and whether their expression is affected by ABA, RNAs were isolated from fibrillin sense and anti-sense transgenic lines. RT-PCR, real time PCR and Northern blot analysis were used to analyse the alteration of gene expression at the transcriptional level.

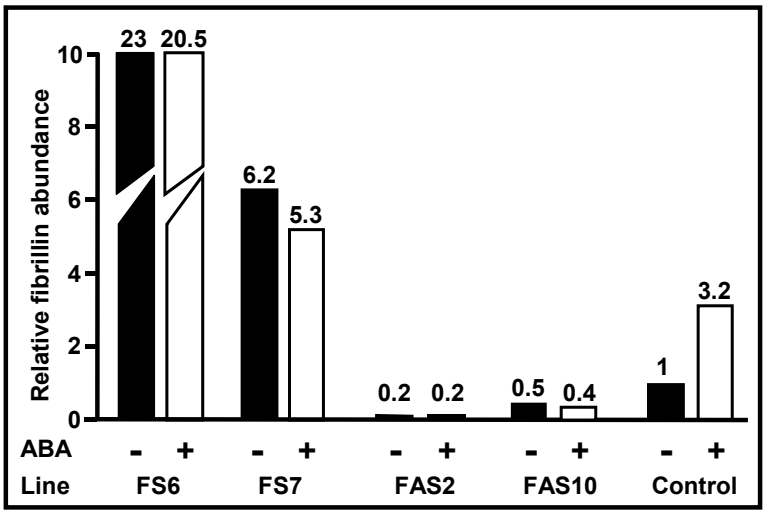


Figure 35. Analysis of the expression of the fibrillin mRNA in the fibrillin transgenic *Arabidopsis* by Real Time PCR.

Total RNAs were isolated from seedlings of fibrillin sense and anti-sense lines and control 35S-GUS treated with or without 30 µM ABA for 24 hours. First strand cDNA was synthesized by using M-MuLV reverse transcriptase. 1 µl cDNA of each was used as the template for PCR. Actin cDNA was used as internal standard. In the control without ABA treatment, normalized PCR product of fibrillin was 12.8 pg per pg actin. Repetition of the analysis confirmed the results. Fibrillin abundance in fibrillin transgenic lines is expressed in relation to the control without ABA treatment. FS6, FS7: fibrillin sense lines 6 and 7, respectively; FAS2, FAS10: fibrillin anti-sense lines 2 and 10, respectively; control: 35S-GUS tansgenic line.

3.3.4.6.1 Fibrillin transcripts in over-expression and RNA interference lines

Two independent fibrillin sense and two independent anti-sense lines were used to analyse the expression of fibrillin at the transcriptional level. Real time PCR as shown in Figure 35 indicated that the fibrillin which was normalized to the expression level of the actin gene was over-expressed in sense lines by a factor of more than 20 (in line FS6) and more than 3 (in line FS7) compared to the control line (35S-GUS transformant line). The relative products were 284 pg and 74.4 pg per pg actin in fibrillin sense line FS6 and FS7, respectively. The relative products of fibrillin in the control line was 12.8. The expression

of fibrillin was decreased in anti-sense lines by a factor of 6 and 2 in fibrillin anti-sense line FAS2 (2.8 pg/pg actin) and line FAS10 (6.3 pg/pg actin) compared to the control 35S-GUS transformant line, respectively. Repetition of the analysis gave comparable results. In a second, independent approach, the mRNA abundance was quantified by RT-PCR (Figure 36) and normalized using the product of actin as an internal control. It showed that fibrillin was over-expressed in the sense lines (FS6) by about 400 times and was decreased in the anti-sense line (FAS10) by a factor of 2.5. The results of Northern blot analysis (Figure 37) also gave a very strong hybridization signal in fibrillin sense line FS6 and a relative strong hybridization signal in sense line FS7. There was a very weak hybridization signal in the control line, but no hybridization signal in either anti-sense lines. The results from RT-PCR, real time PCR and Northern blot clearly demonstrated that fibrillin was strongly over-expressed in over-expression lines and decreased in fibrillin anti-sense lines.

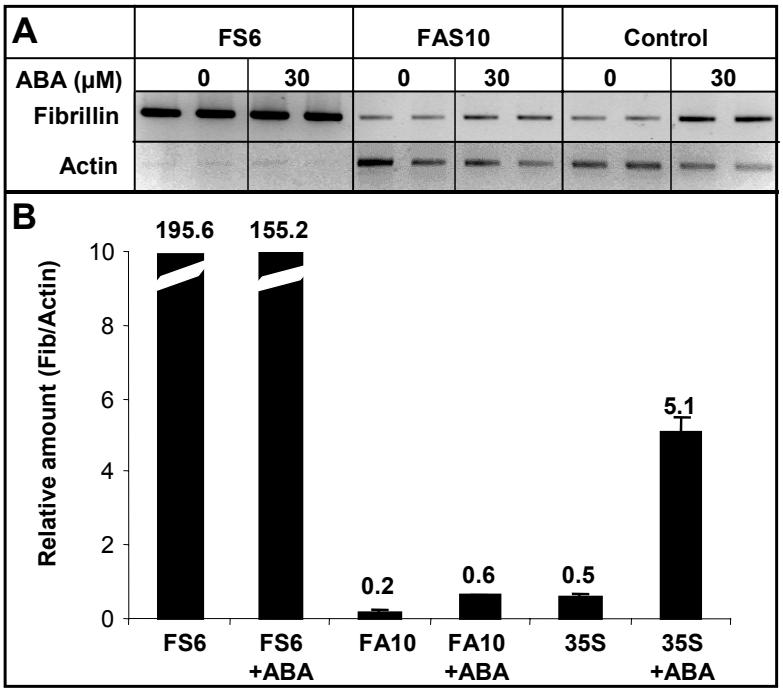


Figure 36. Ectopic expression of fibrillin in stable lines by the effect of ABA--- Transcriptional level analysis.

Total RNAs were isolated from seedlings of fibrillin sense and anti-sense lines, and control 35S-GUS plants treated with or without 30μM ABA for 24 hours. 2.5 μg total RNA of each line was used as template for reverse transcription. **A:** RT-PCR analyses were normalized to actin, two independent PCR reactions gave similar results. **B:** the quantitative analysis of the PCR signal shown in **A** performed with imaging software (Molecular Analyst, BioRad).

3.3.4.6.2 The effect of ABA on fibrillin over-expression and RNA interference lines

In the presence of 30 µM ABA, the expression of fibrillin was induced in the control line (35S-GUS transformant line), for example, by a factor of 10 in the analysis by RT-PCR (Figure 36) which is less reliable than Real Time PCR, where ABA-mediated induction was only 3 (Figure 35). The result was similar to those for wild-type plants (Figure 13) demonstrating that endogenous fibrillin is induced by ABA at the transcriptional level. However, the expression of fibrillin in over-expression lines was not affected by ABA, rather reduced to some degree. The expression of fibrillin in line FS6 was reduced by 11% and 20 % in the presence of 30 µM ABA according to the results from Real Time PCR and RT-PCR, respectively. The expression of fibrillin in sense line FS7 was also reduced by 14% after ABA treatment (Figure 35). Northern blot analysis also showed that the hybridization signal in the 30 µM ABA treatment was weaker than without ABA in both fibrillin sense lines (FS6 and FS7), whereas the hybridization signal in the control 35S-GUS transformant line exposed to ABA was clearly stronger than without ABA. The expression analysis of fibrillin in anti-sense line by Northern blot revealed the absence of any detectable hybridization signal (Figure 37).

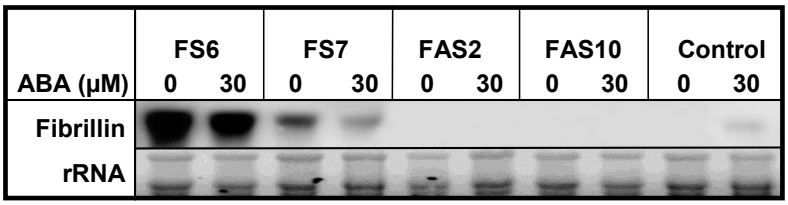


Figure 37. RNA gel blot analysis of fibrillin overexpression in fibrillin sense and anti-sense lines.

Transcript levels of fibrillin-upregulated (FS6 and FS7) and down-regulated (FAS2 and FAS10) lines in comparison to 35S::GUS transgenic control. Total RNAs were isolated from seedlings after exposure to 30µM ABA for 24 hours and fibrillin transcripts were analysed. Approximately 5µg total RNA from transgenic plants 35S::fibrillin sense and anti-sense lines were loaded. The fibrillin probe was labelled with digoxygenin-11-dUTP through PCR reaction. Ethidium bromid staining of RNA was used to indicate the RNA amount.

3.3.4.6.3 The expression of fibrillin homologous gene in transgenic lines

In the genome of *Arabidopsis* there are two fibrillin genes, the so far analyzed fibrillin (AF075598) and a second fibrillin-2 gene (AL021712) which is located on chromosome 4 and has 76% amino acid identity to fibrillin. In addition, there is a fibrillin-like protein with 48% amino acid identity to fibrillin located on chromosome 2 (AC005314) (Figure 38-A).

RT-PCR was used to investigate whether the expression of fibrillin-2 was affected by ABA or by overexpression of fibrillin in sense and anti-sense lines. The results revealed that the expression of endogenous fibrillin-2 gene was not regulated by ABA at the transcriptional level (Figure 38). In the control 35S-GUS transformant line, the PCR products for fibrillin-2 was comparably with or without ABA (0.97:1). However, expression of fibrillin was induced again as shown before by a factor of more than 3 (Figure 38-B,C). Interestingly, expression of the fibrillin-2 was not changed in fibrillin over-expression or knock-out lines and was also not changed after the transgenic lines were treated with ABA. As shown previously, the expression of fibrillin was deregulated compared to the control, however, 10 folder higher in fibrillin sense line and 2 folder lower in fibrillin anti-sense line (Figure 38-B,C).

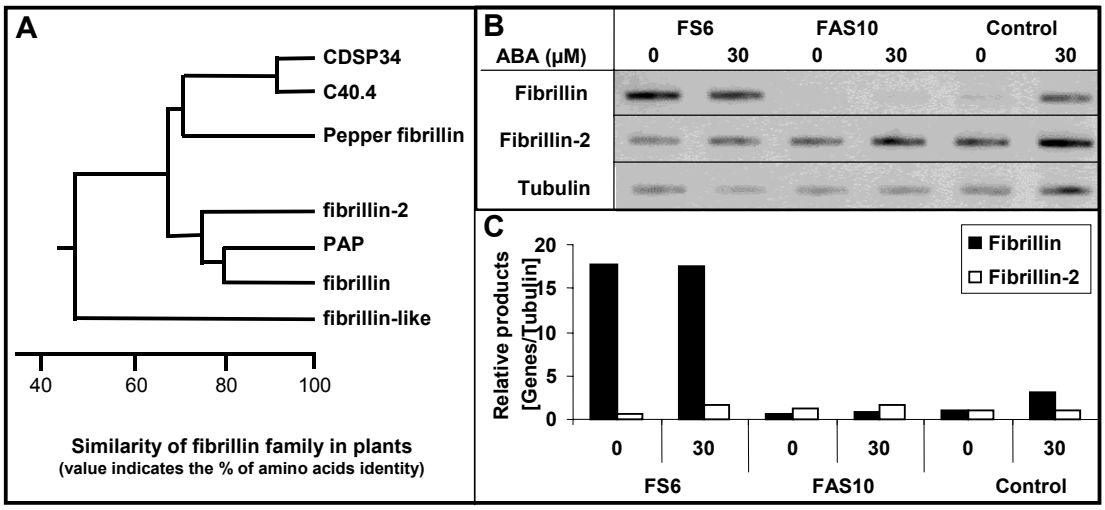


Figure 38. The effect of ABA on fibrillin homologous genes in transcriptional level.

A: Alignment tree shows the comparison of fibrillin family in plants. Of them three fibrillin-related genes are present in *Arabidopsis* and are located on chromosome 2 and 4. Fibrillin and fibrillin-2 (fibrillin precursor-like protein, chr.4) have 76% amino acid identity. Fibrillin and fibrillin-like protein have only 48% similarity. Fibrillin also shows 79%, 64%, 60% and 60% amino acid identity to PAP, C40.4, CDSP34 and pepper fibrillin, respectively. **B, C:** transcriptional analysis showed that the overexpression of fibrillin gene did not affect the expression of fibrillin-2 gene and ABA influences only the expression of fibrillin. **B:** RT-PCR products of fibrillin and fibrillin–2 were of expected sizes; **C:** quantitative analysis of PCR products shown in (**B**) by normalization to tubulin expression using imaging software Molecular Analyst (Biorad). FS6: fibrillin sense line; FAS10: fibrillin anti-sense line; control: 35S-GUS transformant line.

3.3.4.6.4 The expression of other ABA-regulated genes in fibrillin transgenic lines

Several ABA-regulated genes were selected to determine whether their expression at the transcriptional level was affected in fibrillin sense and anti-sense lines. The expression of rab18 (Lang and Palva,1992), ABI1, ABI2, HB6 (Himmelbach et al., 2002) and NCED3 (9-*cis*-epoxycarotenoid dioxygenase-3) (Iuchi et al., 2001) were not changed by either fibrillin over-expression or by down-regulation in anti-sense lines (Figure 39). Several

repetitions of this analysis showed that the crucial enzyme in the ABA biosynthesis pathway, NCED3, was up-regulated by exogenous ABA at the transcriptional level in all plant lines examined.

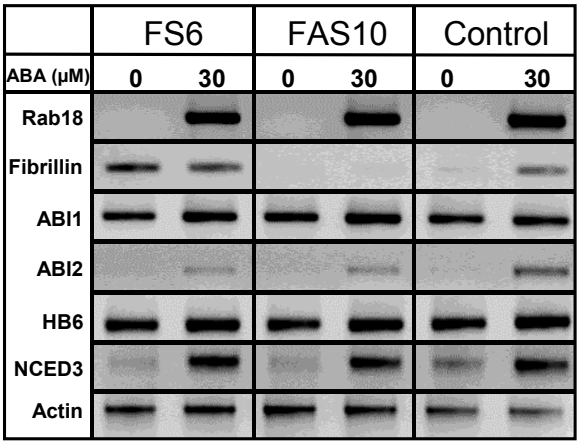


Figure 39. The expression of ABA-regulated genes in fibrillin sense and anti-sense lines at the transcriptional level.

Total RNAs were isolated from seedlings of fibrillin sense (FS6) and anti-sense (FAS10) lines and control 35S-GUS treated with or without 30 μM ABA for 24 hours. 2.5 μg RNA of each lines was used as template for reverse transcription of ABA regulated gene RAB18. RT-PCR analyses were normalized to actin abundance. ABA-regulated genes were amplified with specific primers and their products were of expected length.
FS6: fibrillin sense line; FAS10: fibrillin anti-sense line; control: 35S-GUS transformant line.

3.3.5 Cellular localization of fibrillin-GUS fusion protein

3.3.5.1 Cellular localization of fibrillin-GUS fusion protein

In order to localize the fibrillin intracellular, a fibrillin-GUS fusion protein was ectopically expressed in plant cells. Cell cultures from roots and leaves of *Arabidopsis thaliana* were incubated with *Agrobacteria* having the pPCV812::FIB-GUS construct (plasmid constructs as shown in Figure 87 and 88). To detect the expression of fibrillin-GUS fusion protein in transfected cells, two different methods were used, one with fixation of the plant cells (1% formaldehyde and 0.15 M sodium phosphate buffer, pH 7.0) and another without fixation, instead just washing with 0.1 M sodium phosphate buffer (pH7.0) before adding X-gluc. The blue product of GUS-mediated X-gluc hydrolysis was detected using the light microscope. Counterstaining nuclei with DAPI was used to identify the location of nuclear DNA. As shown in Figure 40-1-A, B, C, D the fibrillin-GUS fusion protein was detected in the cytoplasm either as intracellular plates or bands in the experiment without fixation. The plates or bands structure are reminiscent to the carotene crystals which are formed in

chromoplasts in plant cells (Braune et al., 1999). This result in view of the proposed model for fibril architecture, could indicate accumulation of carotenoids in the center of the fibrils structures which are surrounded by a layer of polar lipids, which in turn are surrounded by an outer layer of fibrillin (Deruére et al., 1994b). The expression of the fibrillin-GUS fusion protein was not observed in nuclei nor plastids but only in cytoplasm. According to the counterstaining by DAPI, there was no GUS staining in the area of nuclei. After fixation of the cells with 1 % formaldehyde, GUS staining occurred in the cytoplasm but not in the nuclear areas. The GUS staining of fibrillin-GUS fusion protein might correspond with the plastoglobules in chromoplasts. Fibrillin (renamed PAP plastid-lipid-associated protein) is associated with plastoglobules in pepper fruit chromoplasts as determined by an immunocytochemical study (Pozueta-Romero et al., 1997)**.** After adding the substrate X-gluc for several hours, the pattern of GUS staining in the experiment without fixation also changed from the plate or band forms into plastoglobules-form in the cytoplasm. The reason for this is probably that chromoplasts are not stable and disintegrate quickly in the fixation solution or in water. In the negative control (transfected by pPCV812GiGi without GUS gene) there was no GUS staining observed (Figure 40-1-E). In the positive control (transfected by pPCV812-MESHI-GUS), the cells were strongly stained in the GUS reaction, but no plate or band forms were observed in plant cells (Figure 40-1-F).

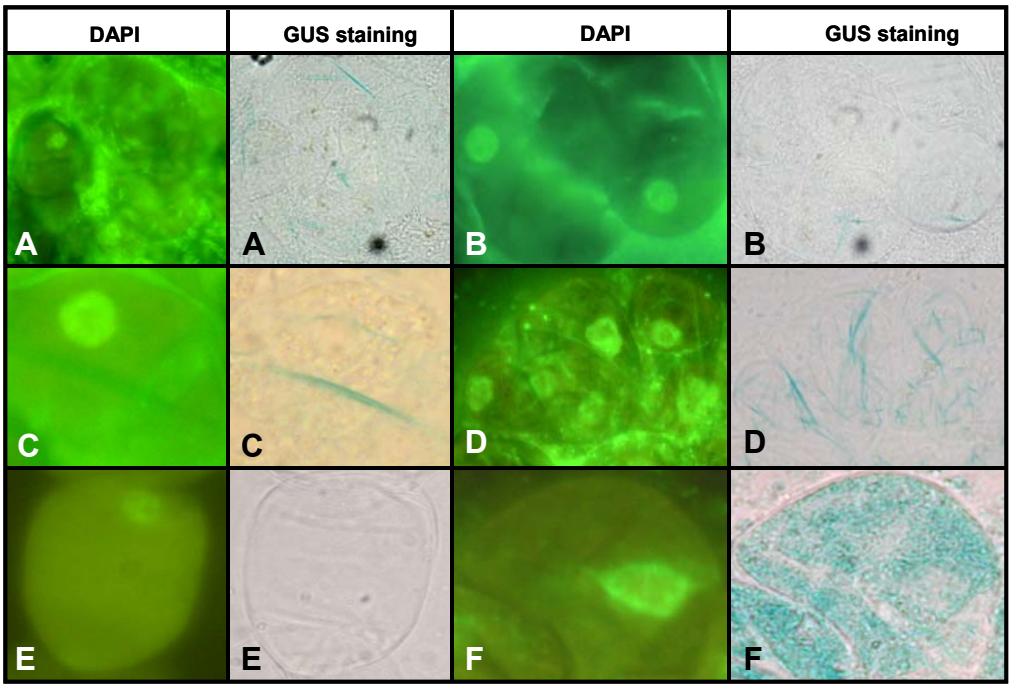


Figure 40-1. Cellular localization analysis of fibrillin using GUS histochemistry .

Cell suspension culture of *Arabidopsis* expressing transiently a fibrillin-GUS fusion protein in fibrillar-like structures. The cells were stained for GUS activity (light microscopic picture). Nucleus were counterstained by DAPI (fluorescence microscopic picture) to visulize the nucleus of plant cells; **A,B,C,D:** GUS staining (right) showed the localization of fibrillin-GUS fusion proteins (pPCVFib-GUS); **E:** negative control (pGiGi); **F:** positive control (pPCVMESHI-GUS). Arrows indicate nucleus (left panels) or fibrillar-like structures (right panels).

Sequential image layers throughout the cell depth also demonstrated that the GUS staining of fibrillin-GUS fusion protein was observed as plate or band forms in the cytoplasm (Figure 40-2).

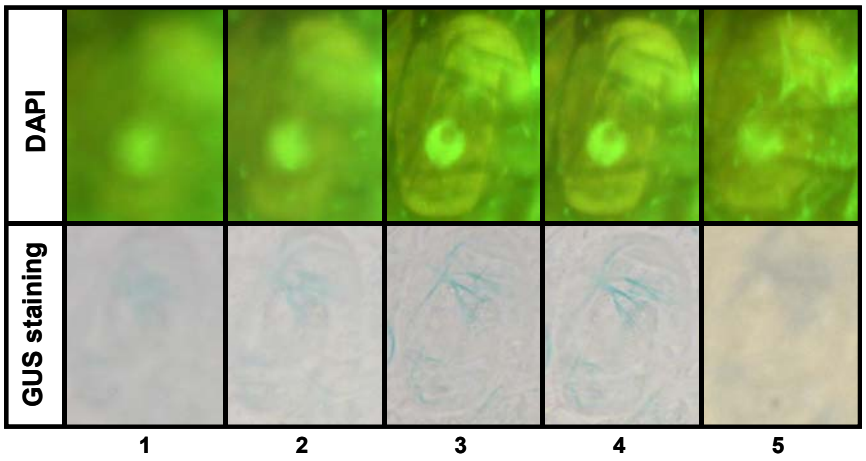


Figure 40-2. Sequential imagelayers of a single plant cell expressing fibrillin-GUS fusion proteins

Cell suspension culture of *Arabidopsis* expressing transiently a fibrillin-GUS fusion protein in fibrillar-like structures. The cells were stained for GUS activity (light microscopic picture, lower panels). Nucleus were counterstained by DAPI (fluorescence microscopic picture, upper panels; 1 to 5: serial images focusing through the cell.

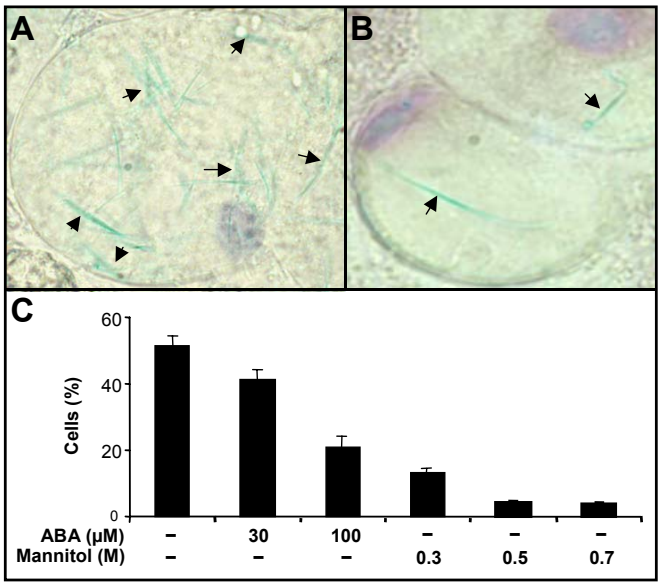


Figure 41. Fibrillar-like structures revealed by fibrillin-GUS fusion protein are regulated by ABA and osmoticum

Cell suspension culture of Arabidopsis expressed transiently a fibrillin-GUS fusion protein. **A,B**: composite pictures of single cells stained for GUS activity (light microscopic picture) and counterstained by DAPI to visualize the nucleus (fluorescence microscopic picture, purple stain) expressing fibrillar-like structures. **A:** Without treatment; **B:** Treatment with 30 µM ABA resulted in a strong decrease of the fibrillar-like structures within the cells. **C:** Fibrillar-like structures in dependence of ABA exposure and osmotic stress. The transformed cell culture was subcultured for treatments with 30 and 100 µM ABA, or 0.3, 0.5 and 0.7 M mannitol for 24h. After staining for GUS activity, samples (n = 3x 100 cells) were analysed for thepercentage of cells which had the fibrillar-like structure(s). Arrows indicate the fibrillar-like structures.

3.3.5.2 Regulation of fibrillin-GUS fusion protein by ABA and osmoticum

Further analysis revealed that fibrillin-GUS fusion proteins were regulated by ABA and osmoticum (Figure 41). After the cell suspension culture of *Arabidopsis* was treated with different ABA and mannitol concentrations (osmotic stress), the number and length of fibrillar-like structures were strongly decreased. As a result, the observable fibrillar-like structures were reduced 20% and 60% after 30 and 100 μM ABA treatments, respectively, and the fibrillar-like structures were hardly to see and reduced by a factor of 3 and 10 after treatment with 0.3 and 0.5 M mannitol, respectively (Figure 41-C).

3.3.6 Analysis of ABA content in fibrillin transgenic *Arabidopsis* lines

To determine whether the ectopic expression of fibrillin in transgenic plants affect on ABA biosynthesis, two fibrillin sense lines (FS6 and 7) and two anti-sense lines (FAS2 and 10) as well as the control 35S-GUS transformant plants were used to test their ABA content. All lines tested showed that the ABA content was about 20 to 30 pmol per gram fresh weight (FW) (Figure 42). The results indicated that there was no significant difference among fibrillin sense, anti-sense lines and the control plant. Therefore, the analysis of ABA content in fibrillin transgenic plants demonstrated that ectopic expression of fibrillin in *Arabidopsis* plant did not altered the concentration of ABA in plants, suggesting that fibrillin does not influence ABA biosynthesis in plant.

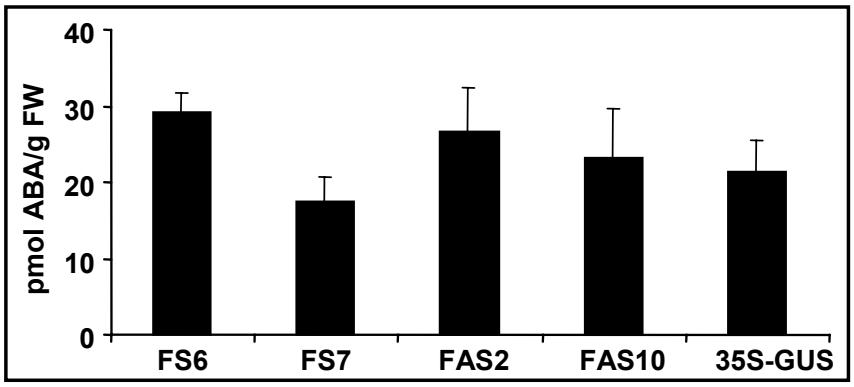


Figure 42. Analysis of ABA content in fibrillin sense and anti-sense transgenic plants.

Leaves from about 3-week old plants were excised and extracted with methanol. The liquid phase was dried in speed vaccuum and then sent to measure the ABA content (Müller A. Uni. Bochum). The results generated from three samples of individual plants (±SD). FS6 and FS7: fibrillin sense line 6 and 7; FAS2 and FAS10: fibrillin anti-sense line 2 and 10; 35S-GUS: control transformant line.

3.4 Characterization of glutamyl tRNA synthetase (AtGluRS) and its role in ABA signal transduction

3.4.1 Expression analysis of AtGluRS regulation by ABA at the transcriptional level

AtGluRS is involved in protein and porphyrins biosyntheses. Until now, there is no report related to the regulation of AtGluRS gene expression in plants. From the protein interaction results shown in 3.2.2, ΔAtGluRS interacted with ABI1 and ABI2, suggesting that AtGluRS has a role in ABA signalling. In order to study whether AtGluRS is regulated by ABA, RT-PCR and Northern analysis were employed. However, there is no obvious change in AtGluRS mRNA abundance in RLD wild-type seedlings after ABA treatment. Both time course and dose-response analysis yielded the same results (Figure 43). Thus, AtGluRS seems not to be regulated by ABA at the transcriptional level.

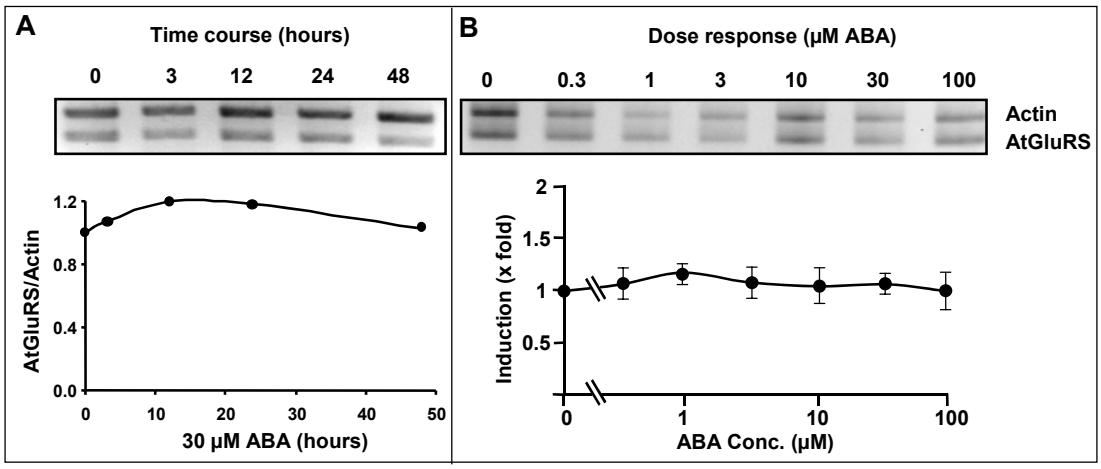


Figure 43. Level of AtGluRS transcripts influenced by ABA.

Total RNAs were isolated from sterile seedlings of *Arabidopsis* ecotype RLD cultivated in liquid medium and analysed by RT-PCR. **A: Time course.** Seedlings were treated with 30 µM ABA for 0, 3, 12, 24 and 48 hours; **B: Dose- response.** Seedlings were treated with 0, 0.3, 1, 3, 10, 30 and 100 µM ABA for 24 hours prior RNA extraction. PCR was performed with AtGluRS cDNA-specific primers and normalized for actin transcripts. Quantification was performed with imaging software Molecular Analyst (Biorad). Upper panels: DNA stained by ethidium bromide in agarose gel; Lower panels: Quantification analysis.

Northern analysis to AtGluRS expression using RNA isolated from *abi1* and *abi2* mutant lines as well as wild-type (La-*er*) was performed. The AtGluRS mRNA level was not affected by ABA in wild-type plants (Figure 44) as already shown in RLD plants. However, in the *abi1* and *abi2* mutant, the mRNA abundance of AtGluRS is lowered by at least a factor of 30 irrespective of the presence of ABA in comparison to wild-type (Figure 44). The expression level of AtGluRS gene in *abi1* was about 30-fold less than that in *abi2* at 30 µM ABA, and about 10-fold less than that in *abi2* and wild-type at 100 µM ABA (Figure

44). The data revealed AtGluRS gene expression is strictly dependent on ABI1 and ABI2 and that ABA can stimulate a recover the expression in the ABA-response mutant.

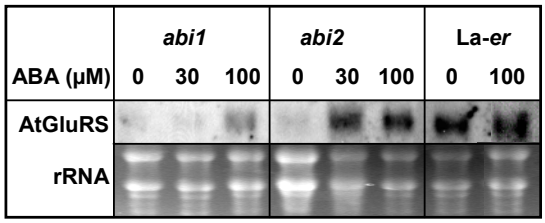


Figure 44. Dependence of AtGluRS expression on ABI1/ABI2.

Total RNA was isolated from the 10 day old seedlings of *Arabidopsis* wild-type Landsberg-*er* and *abi1*, *abi2* plants. Prior RNA isolation seedlings were treated with ABA (0, 30 and 100 µM) for 24 hours. Approximately 20 µg of RNA was loaded per lane and rRNAs were visualised by ethidium bromide staining (lower panel). Digoxigenin-labelled cDNA was employed for Northern analysis of AtGluRS transcripts (upper panel).

3.4.2 Functional analysis --- *in vitro*

3.4.2.1 Expression, purification and detection of GST-AtGluRS fusion protein

The plasmid was constructed as shown in Figure 78-B. To induce the expression of GST fusion proteins, 0.3 mM IPTG for GST-AtGluRS was added into the bacterial culture after the OD_{600} reached 0.5. Silver staining and western blotting of protein extracts from *E. coli* using mouse anti-GST antibody and anti-mouse IgG alkaline phosphatase conjugate showed that the GST-AtGluRS fusion protein was induced by adding 0.3 mM IPTG, but the temperature is crucial for expression. The best incubation temperature for the expression of GST-AtGluRS fusion protein was 25^{o}C. Full-length AtGluRS cDNA is 2,160 bp, so the predicted protein mass is approximately 79 kD. Silver staining and western blotting indicated that the GST-AtGluRS fusion protein was approximately 105 kD (Figure 45-A). The fusion protein was recovered after obtaining all the extract and affinity chromatography. Elution from glutathione sepharose 4B column using 10 mM reduced glutathione elution buffer recovered the purified protein (Figure 45-A). To determine the concentration of GST fusion proteins, silver staining with the BSA as standard showed in Figure 45-B that GST-AtGluRS fusion protein was about 150 ng/µl in eluate 1 (5 µl per lane) with a total of about 150 µg recovered protein.

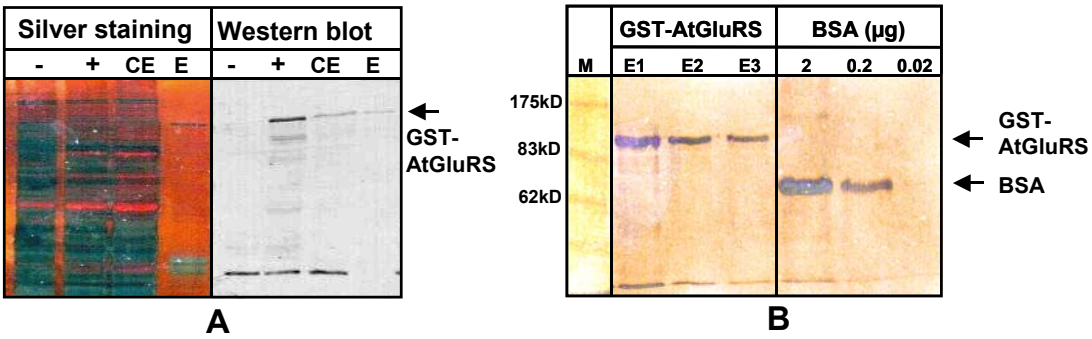


Figure 45. Purification of GST-AtGluRS fusion proteins and determination of protein concentration.

A: Silver staining(left panel) and Western blot (right panel) of protein extracts showed that the GST-AtGluRS fusion protein was induced by IPTG in *E. coli* and purified to near homogeneity. **B:** Purified GST-AtGluRS fusion protein (left panel) was analysed by SDS-PAGE together with a serial dilutions of BSA (right panel) to determine its protein concentration. GST-AtGluRS fusion protein was detected by anti-GST antibody in Western blot. M: protein marker; -: without IPTG; +: with IPTG; CE: crude extracts; E: eluate after purification; E1-E3: eluate fractions after purification; arrows indicate the GST-AtGluRS fusion protein and BSA. M: protein marker.

3.4.2.2 AtGluRS effect on protein phosphatases 2C (PP2C) enzymatic properties

ABI1 and ABI2 are two protein serine/threonine phosphatases of type 2C (EC3.1.3.16) that act as key regulators in the responses of *Arabidopsis thaliana* to ABA (Leung et al. 1994, 1997; Meyer et al. 1994; Rodriguez et al. 1998). Both PP2Cs are characterized by the strict requirement for magnesium or manganese ions for enzymatic activity. Due to the high homology of primary structures of ABI1 and ABI2 (84% identity in catalytic domain and 46% identity in N-terminal extension), their catalytic properties are comparable. In order to study the effect of AtGluRS in ABA signal transduction, ABA-binding property and PP2C enzymatic activity were employed to examine a possible regulatory role of the ABI1 and AtGluRS interaction.

3.4.2.2.1 ABA-binding properties

Interaction between ^{14}C-ABA and BSA (bovine serum albumin):

BSA was at first used as a control to test for unspecific binding of ABA by using an equilibrium dialysis equipment. As shown in Figure 46-A, ABA and test protein were separately added into the two chambers of the instrument separated by a dialyze membrane. ABA can freely diffuse through the semipermeable membrane but the proteins not, so that binding of ABA to protein could be determined by the distribution of radioactivity. In this experiment, 50 µg BSA (0.8 nmol) was added to one side of a container and 0.5 nmol ^{14}C-ABA (2.2 x 10^4 cpm) was added to the other side. The volume in both side was 500 µl, so the concentrations were 1.6 µM and 1 µM for BSA and ABA, respectively. As shown in

Figure 46-B, after incubation for several hours, the radioactivity in the chamber with BSA increased because ^{14}C-ABA diffused through the membrane. Samples were taken to measure the radioactivity at 0, 0.5, 1, 4, 7, 19 and 23 hs. The radiolabel was equally distributed after incubation for 19 hs, and ^{14}C-ABA of the side with BSA reached almost the same level (49%) as the other. If there would have been interaction between ABA and BSA, the radioactivity at the side with BSA would be significantly higher than the other. Hence, there was no binding of ABA to BSA detectable.

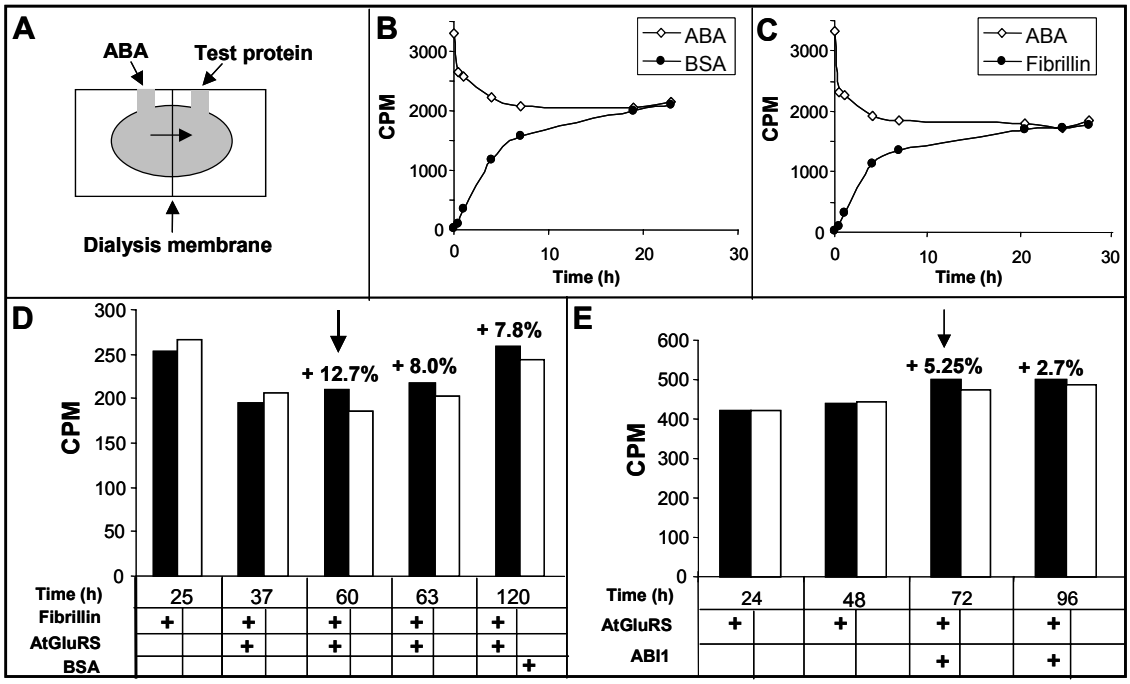


Figure 46. ABA-binding assay. ^{14}C-ABA and test proteins were analysed for binding by equilibrium dialysis (**A**). The instrument consists of two chambers separated by a dialyse membrane so that ABA can freely diffuse through it but protein not. Radioactivities in both sides were measured at different time points. **B:** Interaction between ABA (1µM) and BSA (1.6 µM); **C:** Interaction between ABA (1 µM) and fibrillin (1.6 µM); **D:** Interaction between ABA (0.2 µM) and ABI1 (1.2 µM) as well sequential addition of fibrillin (1.6 µM) at time point 25 h and AtGluRS (0.6 µM) at time point 37 h in the side of ABI1. Arrow indicated that more ABA (12.7%) diffused to the side of ABI1 after AtGluRS was added and incubated for further 23 hs. **E:** Interaction between ABA (0.2 µM) and AtGluRS (0.6 µM) as well addition of ABI1 (1.2 µM) at time point of 48 h. Arrow indicated that more ABA (5.27%) diffused to the side of proteins after incubation for further 24 hs. Solid columns: side of proteins; open columns: side of ^{14}C-ABA. Difference in pippeting the samples was less than 0.5%.

Interaction between ^{14}C-ABA and fibrillin:

Fibrillin was also served to test the interaction with ABA. ^{14}C-ABA (1 µM, 2.2 x 10^4 cpm) and BSA (1.6 µM) was added to one side of the equilibrium dialysis instrument, and then 50 µg GST-fibrillin fusion protein (1.6 µM) was added to the other side. To both sides were added glutathione elution buffer up to a volume of 500 µl. BSA was used to balance the protein content on both sides. As shown in Figure 46-C, samples were taken to measure the radioactivity at 0, 0.5, 1, 4, 7, 20.5, 24.5, and 27.5 hs. Radioactivity in the compartment

with fibrillin reached almost the same level as the side with ^{14}C-ABA after incubation for 20 hs (±10%). This situation did not change after further incubation, suggesting that there is no binding of ABA to fibrillin.

Interaction between ^{14}C-ABA and ABI1, AtGluRS, fibrillin:

In a further investigation of the influence of the interaction between ABI1, AtGluRS or fibrillin on ABA binding to proteins, ^{14}C-ABA (5600 cpm, 0.2 µM) and elution buffer for His-tag protein (300 µl) were added into one side of the container, the other side had 30 µg ABI1 protein (1.2 µM). Both sides contained 30 mM $MgCl_2$, 10 mM ATP, 1 mM DTT and 1 µM azide. After 25 hs, the radioactivity in the side with ABA was a little bit more than that in the side with ABI1 (data not shown). It was the same as the test experiments (Figure 46-B and C). Then 60 µg fibrillin (1 nmol) proteins were added to the side of the ABI1 protein. The same volume of glutathione elution buffer was added to the other side to keep the same ion concentration. After incubation for additional 12 hs, the radioactivity was 51.5% in the side with ABA and 48.5% in the container with ABI1 and fibrillin (Figure 46-D), indicative of no ABA-binding to the protein fraction.

Subsequently, 30 µg AtGluRS (0.3 nmol) was added to the side of ABI1 and fibrillin. After incubation for a further 23 hours, however, the distribution of the radioactivity between both sides changed to 47% in the side with ^{14}C-ABA and 53% in the side with ABI1, fibrillin and AtGluRS. The distribution of ABA was 12.7% in favor of the side of proteins in the presence of AtGluRS (Figure 46-D). This result indicated that about 12 pM ABA was shifted against the random distribution of ABA supporting binding of ABA to the protein fraction on a stochiometric basis of 1:1 the redistributed ABA corresponds to 2% ABA-bond AtGluRS. After another 3 hours, two samples from each side were taken and measured. The ratio did not change and remained at about 48% of radioactivity in the side with ^{14}C-ABA and 52% in the side with ABI1, fibrillin and AtGluRS (Figure 46-D). The same protein amount was added by addition of BSA to the side of ABA to balance the protein level of both sides. The radioactivity ratio did not change after the incubation for further 53 hours. ABA level remained 7.8% higher in the side of AtGluRS than in the side of BSA (Figure 46-D). The phenomenon indicated that ABA perhaps bind to AtGluRS or protein complex and the binding capacity of AtGluRS or protein complex to ABA was reduced as the duration of incubation. Aliquots from both sides were tested for microbial contamination irrespective of the presence of azide in the buffer solution. After 4 day incubation at room temperature, there were indeed no colonies formed on LB plates. Thus, the change in the ratio of radioactivity between the two sides of the membrane seems to reflect ABA binding to protein. The results above suggested that AtGluRS probably affects ABA binding to protein. This effect may reflect interaction of ABI1, AtGluRS and ABA, or AtGluRS itself may bind ABA.

To determine the effect on the ABA-binding to AtGluRS or to protein complex, another experiment was employed to discern the possibility. In this experiment, AtGluRS (0.6 μM) was at first added to one side of the container and another side was ABA (0.2 μM). After incubation for 24 and 48 hs, no differences were detected (Figure 46-E). Then ABI1 (1.2 μM) was added to the protein side. After incubation for a further 24 hs, it showed that the radioactivity in the side with proteins was 5.25% more than that in the side with ABA (Figure 46-E). After incubation for 4 days, there was also a detectable difference with more radioactivity in the side with proteins (Figure 46-E). Besides, one supplemental experiment also demonstrated that more ABA diffused to the side with ABI1 and AtGluRS, whereas, no difference was detected if ABI1 and AtGluRS were separately added into different side of the container (data not shown). From above, it suggested that ABA perhaps binds to the complex of ABI1 and AtGluRS.

3.4.2.2.2 PP2C enzymatic activity

The activation of protein phosphatase 2C is dependent on Mg^{2+} or Mn^{2+} and pH (Leube et al. 1998; Rodriguez et al. 1998; Meinhard et al. 2002). In this work, ABI1 protein was used to study the reactivation of PP2C enzymatic activity in the presence of fibrillin and AtGluRS and methylumbelliferyl phosphate (MUP) was used as substrate. The reaction is preferentially Mn^{2+} dependent (Meinhard et al., 2002). The principle of the assay is the exchange of Mg^{2+} by Mn^{2+} in the catalytic domain thereby activating ABI1. Then the dephosphorylation of MUP by ABI1 resulted in the formation of fluorescent MU (Figure 47-A).

Because purified fibrillin and AtGluRS were fused to glutathione S-transferase (GST), GST is used as the control in this assay. Incubation of 50 ng ABI1 (0.1 μM) with buffer or buffer containing 250 ng of GST (1 μM), 600 ng of fibrillin (1 μM), 200 ng of AtGluRS (0.2 μM) or both fibrillin (1 μM) and AtGluRS (0.2 μM) fusion proteins did not reveal any significant changes in PP2C enzymatic activity in the presence of 5 mM $MnCl_2$ (Figure 47-B). After treatment with 30 μM ABA, the PP2C activity was reduced 17.5% compared to the results without ABA treatment (Figure 47-B). Several independent repetitions yielded reproductive result. Interestingly, in another experiment with 1 mM $MnCl_2$ differences of ABI1 activity was detected in the presence of AtGluRS fusion protein. The enzymatic activity of ABI1 was reduced by factor 5.8 in the presence of AtGluRS, and after treatment with 30 μM ABA, the activity was reduced 4 times compared to the control and incubations with other fusion proteins (Figure 48). It seems that fibrillin and ABA had no or no profound effect on the activation of ABI1, so further investigation focused on the relationship between ABI1 and AtGluRS was carried out.

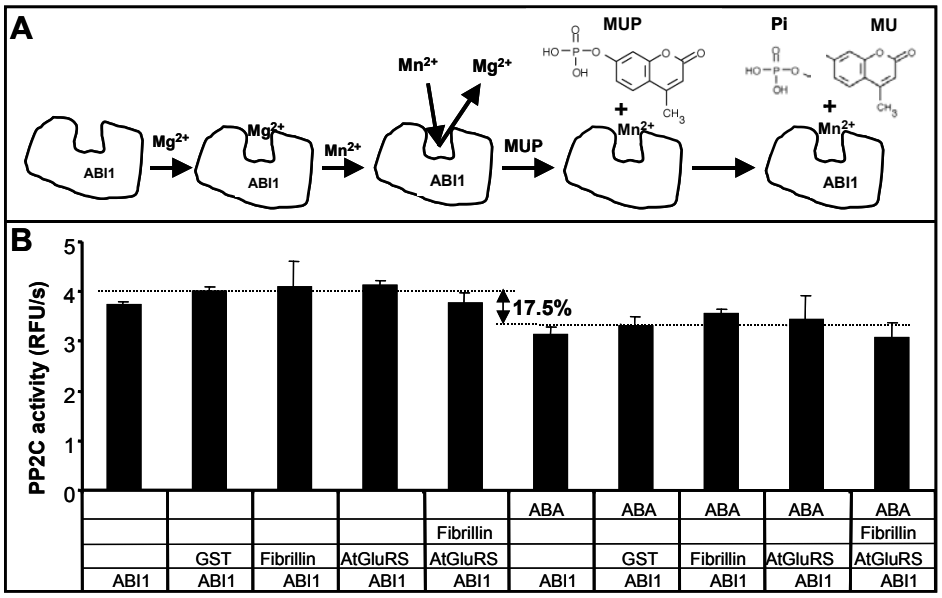


Figure 47. PP2C enzymatic assay with MUP as substrate.

ABI1 (0.1 µM) protein was used to assay the enzymatic activity of the protein phosphatases 2C (PP2C) with methylumbelliferyl phosphate (MUP, 1 mM) as substrate and Mn^{2+} (5 mM) as the activating ion for ABI1. **A:** The principle of the assay is the exchange of bound Mg^{2+} by Mn^{2+} in ABI1 at first and then the Mn^{2+} -dependent dephosphorylation of MUP by ABI1; **B:** PP2C enzymatic assay was carried with 5 mM of Mn^{2+} with or without 30 µM ABA. GST (1 µM), fibrillin (1 µM) and AtGluRS (0.2 µM) were used as effectors. Data are means ± SD (n=3). Dashed lines represent the difference of PP2C activity after subjected to ABA.

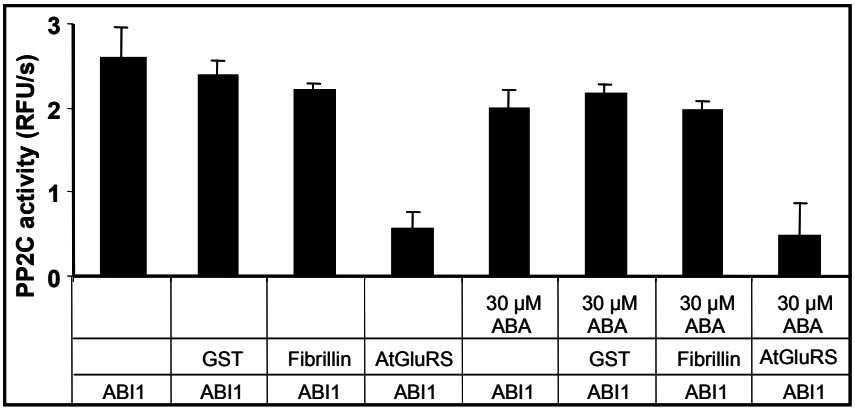


Figure 48. Inactivation of Arabidopsis ABI1 by AtGluRS(I).

The enzymatic activity of ABI1 was measured in the presence of different proteins and 30 µM ABA with 1 mM MUP as substrate at 1 mM of Mn^{2+}. Protein concentrations were as same as indicated in Figure 47. Data are means ± SD (n=3).

To study the effect of AtGluRS on the activation of ABI1, several control experiments were performed. The PP2C activity did not change significantly after incubation of ABI1 with GST, elution buffer for GST protein (GEB buffer) or protease inhibitor (Figure 49). However, PP2C activity was reduced by a factor of 3 (Figure 49-B) to 10 (Figure 49-D) in

the presence of AtGluRS. In contrast, AtGluRS heated at 95°C for 10 min had no apparent effect on ABI1 activity. Thus, only functional AtGluRS was able to reduce the activity of PP2C.

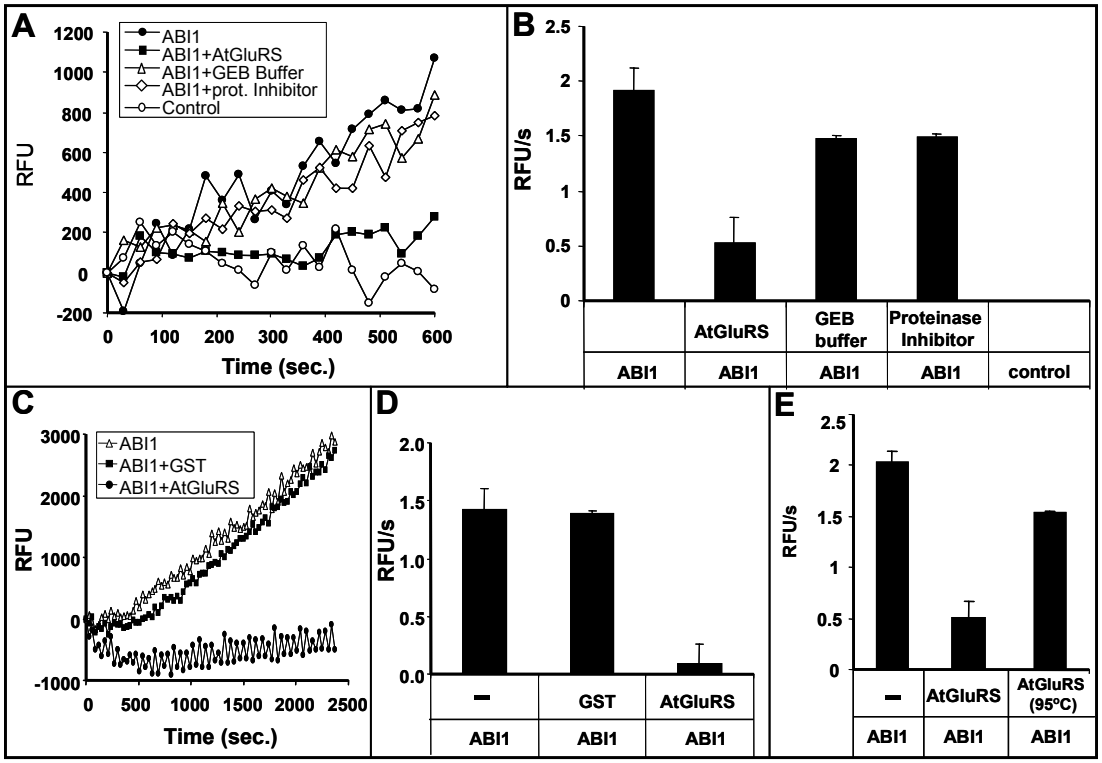


Figure 49. Inactivation of Arabidopsis ABI1 by AtGluRS (II).

The enzymatic activity of ABI1 was measured in the presence of AtGluRS as well as other potential factors. **A, B:** PP2C enzymatic activity of ABI1 influenced by AtGluRS, elution buffer for GST protein (GEB buffer) or protein inhibitor; **C, D:** PP2C enzymatic activity of ABI1 in the presence of GST or AtGluRS; **E:** Comparison of PP2C enzymatic activity of ABI1 in the presence of AtGluRS or AtGluRS heated by 95°C. Data are means ± SD (n=3). **A, C:** time courses of **B** and **D**, respectively; **B, D, E:** 10 minute preincubation and 10 minute mesurement for **B** and 40 minutes for **D** and **E**. 1 mM MUP was used as substrate and 1 mM Mn^{2+} as metal ion. Protein concentrations were as same as in Figure 47.

Because the activation of protein phosphatases requires manganese ions, different manganese concentrations were used to investigate the enzymatic activity of ABI1 in the presence of AtGluRS. Results indicated that the enzymatic activity of ABI1 was reduced by more than a factor of 3 at manganese concentrations lower than 1 mM, for example, by a factor of 3, 4 and 6 at 1, 0.5 and 0.1 mM manganese ion concentrations, respectively (Figure 50-A). Whereas, there was no any alteration of PP2C activity if the concentration of manganese was greater than 2 mM (Figure 50-A). This result corresponded with the results shown in Figures 47, 48 and 49.

A key factor affecting the rate of a reaction catalyzed by an enzyme is the substrate concentration. To clarify the effect of substrate concentration on the PP2C activation, different MUP concentrations were employed to study the inhibition of the activation of

ABI1 by AtGluRS in more detail. First, the optimal Mn^{2+} concentration for observing the AtGluRS effect on ABI1 was determined (Figure 50-A). For subsequent analysis, 1 mM Mn^{2+} was used. As shown in Figure 50-B, the enzymatic activity of ABI1 was greatly inhibited in the presence of AtGluRS. The Michaelis constant (K_m) was about 0.7 mM of MUP for ABI1, but the K_m for ABI1 with AtGluRS was much higher (more than 2 mM). The Lineweaver-Burk plot also showed that AtGluRS was an inhibitor of the enzymatic activation of ABI1 (Figure 50-C).

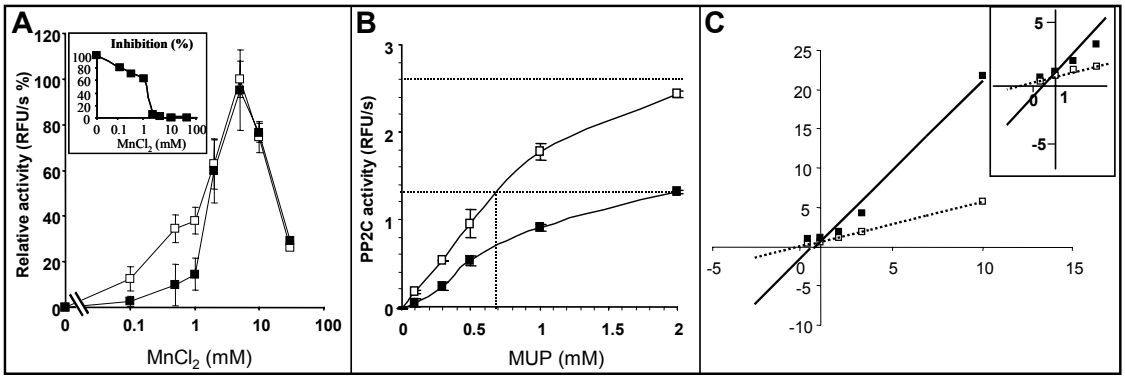


Figure 50. Inactivation of *Arabidopsis* ABI1 by AtGluRS (III).

The enzymatic activity of ABI1 was measured in the presence of AtGluRS at different Mn^{2+} concentrations (**A**) and substrate (MUP) concentrations (**B**). **C:** Lineweaver-Burk presentation of data shown in (**B**). Substrate MUP was 1 mM in (**A**) and Mn^{2+} was 1 mM in (**B**). Small figure in (**A**) shows the inhibition (%) of PP2C activity by AtGluRS. The activity of PP2C indicated in (**A**) is generated from three independent experiments and represented in relative activity (RFU/s %). The experiment was repeated three times with different preparation of ABI1 and AtGluRS used here. Data are means ± SD (n=3). Reaction mixtures were preincubated for 10 minutes. 0.1 µM ABI1 and 0.2 µM AtGluRS were used for each reaction. ABI1: open squares; ABI1+AtGluRS: closed squares.

Alternatively, phosphocasein was also employed as a substrate in this work to investigate the activation of ABI1 in the presence of AtGluRS.

Casein was labeled by γ–^{33}P ATP according to Leube et al (1998). Partially dephosphorylated casein (Sigma) was used for labeling with γ–^{33}P ATP, and protein kinase A in the presence of 10 mM Mg^{2+}. After gel infiltration to remove unincorporated ATP, fractions were collected and measured by scintillation counting. The labeling efficiency for phosphocasein of approximately 8% total radioactivity (100 µCi) was achieved (Figure 51-A). To determine the background contamination of free Pi in the casein fraction, 20% TCA was used to precipitate the protein. The results showed that the free phosphate or non-precipitable phosphopeptide level was only about 0.3% of total radiolabel (Figure 51-B). Addition of BSA prior precipitation of the phosphocasein did not improve the recovery (Figure 51-B).

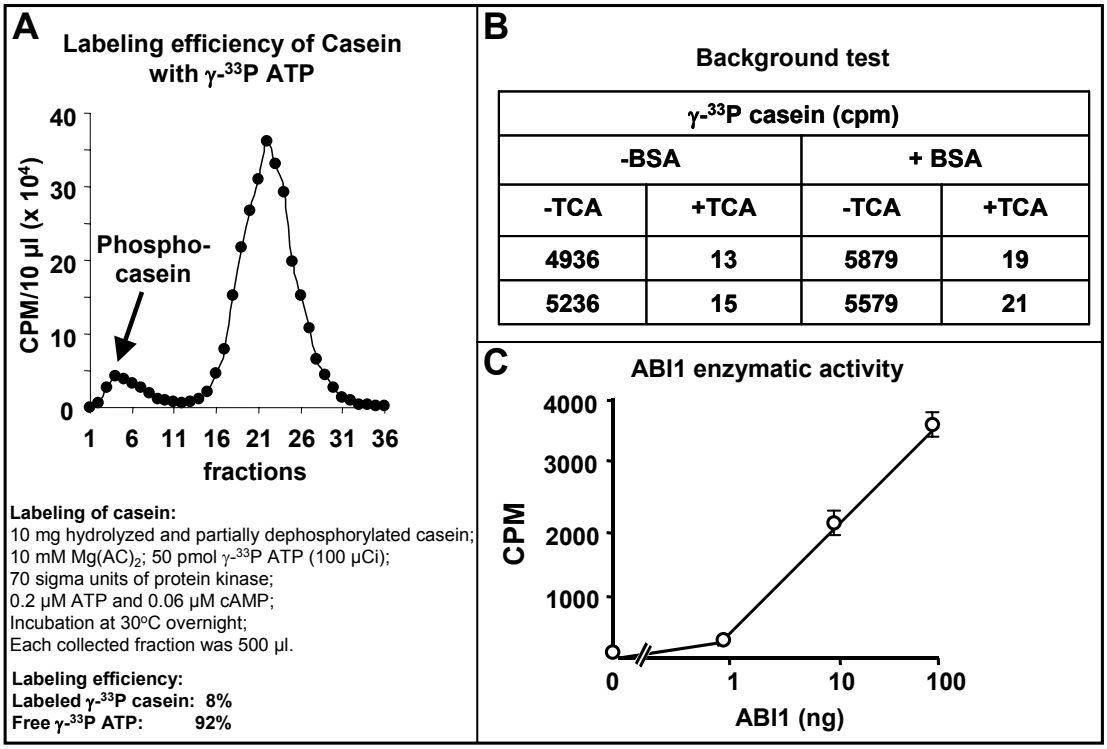


Figure 51. Labelling of casein with γ-^{33}P ATP and protein phosphatase activity test.

A: Reaction mixture for labeling of casein with γ-^{33}P ATP as indicated. Gel infiltration was used to remove the free ATP. 10 µl of each fractions were measured by scintillation counting and the labeling efficiency was determined according to ratio of the amount of labeled casein to total amount of γ-^{33}P ATP. Arrow indicates the peak of labeled casein; **B:** determination of the background level of free P_i after precipitation of γ-^{33}P ATP labeled casein with 20% TCA. BSA (10 µg) was used to improve the precipitation of the casein. The value indicates the amount of cpm measured in the supernatant of aliquote taken; **C:** pilot experiment of ABI1 enzymatic activity in the presence of 10 mM Mg^{2+}.

With casein as substrate, the dependence of ABI1 and ABI2 activation on Mg^{2+} yielded a bell-shaped curve, with half-maximal activity in the presence of approximately 1 mM Mg^{2+} and maximal activity at 10 mM Mg^{2+} (Meinhard et al. 2002; Leube et al. 1998). In order to assay the ability of ABI1 to dephosphorylate casein, phosphocasein (1.2 x 10^4 cpm) were incubated with 1, 10 or 100 ng of ABI1 in the presence of 10 mM Mg^{2+} at pH 7.5. The results showed that 1 ng of ABI1 dephosphorylated casein to 2.3% (273 out of 1.2 x 10^4 cpm), 10 ng and 100 ng of ABI1 dephosphorylated 14.6% (1757 out of 1.2 x 10^4 cpm) and to 27.9% (3345 out of 1.2 x 10^4 cpm) in 15 min, respectively (Figure 51-C, n=3). Based on these results, 30 ng of ABI1 was selected for further activation studies of ABI1 in the presence of AtGluRS.

To investigate the effect of AtGluRS on the activation of ABI1 to dephosphorylate phosphocasein, 1 mM and 10 mM of Mg^{2+} were used to detect a possible difference of the enzymatic activity of ABI1 in the presence of AtGluRS. With MUP as substrate, the

activation of ABI1 depended on Mn^{2+} concentration, and the difference between ABI1 and ABI1 inhibited by AtGluRS was detected at low Mn^{2+} concentration. With casein as substrate, however, no difference was observed in the activity to dephosphorylate phosphocasein of ABI1 with or without AtGluRS after pre-incubation of the mixture with 1 and 10 mM Mg^{2+} (Figure 52-A). After pre-incubation of ABI1 and AtGluRS without Mg^{2+}, however, the ability of ABI1 to dephosphorylate phosphocasein was inhibited by AtGluRS in both 1 mM and 10 mM Mg^{2+} by factor 2 in 7.5 minutes (Figure 52-B). In the presence of different concentration of Mg^{2+}, the inhibition of ABI1 activation by AtGluRS was confirmed (Figure 52-C). After AtGluRS protein was denatured by heating at 95°C for 10 minutes, there was no effect of AtGluRS on the activity of ABI1. The results indicated that AtGluRS induced interferences with ABI1.

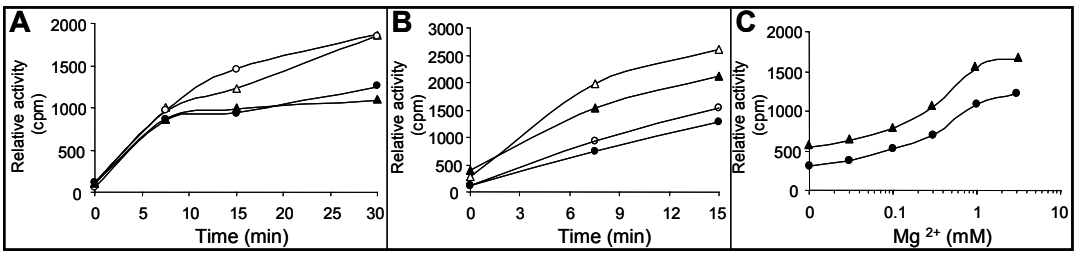


Figure 52. Inactivation of Arabidopsis ABI1 by AtGluRS with phosphocasein as substrate.

Enzymatic activity of ABI1 (0.06 µM) was measured with (**A**) pre-incubation with 1 mM and 10 mM Mg^{2+} and (**B**) preincubation without Mg^{2+}; ABI1 in 1 mM Mg^{2+} (closed triangles) and 10 mM Mg^{2+}(open triangles); ABI1 with AtGluRS (0.1 µM) in 1 mM Mg^{2+} (closed circles) and 10 mM Mg^{2+} (open circles); enzymatic activity of ABI1 was also measured at dfferent Mg^{2+} concentrations (**C**). ABI1 (closed triangles), ABI1 with AtGluRS (closed circles).

3.4.3 Functional analysis-*in vivo*

3.4.3.1 Regulation of AtGluRS promoter by ABA in Arabidopsis thaliana

Up to now, there is no study about the relationship between the AtGluRS and ABA signal transduction. From the results presented in 3.2.2, it can be concluded that there is a strong interaction between AtGluRS and ABI1 or abi1. To analyse the function of AtGluRS in ABA signal transduction, the AtGluRS gene was isolated from an Arabidopsis genomic cosmid library (Figure 83). Subsequently, its promoter was fused to GUS and luciferase reporter genes (Appendix, Figure 84). These constructs were first used to study the expression of the AtGluRS promoter in protoplasts of *Arabidopsis thaliana*.

After cotransfection of protoplasts with the reporter plasmid AtGluRS promoter-LUC (20 µg) and 35S-GUS plasmids (50 µg), the luciferase activity driven by the AtGluRS promoter was determined in the presence of increasing ABA concentrations (Figure 53). In the presence of 30 µM ABA, the luciferase expression was increased by 3 fold. Further

experiments revealed that the relative LUC activity was induced when either the protoplasts (Figure 54-A) or plants prior to protoplast isolation (Figure 54-B) were treated with ABA (30 µM) (normalized to 35S-GUS activity). The results were generated in three independent transfection experiments.

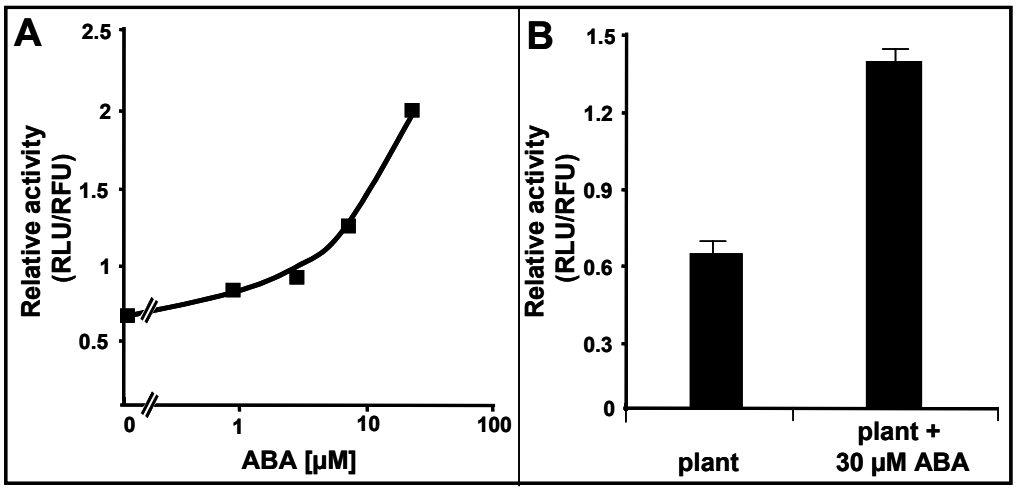


Figure 53. Regulation of AtGluRS promoter activity by ABA in protoplasts.

A.B: ABA-dependent activation of the AtGluRS promoter driving luciferase expression. *Arabidopsis* protoplasts were transfected with the reporter construct and a constitutively expressed β-glucuronidase gene. The protoplasts were either treated with different ABA concentrations for 24 h after transfection **(A)** or the plants had been ABA-treated (24h) prior protoplast isolation **(B)**. 20 µg of reporter construct and 50 µg of 35S-GUS construct were used for cotransfection. The luciferase activity was normalized to GUS expression. The data represent the mean of value from three different cotransfections (± SD).

An interesting phenomenon of this study is shown in Figure 54. Transient expression of AtGluRS promoter with luciferase influenced the expression of the 35S-GUS expression. Several experiments revealed that the activity of 35S constitutive promoter driven reporter gene was greatly increased in the presence of the AtGluRS promoter in transient expression analysis using the protoplast system. In Figure 55-A, protoplasts were isolated from plants which were pretreated with 30 µM ABA. 35S-GUS plasmids (50 µg) and GPR-LUC, FPR-LUC or 35S-LUC (20 µg) were used to co-transfect the protoplasts. GUS activity was induced in the presence of the AtGluRS promoter as much as 6 fold when cotransfected with FPR-LUC and 20 fold when cotransfected with 35S-LUC from three independent transfections. The same results were obtained after cotransfected protoplasts were treated with 0, 1, 3, 10 or 30 µM ABA overnight. Data from three independent experiments documented the stimulation of GUS activity by 20-30 folds in the presence of GPR-LUC in comparison to the presence of FPR-LUC or 35S-LUC (Figure 54-B). In order to confirm this amazing phenomenon, 35S-LUC plasmids were used instead of 35S-GUS to

co-transfect protoplasts with GPR-GUS constructs. In addition, the protoplasts were isolated from wild-type (RLD), *abi1* and *abi2* mutants, and either plant were treated with 30 µM ABA before protoplast isolation or the protoplasts were exposed to the plant hormone after cotransfection. Figures 55-A and -B show that the expression of the luciferase reporter gene was also dramatically induced in the presence of the AtGluRS promoter in protoplasts which were isolated from wild-type or *abi1* plants pretreated with 30 µM ABA. The same response was observed with expression of the luciferase reporter gene in wild-type, *abi1* and *abi2* protoplasts treated with 30 µM ABA after cotransfection (Figure 55-C, -D and -E). The expression of the luciferase gene under the control of constitutive 35S promoter was more than 200 fold higher in the presence of the AtGluRS promoter. The results stem from three independent co-transfections and suggest a positive role of regulatory control elements present on the AtGluRS promoter on the activity of viral 35S promoter.

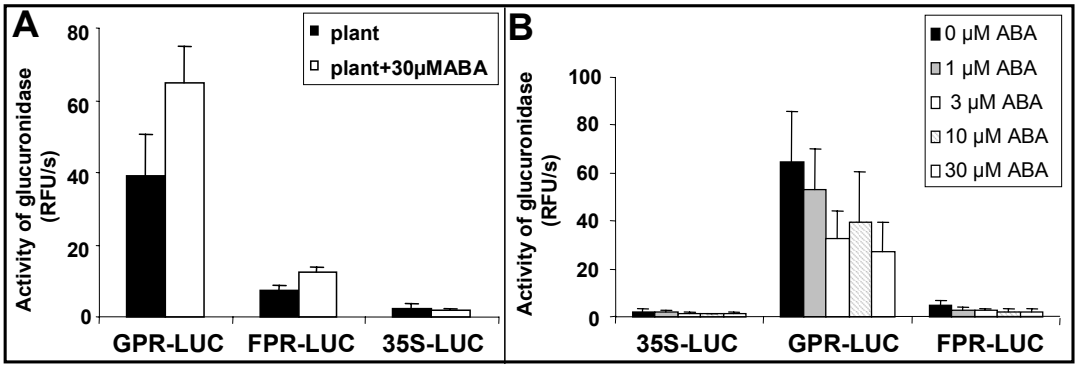


Figure 54. Expression level of the constitutive 35S promoter-GUS reporter gene influenced by AtGluRS promoter in protoplasts (I).

35S promoter expression construct driving β-glucuronidase was co-transfected with 20 µg of GPR-LUC, FPR-LUC or 35S-LUC plasmids. GUS activity (RFU/s) was induced by the AtGluRS promoter reporter constructs. **A:** plants were treated with 30 µM ABA for one days before the isolation of protoplasts. **B:** protoplasts were treated with different ABA concentration overnight. GPR-LUC: luciferase reporter gene under the control of AtGluRS promoter; FPR-LUC: luciferase reporter gene under the control of fibrillin promoter; 35S-LUC: luciferase reporter gene under the control of constitutive 35S promoter. 35S-GUS: β-glucuronidase reporter gene under the control of the constitutive 35S promoter.

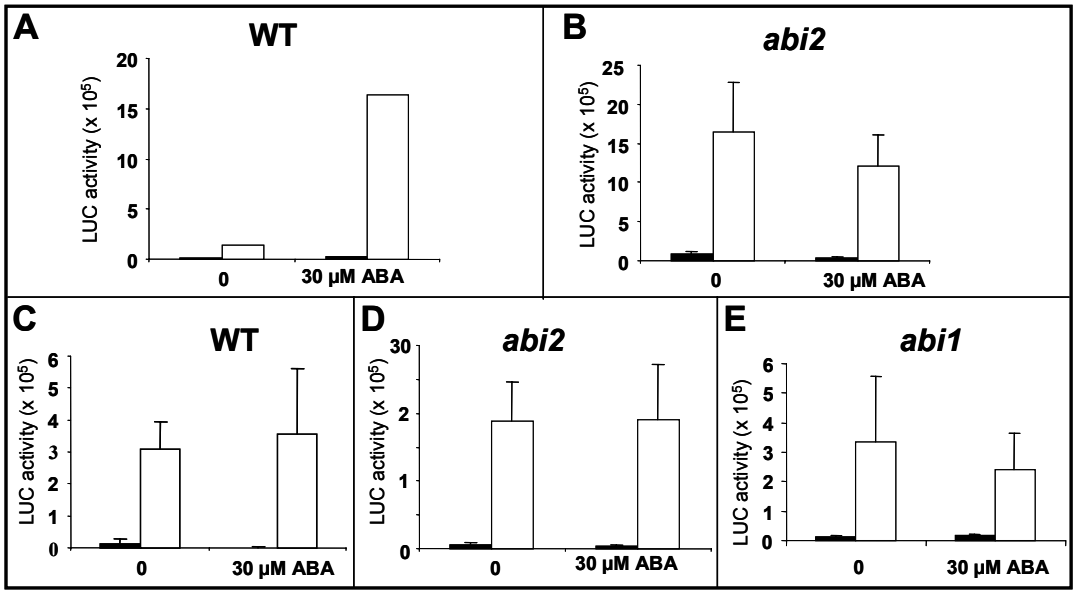


Figure 55. Expression level of constitutive 35S promoter influenced by AtGluRS promoter in protoplasts (II).

The reporter construct consisting of the constitutive 35S promoter driving luciferase gene was co-transfected with AtGluRS promoter (20 µg) driving β-glucuronidase. LUC activity was determined in dependence of the presence of AtGluRS promoter construct. **A-B:** plants were treated with 30 µM ABA for one day before isolation of protoplasts; **C-E:** protoplasts were treated with 30 µM ABA overnight. Closed columns: control, transfection with 35S-LUC constructs: Open columns: cotransfection with 20 µg of GPR-GUS plasmids. WT: wild-type (La-*er*); *abi1* and *abi2*: mutant plants

3.4.3.2 Transient expression of AtGluRS: Influence on ABA signal transduction

An efficient method to study the signal transduction pathway (Downward, 2001) is over-expression of specific proteins and subsequent analysis of possible effects. Protein interaction analysis in the yeast demonstrated that there was a strong protein-protein interaction between ABI1 and AtGluRS. To determine the effect of AtGluRS on ABA signal transduction, AtGluRS was overexpressed under the control of the CaMV 35S promoter in *Arabidopsis* protoplasts. The vector used in this work was pBI221 (Jefferson, 1987). The constructs for protoplast expression are illustrated in Appendix (Figure 86).

The results obtained indicated that AtGluRS might be a negative regulator of the ABA signal transduction pathway. As shown in Figure 56, the ectopic expression of the effector AtGluRS inhibited the expression of the two ABA-regulated reporter genes, rab18-LUC normalized by aequorin and Lti65-LUC normalized by GUS. The expression of the reporter gene rab18LUC was inhibited by a factor of more than 6 at 0.3 µM ABA and a factor of 2

at 3 µM ABA after cotransfection with AtGluRS (20 µg) (Figure 56-A). At 3 and 10 µM ABA concentrations, the expression of Lti65LUC reporter gene was reduced by a factor of 2 and 1.7 after cotransfection with AtGluRS (20 µg) compared to the expression level without the effector, respectively (Figure 56-B).

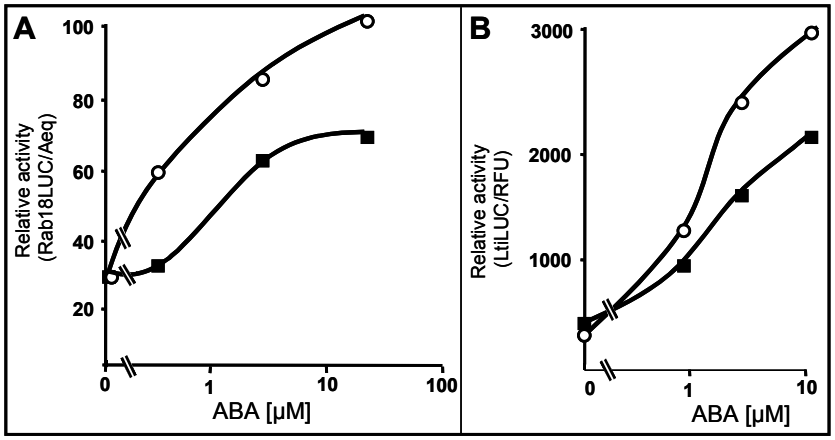


Figure 56. Inhibition of ABA-responsive promoters by AtGluRS in transient expression system.

Expression of the luciferase reporter gene driven by ABA-regulated promoters in the presence of AtGluRS. AtGluRS expressing plasmid (20 µg) as effector in the regulation of **(A)** Rab18-LUC which was normalized by aequorin (LUC/Aeq) and (**B)** Lti65-LUC that was normalized by 35S-GUS (RLU/RFU) in *Arabidopsis* protoplasts. Protoplasts were treated with different concentrations of ABA. The value are indicated for the control (open circles) and ectopic AtGluRS expression (closed squares). Three experimental repetitions yielded comparable results.

3.4.4 Analysis of stable transgenic plants

3.4.4.1 Ectopic expression of AtGluRS in Arabidopsis

To analyse the effect of ectopic AtGluRS expression in plants, AtGluRS sense and anti-sense constructs were cloned into the binary vector pBI121 (Jefferson, 1987) under the control of the 35S promoter to generate transgenic plants (see Appendix, Figure 86). Those constructs were introduced into *Arabidopsis* plants (RLD) *via Agrobacterium*-mediated transformation. Nine independent primary sense lines and nine independent primary anti-sense lines of AtGluRS transgenic plants were analyzed. Five primary transgenic lines were selected and brought to the T_2 generation. Selected sense and anti-sense lines were chosen for further analysis and were segregating for the anti-sense and sense genes.

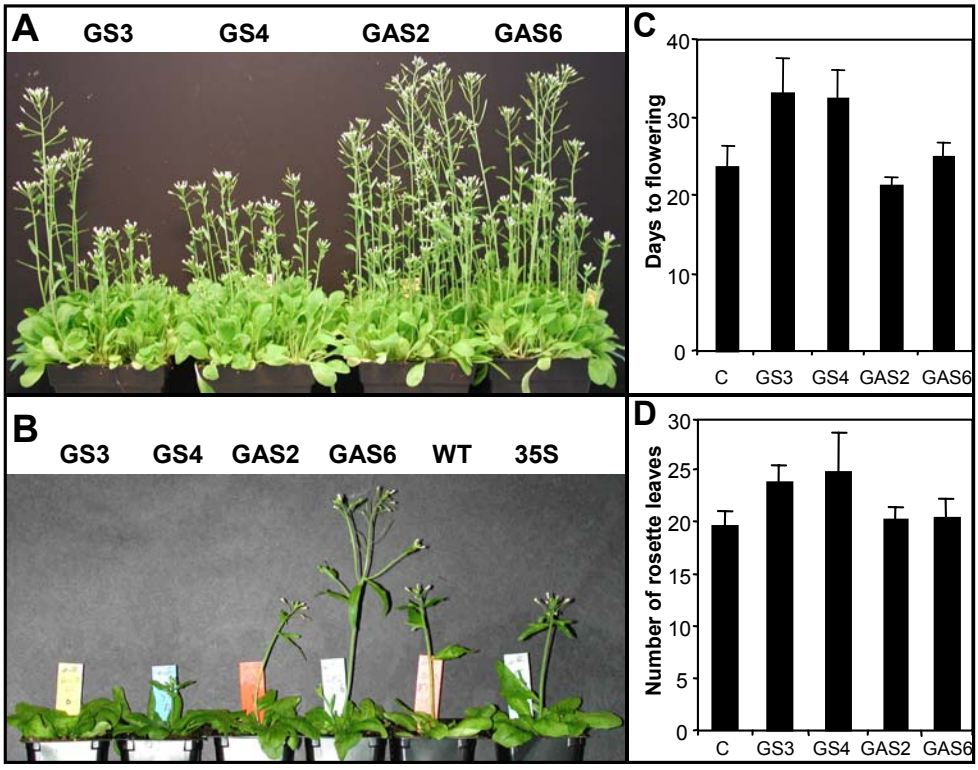


Figure 57. Phenotype of AtGluRS transgenic plants.

A, B: Phenotypic alteration of AtGluRS sense and anti-sense transgenic plants 4 weeks after sowing; **C:** days to flowering; **D:** number of rosette leaves at initiation of flowering. GS3, GS4: AtGluRS sense lines 3 and 4; GAS2, GAS6: AtGluRS anti-sense lines 2 and 6; WT: wild-type (RLD); 35S: 35S-GUS as controls (background RLD).

3.4.4.1.1 Phenotype of AtGluRS transgenic plants

Phenotypic alterations between AtGluRS sense and anti-sense transgenic lines were observed in flowering time and number of rosette leaves (Figure 57). As shown in Figure 57-C, the onset of reproductive development under standard conditions (16 hs light/ 8 hs dark at 100 µE photon/m^2.s) was delayed in AtGluRS sense lines (GS3 and GS4), which flowered in average after 33 days, as compared to 35S-GUS transgenic control plants, which flowered after 24 days. In contrast, there was no difference observed between AtGluRS anti-sense lines and control plants. The number of rosette leaves was increased in AtGluRS sense lines, with 24 rosette leaves on average, but only 17.5 in control lines and 19 in AtGluRS anti-sense lines as shown in Figure 57-D. There were no differences in auxiliary inflorescences and secondary inflorescences, but the main stems in transgenic

sense plants were further developed than in the control and anti-sense transgenic plants. The typical appearance of AtGluRS sense and anti-sense transgenic plants are documented in Figure 57-A and B.

3.4.4.1.2 Physiological analysis of AtGluRS transgenic plants for ABA responses

To analyse the physiological responses of ABA-mediated inhibition of germination and vegetative growth as well as control of stomatal aperture, AtGluRS sense and anti-sense plants were randomly selected which have 3:1 segregation ratio of the transgene. For germination assays and stomatal response two sense lines and two anti-sense lines while 5 sense lines and 5 anti-sense lines were chosen for root growth assay. As shown in Figure 58-A, AtGluRS sense lines were by a factor of 4 more sensitive to ABA than AtGluRS anti-sense lines and control plants. The IC_{50} value of half-maximal inhibition by exogenous ABA was 0.25 µM in the AtGluRS seedlings after 4 days of inhibition. AtGluRS anti-sense lines were to some degree also more sensitive to ABA than the control by a factor of less than 1.5 in the IC_{50} value (Figure 58-B).

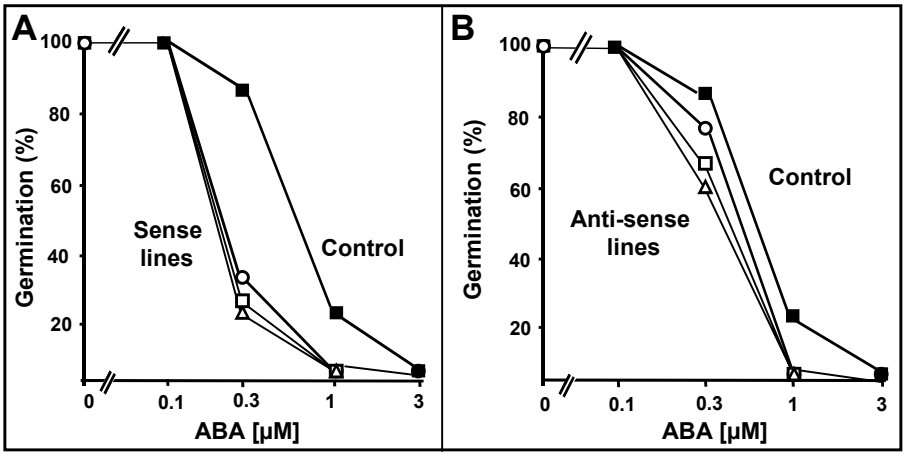


Figure 58. Germination assay of AtGluRS transgenic seeds.

Seeds transgenic for AtGluRS sense (**A**) and anti-sense (**B**) constructs were allowed to germinate on MS plates at different ABA concentrations. The germination of seeds was measured after 4 days. 35S-GUS control: closed squares; AtGluRS sense lines 3, 4 and 9: open triangles, squares and circles in (**A**), respectively; AtGluRS anti-sense lines 2, 6 and 10: open triangles, squares and circles in (**B**), respectively.

The germination assay indicated that AtGluRS overexpression lines were hypersensitive to ABA but AtGluRS anti-sense lines were not significantly altered compared to the transgenic control. Interestingly, root growth inhibition by ABA was indistinguishable in all lines tested (Figure 59).

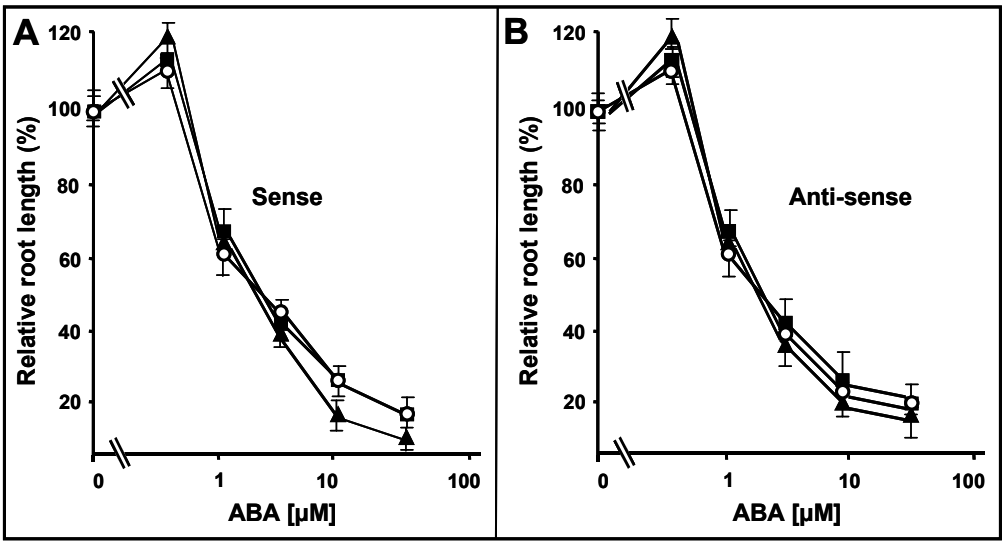


Figure 59. Root growth assay.

Four-day-old seedlings grown under sterile condition were transferred onto solid medium with various ABA concentrations. Root growth within 4 days after transfer was determined (n=20, SD 15%). In the absence of ABA, root growth equalled 14.0 1.8, 12.0 1.6, 14.0 1.2, 13.0 1.5 and 14.6 0.8 mm for the control, AtGluRS sense 3, sense 4, anti-sense 2 and anti-sense 3 lines, respectively. The values are indicated 35S-GUS control (open squares), AtGluRS sense lines 3 and 4 (closed triangles and squares in **A,** respectively) and AtGluRS anti-sense lines 2 and 3 (closed triangles and squares in **B,** respectively).

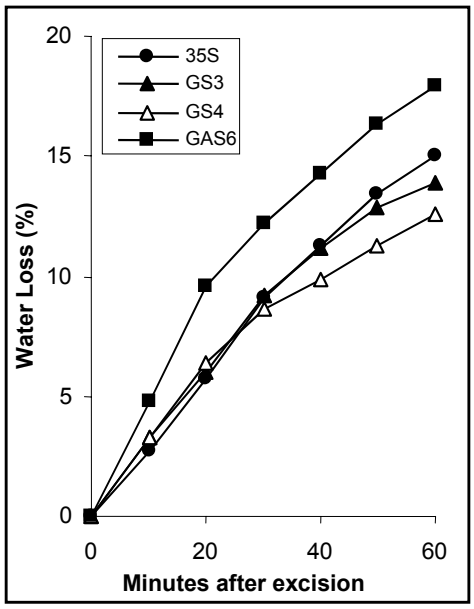


Figure 60. Water loss of AtGluRS transgenic plants.

Water loss of excised leaves as an indication of stomatal aperture. Comparable developmental leaves (n=6) from 4-week-old plants were excised and the loss of the fresh weight was measured at ambient conditions.

Analysis of stomatal regulation, however, supported a role for AtGluRS also in vegetative responses to ABA. Leaves of the same age (4-week old) and size from AtGluRS over-expressing plants, anti-sense plants and control plants were tested (Figure 60). The difference between AtGluRS anti-sense and sense lines in water loss was more than 30% in one hour excised from the plants. The loss of water in one hour was 15% of initial leaf weight in the control line while 17.5% in AtGluRS anti-sense line and 13.3% in AtGluRS sense lines. The differences are significantly and the data provide further support to the results of the germination assay that AtGluRS sense plants were hypersensitive to ABA.

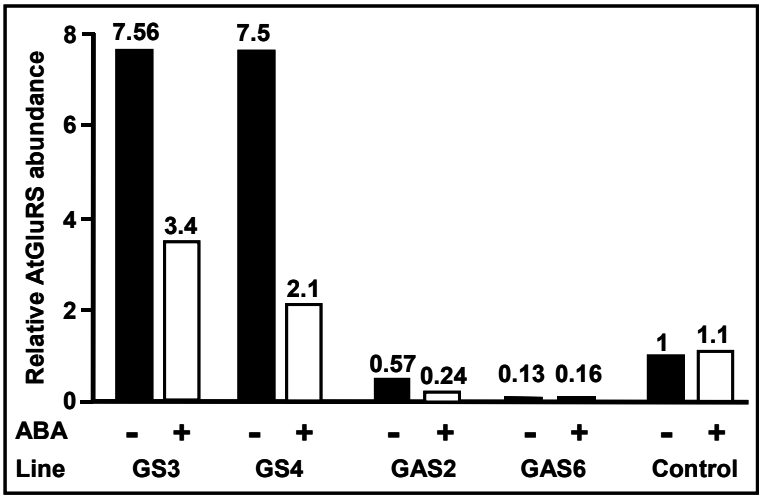


Figure 61: *Arabidopsis* with specifically altered levels of AtGluRS transcripts --analysis by real time PCR.

Transcript levels of AtGluRS sense and anti-sense lines in comparison to 35S-GUS transgenic control. Total RNAs were isolated from seedlings after exposure to 30µM ABA for 24 hours. First strand of cDNA was synthesized by M-MuLV reverse transcriptase and used as template for PCR. The modulation of AtGluRS transcript levels in AtGluRS sense and anti-sense lines was quantified by real time RT-PCR with actin transcripts as an internal standard. The AtGluRS level is expressed relative to the untreated control (set to 1) that yielded 3.8 pg AtGluRS cDNA product per pg actin cDNA fragment. GS3, GS4: AtGluRS sense line; GA2, GA6: AtGluRS anti-sense line; control: 35S-GUS transformant line.

3.4.4.2 Transcriptional analysis of AtGluRS transgenic lines

3.4.4.2.1 Expression of AtGluRS in over-expression and RNA interference lines

Three independent AtGluRS sense and four independent anti-sense lines were used to analyse the expression of AtGluRS at the transcriptional level. Real time PCR (Figure 61) showed that the AtGluRS mRNA was over-expressed in sense lines by a factor of more than

7 in both sense lines analyzed (GS3 and GS9) compared to the control line (35S-GUS transformant line). In addition, the expression of AtGluRS was decreased in anti-sense lines by a factor of 2 (GAS2) and 7 (GAS6) compared to the control line. The relative products of AtGluRS were about 28 pg per pg actin PCR product in both AtGluRS sense lines and 2.2 pg and 0.5 pg per pg actin in AtGluRS anti-sense line 2 and line 6, respectively, 3.8 pg in the control line. The repetition of the analysis generated reproducible results. RT-PCR (Figure 62), normalized by using actin transcript as an internal control, revealed that AtGluRS was over-expressed in AtGluRS sense lines (GS3 and GS4) by a factor of more than 3.5 and decreased in anti-sense line (GAS2) by a factor of 1.7 compared to the control line. Northern blot analysis (Figure 63) also showed a very strong hybridization signal for the AtGluRS transcripts in sense line 3 (GS3) and relatively strong hybridization signal in sense line 9 (GS9). There was a weak signal in the control line, and very weak one in both anti-sense lines (GAS3 and GAS4). The results from RT-PCR, real time PCR and Northern blot clearly demonstrated that AtGluRS is over-expressed in over-expression lines and decreased in anti-sense lines

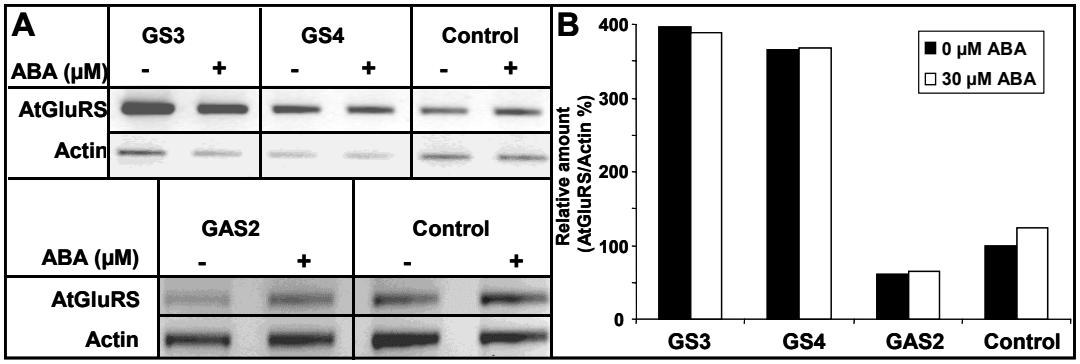


Figure 62. Ectopic expression of AtGluRS in stable lines in the presence of ABA---transcriptional level analysis.

Total RNAs were isolated from seedlings of AtGluRS sense and anti-sense lines and the control treated with or without 30μM ABA for 24 hours. 2.5μg RNA of each lines was used as template for reverse transcription. **A:** RT-PCR analyses were normalized by actin transcript, two independent PCR reaction for each gave similar results. Upper panel: AtGluRS sense transgenic lines and the control; lower panel: AtGluRS anti-sense transgenic line and the control. **B:** The quantitative analysis of the PCR bands was performed by imaging softeware (Molecular Analyst, BioRad). GS3, GS4: AtGluRS sense lines; GAS2: AtGluRS anti-sense line; Control: 35S-GUS plant.

3.4.4.2.2 The effect of ABA in AtGluRS over-expression and RNA interference lines

In the presence of 30 μM ABA, the abundance of AtGluRS transcript was more or less not elevated by ABA in the control line (factor 1.1). The result was similar to that in wild-type plants (Figure 43) which indicated that the endogenous AtGluRS was not regulated by ABA at the transcriptional level. In these experiments, there was no difference in the expression of the AtGluRS in over-expression lines with or without ABA from RT-PCR (Figure 62). However, Real Time PCR revealed that AtGluRS specific PCR products were reduced by a factor of more than 2 in both AtGluRS sense lines after ABA treatment (Figure 61). Northern blot analysis also showed that the hybridization signal was reduced in one AtGluRS sense line (GS9) after treatment with 30 μM ABA, but the signal was still slightly stronger than the ABA treated control line. The expression analysis of AtGluRS in anti-sense lines revealed there were no detectable differences generated by ABA treatment (Figures 61, 62 and 63).

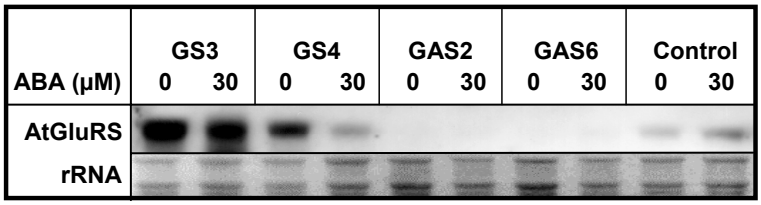


Figure 63. RNA gel blot analysis of AtGluRS transcript abundance.

Approximately 5μg total RNA extracted from AtGluRS sense and anti-sense lines were analysed for AtGluRS transcript abundance together with the control line. Total RNAs were isolated from seedlings treated with or without 30 μM ABA for 24 hours. The AtGluRS specific probe was generated by incorporation of digoxigenin-11-dUTP through PCR reaction. The EtBr-stained rRNA signal indicated comparative RNA loading. GS: AtGluRS sense line; GAS: AtGluRS anti-sense line; control: 35S-GUS transformant line.

3.4.4.2.3 The expression of ABA-regulated genes in AtGluRS transgenic lines

To investigate the expression of ABA-regulated genes in AtGluRS sense and anti-sense lines, several indicated genes were selected and their expression analyzed at the transcriptional level. The PCR product was normalized using actin as an internal control (Figure 64)

HB6 is an ABA-regulated gene in *Arabidopsis thaliana* (Himmelbach et al., 2002). In all three AtGluRS anti-sense lines, the expression of HB6 was decreased by a factor of 2 in both GAS1 and GAS6 lines compared to the control 35S-GUS transformant line and by a

factor of more than 3 compared to AtGluRS sense line 3, one line (GAS2) did not show a difference compared to the control. After treatment with 30 µM ABA, the expression of HB6 in GAS2 was reduced by a factor of 2 compared to the control and by a factor of 3.4 compared to the AtGluRS sense line, but no apparent difference in the other two anti-sense lines. NCED3 is a key enzyme in the biosynthesis of ABA and regulated by drought stress (Iuchi et al., 2001) and ABA (Seki et al., 2002). The expression of NCED3 was decreased by a factor of more than 2 (in GAS2 and GAS10) and 3 (GAS1) compared to AtGluRS sense line and the control line (Figure 64). The expression of another ABA-regulated gene, Lti65 (Nordin et al., 1993), was also decreased by a factor of 6 (GAS2) and more than 2 in AtGluRS anti-sense lines 2 and 1, respectively, compared to the control after ABA treatment (Figure 64). According to this result, it suggests that AtGluRS is probably involved in the transcripts of ABA-regulated genes.

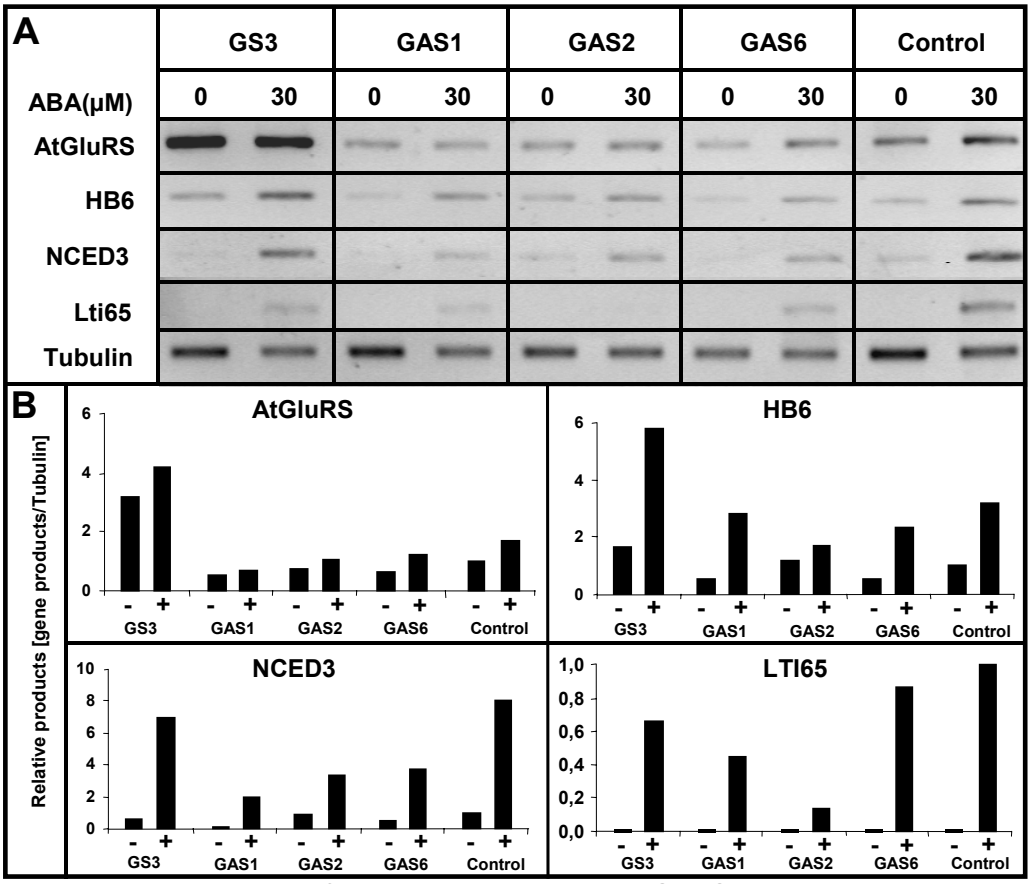


Figure 64. The expression of ABA-regulated genes in AtGluRS sense and anti-sense lines at the transcriptional level.

Transcript levels of ABA-regulated genes in AtGluRS anti-sense lines (GAS1, GAS2 and GAS6) in comparison to one AtGluRS sense line (GS3) and the control 35S::GUS transformant line. Total RNAs were isolated from seedlings after exposure to 30µM ABA for 24 hours and ABA-regulated gene transcripts were analysed. **A:** The RT-PCR products of AtGluRS and different ABA-regulated genes were of expected sizes; **B:** The quantitative analysis of the PCR bands were measured by imaging software (Molecular Analyst, BioRad) and normalized by tubulin transcript level.

3.4.4.2.4 The effect of AtGluRS overexpression on RNA splicing of ABI1 and ABI2

Gene expression can be also controlled at the mRNA processing step as well as during transcription and translation. From the results in Figure 65, the processing of pre-mRNA to mature mRNA of two ABA-regulated genes, ABI1 and ABI2, did not work properly in AtGluRS sense line 3. Predicted cDNA fragments were 350 bp for ABI1 and 870 bp for ABI2 from wild type mRNA, however, the fragments amplified by RT-PCR in GS3 were 440 bp and 1,100 bp for ABI1 and ABI2, respectively (Figure 65-C). The products were of the same length as the genomic DNA for both genes. There is a 93 bp intron in the fragment amplified by the specific primers for ABI1, and there are two introns of 87 bp and 130 bp length for the fragment amplified by specific primers for ABI2 (Figure 65-A). However, mRNA of other ABA-regulated genes, such as HB6, Rab18, Lit65S, NCED3, and also AtGluRS and fibrillin, all having one or two introns between the specific primers in the genomic DNA sequences, were processed normally. Fragments amplified by RT-PCR yielded only the predicted mature cDNA length. Thus, introns of ABI1 and ABI2 could not be efficiently spliced out in the over-expression line. After the AtGluRS over-expression line was treated with ABA (30 µM, 24h), splicing of ABI1 and ABI2 RNA worked properly (Figure 65). This phenomenon was seen only in AtGluRS sense line 3 (GS3) and was not observed in other AtGluRS sense lines. AtGluRS was, however, much stronger over-expressed in GS3 line than in the other AtGluRS sense lines, suggesting links between the inhibition of proper processing of ABI1 and ABI2 mRNA and AtGluRS expression above a certain threshold level. ABA, however, stimulates or rescues the processing capacity of the plant.

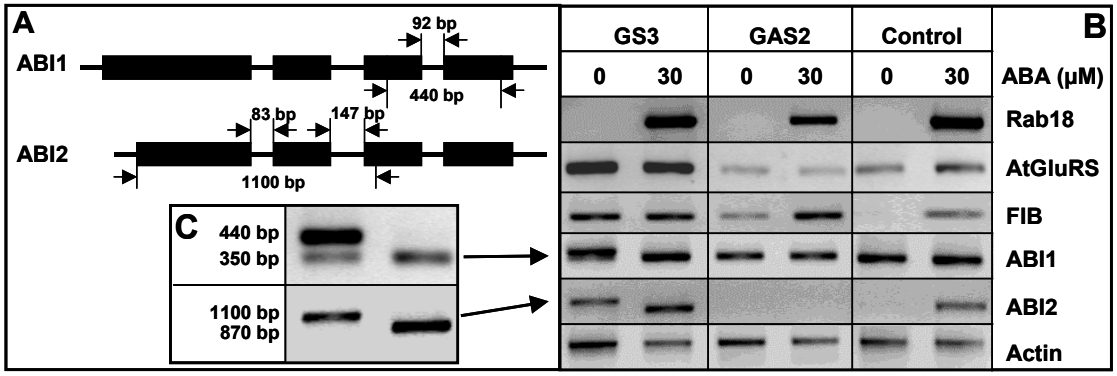


Figure 65. The effect of RNA splicing of ABI1 and ABI2 transcript by overexpression of AtGluRS in plant.

ABI1 and ABI2 cDNA fragments were amplified of 350 bp and 870 bp in length respectively. A 90 bp intron in ABI1 and two introns with 83 and 147 bp in ABI2 are presented in the primary transcripts from the genomic DNA and in amplified cDNA fragments (**A, B**). The RNA splicing occured imperfectly in the AtGluRS sense line (GS3) but did not affect the expression of other ABA regulated genes (**C**). For example: AtGluRS, 430 bp for mature cDNA and 560 bp for genomic DNA sequences; fibrillin, 330bp for mature cDNA and 420 bp for genomic DNA sequences; actin, 570 bp for cDNA and 670 bp for genomic DNA sequences.

4 Discussion

Arabidopsis thaliana is estimated to have at least 2,000 ABA-regulated genes. However, only a few of them have been identified and characterized (reviewed by Finkelstein et al., 2002), which limits our understanding of ABA action at the molecular level. Therefore, identification and characterisation of the novel ABA-responsive genes in *Arabidopsis* genome may lead to new targets and functions for the known regulators or to even new classes of regulators.

The work presented in this thesis includes: (1) identification of new cellular interaction partners of ABI2 using yeast two-hybrid screening of *Arabidopsis thaliana* cDNA libraries; (2) verification and quantification of positive interaction partners of ABI2 with different deleted and mutated versions of ABI1 and ABI2; (3) characterisation of two positive candidates identified by yeast two-hybrid screening, fibrillin and AtGluRS of *Arabidopsis thaliana*, with respect to their functions in ABA signal transduction *in vitro* and *in vivo*; (4) investigation of gene expressions of fibrillin and AtGluRS at the transcriptional level including ectopic expression *Arabidopsis* transgenic lines; (5) cellular localization of fibrillin-GUS fusion protein in plant cells.

4.1 *Identification of new cellular interaction partners of ABI2 using the yeast two-hybrid system*

Since the establishment of yeast two-hybrid system (Fields and Song, 1989), its development provided a genetic means to identify proteins that physically interact *in vivo*. The two-hybrid system is frequently not only used to test interactions between known proteins, but it has also been considered as one of the most efficient and popular tools to screen libraries in order to isolate and identify genes that encode interacting partners for a protein of interest (Chien et al., 1991). A major advantage using the two-hybrid approach for screening a cDNA library is the immediate availability of the gene of interest for DNA sequence analysis.

Primary identification of interacting partners of ABI2

According to previous studies, ABI2 protein displays the same characteristic modular architecture as the homologous protein ABI1 with 86% amino acid identity in the C-terminal domains. Their N-terminal domains are less conserved (49% identity) and display no extensive similarity to other known proteins (Meyer et al., 1994; Leung et al., 1997; Rodriguez et al., 1998). Both ABI1 and ABI2 have PP2C catalytic activity (Meyer et

al., 1994; Bertauche et al., 1996; Leung et al., 1997; Rodriguez et al., 1998). The mutant forms, abi1 and abi2, show the markedly reduced phosphatase activity because of the substitutions of Gly-to-Asp at the equivalent positions in ABI1 (Gly-180) and ABI2 (Gly-168) phosphatase domains, respectively (Meyer et al., 1994; Leung et al., 1997; Rodriguez et al., 1998). Homeodomain protein (HB6) was identified using ABI1 as a bait protein to screen the *Arabidopsis* cDNA library with the yeast two-hybrid system (Leube 1994). The interaction between HB6 and ABI1 is mediated by the catalytic center of protein phosphatase domain of ABI1 and the aminoterminal domain of HB6 containing part of the DNA-binding site (Himmelbach et al., 2002).

In this study, thirteen interacting candidates of ABI2 were identified from *Arabidopsis* cDNA Ohio and Clontech libraries using the yeast two-hybrid system. Analysis of protein sequences encoded by the identified cDNA revealed that the primary interacting candidates of ABI2 included different known and unknown proteins of *Arabidopsis*. And all of the interacting proteins had partial or nearly complete sequences with high similarity compared to those in the Arabidopsis databank (Tabs. 4, 5, 6).

According to the sequencing results (see Appendix 5.2), most of the interacting candidates of ABI2 are in frame to the GAL4 sequence except catalase and HB6. The cDNA sequences of catalase and HB6 in the open reading frame of GAL4 would encode for short peptides (WNSDPRGHEGHTPICEIHTVSPKNRKRRRIRIKIVFKHHGSIQGSSFKRS) and (SRGS EFAAASTRPRRPLSLSFGLSQSISLSLFFLI) which have no identity with any other protein in the databank. Interestingly, no interaction is detected between ABI2 and catalase after cloning catalase (N-terminal part about 200 a.a.) into the correct open reading frame. One possible explanation is that the short unknown peptides could interact with ABI2 protein in the yeast two-hybrid assay. Another explanation would be the presence of its nuclear localisation sequence (NLS). For example, HB6 has its own NLS with which HB6 can be imported into nuclear so that the reporter gene is activated.

Verification and quantitative analysis of protein-protein interaction

The quantitative β-galactosidase assay provided more details about the interactions of interacting candidates with ABI1 and ABI2 and their deleted and mutated versions thereof as summarized in Table 7.

In Table 7, the candidates can be divided into 5 groups according to their different interaction patterns with ABI2 and ABI1. Group I includes fibrillin which displays only strong interaction with wild-type form of ABI2. Group II includes ΔAtGluRS which has a stronger interaction with ABI1 than ABI2 but cannot discriminate between catalytically active and inactive forms of ABI1. The ΔAtGluRS cDNA encoded for an N-terminal

fragment (262 out of 719 amino acids) of full length AtGluRS. Group III includes two unknown proteins (AB025622 and AC023628) which have a strong preference for ΔABI1 compared to ABI2. Group IV encompasses one unknown protein (ATAC006587) and peroxiredoxin which have weak interaction with ABI2 as well as ΔABI1. Group V includes the candidates which have only weak interaction with ABI2 but the interaction cannot be quantified by liquid assay.

Table 7. Summary of putative ABI2 interacting partners

Positive candidates (AD/prey)			Quantitative β-galactosidase assay with different BD versions							
Group	Description	Accession	Empty BD	ABI2	abi2	ΔABI2	Δabi2	ΔABI1	Δabi1	NAP	NCO
I	Fibrillin	AF075598	-	++	-	-	-	-	-	-	-
II	ΔAtGluRS	AF067773	-	++	-	-	-	++++	++++	+++	++
III	unknown protein	AB025622	-	++	-	-	-	+++	++	-	-
	unknown protein	AC023628	-	+++	-	+	-	++++	+	-	-
IV	Unknown protein	ATAC006587	-	-/+	-	-	-	-/+	-/+	-	-
	Peroxiredoxin	CAA71503	-	-/+	-	-	-	-/+	-/+	-	-
V	Ath mitochondrial genome	MIATGENB	-	-/+	-	-	-	-	-	-	-
	Erd15	ATHERD15	-	-/+	-	-	-	-	-	-	-
	RibA	ATHJ0053	-	-/+	-	-	-	-	-	-	-
	hypothetical protein	AF007271	-	-/+	-	-	-	-	-	-	-
	Ath Cab binding protein	CAA39534	-	-/+	-	-	-	-	-	-	-

Interaction data of yeast two-hybrid system:

-: No interaction, β-gal unit is below 0.2; **-/+**: Weak interaction, 0.2 to 0.5 β-gal units; **+**: Interaction from 0.5 to 3 β-gal units; **++**: Strong interaction, 3 to 5 β-gal units; **+++**: Very strong interaction, 5 to 10 β-gal units; **++++**: Over 10 β-gal units. All BD versions fused to pGBT9 Gal4 BD. Empty BD: no insert; ABI2: full length ABI2; abi2: full length abi2 ($168^{G\ to\ D}$ in ABI2); ΔABI2: 95-423 a.a. of ABI2; Δabi2: 95-423 a.a. of abi2; ΔABI1: 123-434 a.a. of ABI1; Δabi1: 123-434 a.a. of abi1 ($180^{G\ to\ D}$ in ABI1); NAP: non active PP2C ($177^{D\ to\ A}$ in ΔABI1); NCO: N-terminus of ABI1 from 1-180 a.a. a.a.: amino acid.

In this work, N-terminal deleted versions (123-434 a.a.) of ABI1 were used for the interaction studies in the yeast two-hybrid system. Full length ABI1 cDNA fused to GAL4 DNA-binding domain (BD) cannot be used in yeast two-hybrid system because it strongly activates the *LacZ* reporter expression. However, truncation of amino acids 1-110 reduced transcription activity to background levels (Himmelbach et al., 2002).

As group I and group II interacting candidates, fibrillin and ΔAtGluRS display distinct differences in the interaction with ABI1 and ABI2. Fibrillin interacts only with full length ABI2 (Fig. 11-A). However, it fails to interact with abi2 and ΔABI2, indicating an important role for both the PP2C active center and the N-terminal domain. ΔAtGluRS has stronger interaction with ΔABI1 than ABI2. In addition to very strong interaction with ΔABI1 and its mutated form, ΔAtGluRS revealed strong interaction with the N-terminus of ABI1 (NCO, 1-180 a.a.) and the non-active ABI1 version NAP ($177^{D\ to\ A}$ in ΔABI1) (Fig. 11-B). The results suggest that the interaction between ABI1 and ΔAtGluRS depends on neither PP2C activity nor the N-terminus of ABI1 but on the C-terminal domain of ABI1. However, there is no detectable interaction between full length AtGluRS and ABI1 or ABI2. The interpretation is that the N-terminal domain of AtGluRS is sufficient to interact with ABI1 or ABI2 similar to the interaction of RAS (guanine nucleotide-binding protein) and RAF (downstream effector for RAS) which also required only the N-terminal domains of RAF (Aelst et al., 1993). However, in the presence of full length AtGluRS protein GAL4-BD and AD cannot be brought into close physical proximity in the promoter region so that the DNA-BD and AD peptides do not directly interact with each other and, therefore, cannot activate the responsive reporter genes.

Two unknown putative proteins (AB025622, AC023628) in group III display strong preference with ΔABI1 compared to ABI2. In addition, they showed also weaker interaction with Δabi1 than with ABI2 and ΔABI1. The analysis suggests that the interactions between PP2Cs and interacting candidates in group III depend on both PP2C activity and the N-terminal domain of ABI2. However, minor structural difference in C-terminal domains between ABI1 and ABI2 lead to their minor difference in the interaction with group III candidates. Δabi1 with reduced PP2C activity has also reduced interaction with these candidates. Sequence data (Fig. 9) disclose that these two unknown proteins have 77% amino acids identity in their protein sequences including domains similar to pathogenesis-related proteins. Therefore, their structural similarity may lead to similar interaction characteristics with ABI1 and ABI2.

The candidates in group IV have weak interaction with ABI2 as well as with ΔABI1 and Δabi1. Those in group V revealed only weak interaction with ABI2 and these interactions could not be vigorously verified by further quantitative analysis. Weak and transient

two-hybrid interactions are difficult to be detected and quantified using the quantitative β-gal assay. However, there is the report of no direct correlation between β-galactosidase activity in yeast two-hybrid system and the affinity of two interacting proteins *in vitro* (Estojak et al., 1995). Thus, these candidates could be potential interacting partners of ABI2. In order to quantify these types of interaction, more sensitive assay should be used, e.g. assay using chlorophenol red-β-D-galactopyranoside as substrate for β-galactosidase analysis.

In summary, the analysis using quantitative β-galactosidase assay revealed: (1) Most of the ABI2 binding proteins also interact with the ΔABI1; (2) Most of these interacting candidates of ABI2 have no interaction with ΔABI2. These findings suggest an important role of the PP2C domains in establishing protein interactions; (3) ABI1 and ABI2, two homologous genes displaying the same characteristic modular architecture and catalytic activity, even have common interacting partners; (4) Weak interacting partners could not be verified by quantitative β-galactosidase assay.

ABI1 and ABI2 are two homologous proteins with high identity in C-terminal PPase domains (86%) and low identity in N-terminal domains (49%) and display functional redundancy in ABA signalling (Meyer et al., 1994; Leung et al., 1997; Rodriguez et al., 1998). Both PP2Cs play a key role in ABA signal transduction (Merlot et al., 1997). Reduced PP2C activities in *abi1* and *abi2* mutants reduce the plant's responsiveness to ABA (Merlot et al., 1997). According to the analyses in the yeast two-hybrid system, interactions of interacting partners with ABI1 and ABI2 can have similar interaction patterns. These results possibly reflect the fact that both PP2Cs have the similar catalytic activity and functional redundancy. However, their structural difference in N-terminal and PP2C domains lead also to differential interactions. The analyses of interacting proteins in the yeast two-hybrid system reveal either a discrimination of ABI1 and ABI2 and/or discrimination of the enzymatic active PP2C and its catalytically deficient form. Thus, it provides solid evidence for the specificity of protein interaction. The interacting proteins are good candidates to play a role in ABA signalling.

The yeast two-hybrid system is a powerful tool to identify the protein-protein interaction partners of bait (Chien et al., 1991). For example, 14-3-3 proteins have been proven to interact with VP-1 (viviparous-1) in a yeast two-hybrid experiment and *in vitro* (Schultz et al., 1998). Recent analysis revealed that the 14-3-3 proteins interact with components of ABA-induced gene expression machinery (Fulgosi et al., 2003). Hence, further analysis of the interacting candidates of ABI2 identified in the yeast two-hybrid system may lead to the finding and understanding of the new components of ABA signal transduction.

In this work, fibrillin and AtGluRS were selected in order to characterize a possible function in ABA signal transduction. As mentioned above, fibrillin was strongly discriminatory for the catalytically active form and N-terminal domain of PP2C and interacted only with full length ABI2. In contrast, ΔAtGluRS interacted not only with ABI2 but also with deleted or mutated ABI1 and even the N-terminal domain of ABI1. Due to the distinct characteristics of fibrillin and ΔAtGluRS in the protein-protein interaction with ABI1 and ABI2 in yeast, it was interesting to study whether they play a role in controlling ABA responses in *Arabidopsis*.

4.2 *Role of fibrillin in plants*

Fibrillin and its homologous genes

Fibrillin is a 32-kD protein located in fibrils in which carotenoids are stored. It was first identified in bell pepper by Deruére et al. (1994a, b). Proteins belonging to the fibrillin family are present in chromoplastic structures from different species (Deruére et al., 1994b; Vishnevetsky et al., 1996). According to previous studies, fibrillin probably exists in the outer layer of fibril in which carotenoids, surrounded by a layer of polar lipids, accumulate in the center (Fig. 66) (Deruére et al., 1994b). The gene encoding fibrillin is specifically expressed during fruit ripening in *Capsicum annuum*, leading to its accumulation in chromoplasts. One of the two relatively abundant chloroplastic drought-induced proteins (CDSP) in *Solanum tuberosum* plants displays high homology with pepper fibrillin (Gillet et al., 1998) and is preferentially associated with stromal lamellae (Eymery et al., 1999; Pruvot et al., 1996). C40.4, a cDNA clone in potato, has strong homology to bell pepper fibrillin and is located in the chloroplast in association with thylakoid membranes (Monte et al., 1999). There are other fibrillin genes known in *Citrus unshiu* (Moriguchi et al., 1998), in *Arabidopsis thaliana* (AF075598, AL021712 and AC005314), in *Zea mays* (AA979828) and in *Brassica* (Hernandez-Pinzon et al., 1999), suggesting a well-conserved gene family of fibrillin expressed in both di- and mono-cotyledonous plants.

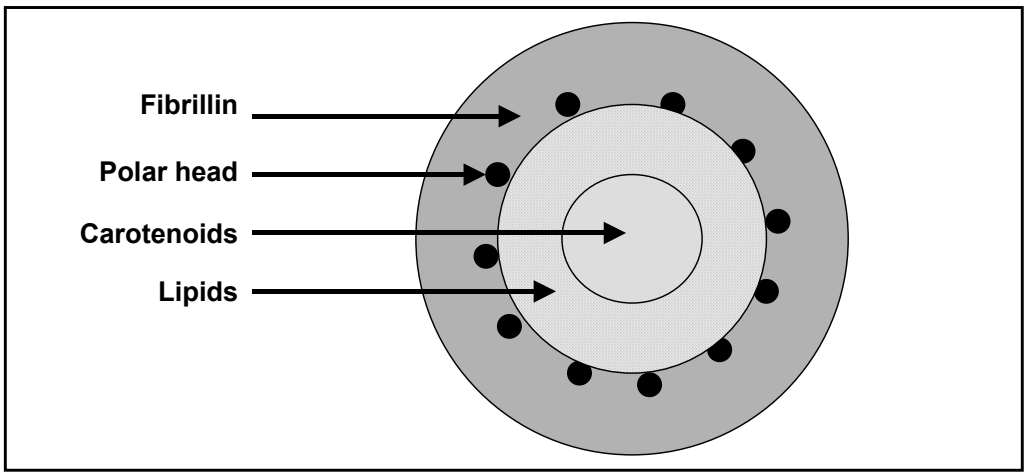


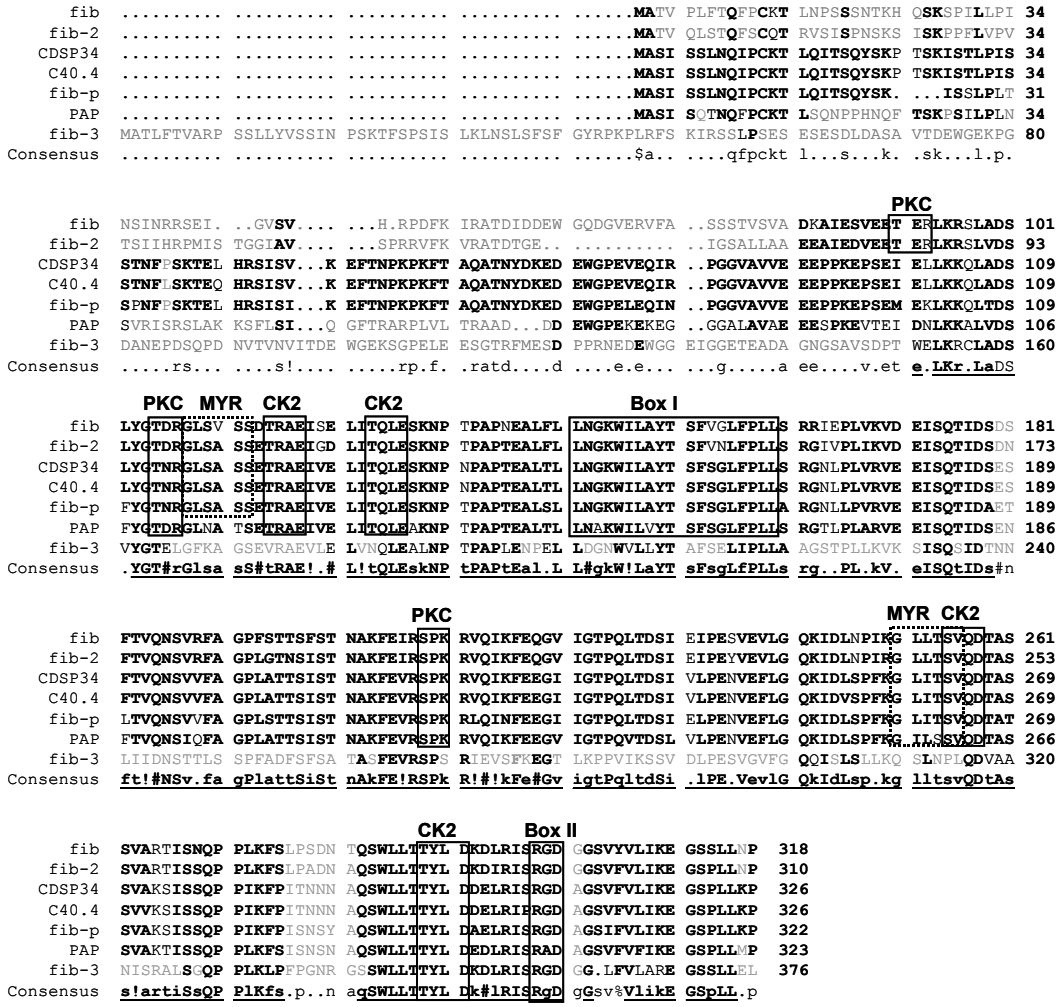
Figure 66. Model of fibril assembly.

The core is occupied by carotenoid that interacts with the acyl residues of the more polar galactolipids and phospholipis (LIP), whose polar head groups (dark circles) interact with fibrillin molecule, which are directly in contact with the plastid stroma. [According to Deruére et al., 1994]

Protein sequence alignment of fibrillin family in plants is presented in Figure 67 and Figure 38-A. Putative fibrillin (fib) (AF075598) has 79% amino acid identity to plastid-lipid associated protein (AF290563) in *Brassica rapa*, 76% identity to fibrillin precursor-like protein (fib-2) in *Arabidopsis thaliana* (AL021712), 64% identity to CHRC (chromoplast-specific carotenoid-associated protein) in *Cucumis sativus* (AF099501), 64% to C40.4 in *Solanum demissum* (AJ131455), 60% identity to pepper fibrillin (AJ131456) in *Caspicum annuum* and 48% identity to fibrillin-like protein (fib-3) in *Arabidopsis thaliana* (AC005314).

According to *in silico* sequence analysis of the fibrillin family, most of them contain a possible helical transmembrane region (Box I), a potential cell adhesion motif (Box II), 4 casein kinase II phosphorylation sites (CK2), two or three protein kinase C phosphorylation sites (PKC) and two N-myristorylation sites (MYR) (Gattiker et al., 2002). Only the motifs of the fibrillin-like protein in *Arabidopsis* (fib-3) are quite different since it contains only one CK2 site and the potential cell adhesion motif. The N-termini of all three fibrillins in *Arabidopsis* have less identity to other fibrillins, whereas their C-termini except for fib-3 show high homology to other fibrillins in plants. This indicates that the fibrillin family has a relatively conserved C-terminal domain.

Figure 67. Protein sequences alignment of fibrillin family in plants.



Amino acid sequence comparison of the putative fibrillin (fib), fibrillin-2 (fib-2) and fibrillin-like protein (fib-3) in *Arabidopsis*, CDSP34 and C40.4 in potato, fib-p (pepper fibrillin) and PAP in bell pepper. Identical amino acids are presented in dark. Box I: possible helical transmembrane region; Box II: the potential cell adhesion motif; PKC: Protein kinase C phosphorylation site; CK2: Casein kinase II phoshorylation site; MYR: predicted N-myristorylation site.

Physical interaction of ABI2 with fibrillin

The yeast two-hybrid data showed that the ability of ABI2 to interact with fibrillin was strictly correlated with PP2C activity and the N-terminal domain (a.a. 1-95) of ABI2. Deletion of the ABI2 N-terminus and reduction of the enzymatic activity by a single point mutation completely abolished the interaction (Fig. 11a), supportive of a specific interaction of fibrillin with ABI2. Further *in vitro* analysis with heterologously expressed

and purified protein confirmed that fibrillin interacted with ABI2 but not with its mutant form abi2 (Figs.17, 18), corroborating the finding of the yeast assay. The *in vivo* and *in vitro* analyses strongly suggest a specific interaction of fibrillin with ABI2 and a discriminative interaction against the catalytically deficient abi2.

Regulation of fibrillin in *Arabidopsis thaliana*

The regulation of the fibrillin promoter was previously studied in pepper, tomato, potato, tobacco and rapeseed but not in *Arabidopsis thaliana*. ABA did not induce the expression of fibrillin in bell pepper (Kuntz et al., 1998). Gillet et al. (1998) reported that CDSP34 mRNA, encoding for a fibrillin-like protein in potato, was induced after exposure to 100 μM ABA for 10 days. According to RT-PCR (Fig. 13-1) and Northern analysis (Figs. 13-2 and 37) in this work, expression of fibrillin is significantly induced by ABA in *Arabidopsis*. The induction can be detected even in the presence of 0.3 μM ABA or after short exposure to ABA. Further analysis demonstrated that the activity of luciferase reporter gene expression controlled by the fibrillin promoter increased in transgenic *Arabidopsis* plant after challenge to ABA (Fig. 26). Recently, a full-length cDNA microarray demonstrated that *Arabidopsis* fibrillin was up-regulated by ABA (Seki et al., 2002). This result is in agreement with the finding in this work that fibrillin gene is up-regulated by ABA at the transcriptional level.

Subsequent Northern analyses of wild-type (La-*er*) and *abi1, abi2* mutant lines confirm that expression of fibrillin is up-regulated by ABA (Fig. 13-2). However, the ABA-induced alteration of fibrillin gene expression is strictly dependent on ABI1 (Fig. 13-2), since the abundance of fibrillin mRNA is much lower in the *abi1* mutant background, indicating an ABI1-dependent signal pathway.

According to the transcription levels, fibrillin is up-regulated by ABA in an ABI1-dependent manner. This either reflects ABA-triggered changes in fibrillin promoter activity or/and alterations in fibrillin mRNA stability. Using the transient expression system, it confirmed the up-regulation of fibrillin promoter of *Arabidopsis* by ABA. In addition, ectopic expression of abi1 and abi2 abrogated the induction of reporter gene driven by the fibrillin promoter (Fig. 20-C). Thus, the expression analyses clearly demonstrated the stimulation of the fibrillin promoter by an ABI1-dependent signal pathway.

In silico analysis of the Arabidopsis fibrillin gene identified several putative *cis* elements controlling its gene expression. Sequencing data mining (Appendix 5.3) revealed a putative ABA-responsive element (ABRE, Marcotte et al., 1989) in the promoter region of fibrillin in *Arabidopsis thaliana* (Fig. 68). The ABRE (CACGTGTC) is located from –142 to –134 bp upstream of the transcription start site of the fibrillin gene. ACGTGGC and ACGTGTC

were proven to be ACGT-containing ABRE (Hattori et al., 2002). Thus, the ABRE in the fibrillin promoter matches the sequence requirement of an ACGT-containing ABRE. The presence of the ABRE-element seems to be responsible for ABA activation of the fibrillin promoter. Other fibrillin genes such as the pepper ortholog lack the ABRE with the consequence that ABA is not involved in regulating the promoter activity (Kuntz et al., 1998).

In addition, there is a coupling element (CE-1) core, which is a *cis* element that is functionally important to ABA-regulated gene expression (Shen and Ho, 1995). One GT-motif and one GC-motif are located in fibrillin promoter. There are one MYB (Myeloblast, Roussel et al., 1979) recognition sequence which is involved in ABA-induced transcription (Abe et al., 1997; Urao et al., 1993) and one DOF-motif (DNA binding with one finger motif) which binds DOF proteins and is involved in tissue-specific and light-regulated gene expression (Yanagisawa and Sheen, 1998). Besides, there are four ABRE cores (ACGT). The ABRE and CE-1 core are located very closely, as well as the GT-motif, GC-motif and MYB recognition sequence in the upstream region of the fibrillin gene in *Arabidopsis thaliana*, suggesting the regulatory region of the fibrillin is located within 500 bp upstream of the transcription start.

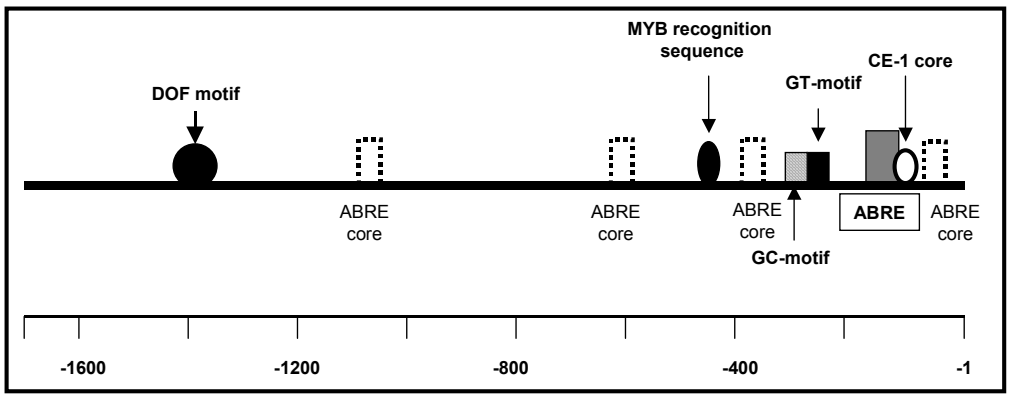


Figure 68. Regulation elements in promoter region of fibrillin in *Arabidopsis thaliana.*

In the 1.7 kb promoter region of fibrillin, there are one ABA-responsive element (ABRE) and 4 ABRE cores, as well as coupling element-1 (CE-1), GT- and GC-motif, myeloblast (MYB) recognition sequence and DOF (DNA binding with one finger) motif. Lower line indicates the nucleotide number upstream of the transcript starting point.

Promoters containing ACGT-containing ABRE can bind bZIP transcription factors but a single copy of an ABRE requires a *cis*-acting coupling element (CE) to permit ABA induction (Shen and Ho, 1997). Thus, the ABRE and CE-1 core in fibrillin promoter region perhaps matches the requirement to achieve ABA induction. The low ABA induction (3 fold) in *Arabidopsis* might be due to the close proximity of ABRE element to the transcriptional

start point (less than 150 bp). Due to sterical hindrance the interaction between a bZIP (basic domain leucine zipper, Izawa et al., 1993) transcription factor and RNA polymerase could be limiting for the initiation of transcription. Normally, the optimal distance for an interaction between activator proteins and RNA polymerase is about 400-500 bp (Mossing and Record, 1986). For a functional analysis of the putative regulatory elements in the fibrillin promoter, deletion and point mutation of ABRE and CE-1 core in fibrillin promoter can be constructed. Fusions of the mutated sequences to reporter genes allow analysis of the ABA-regulation in transient systems or in transgenic *Arabidopsis* plants.

Previous studies suggested that the C40.4 protein, a fibrillin-like protein in potato, is involved in photosynthesis by modulating photosynthetic efficiency and dissipation of excess absorbed light energy within the antenna complex (Monte et al., 1999). The pepper fibrillin promoter is up-regulated by high light intensities (Manac'h et al., 1999). Transient analysis revealed that the fibrillin promoter was induced by light in *Arabidopsis* similar to potato. According to promoter analysis, a DOF-motif could be involved in the light-regulation of fibrillin gene expression. However, the activity of the luciferase gene under the control of the fibrillin promoter was higher by ABA and light together than by light or by ABA alone (Fig. 21), suggesting that light and ABA might act synergistically in activating the fibrillin promoter.

Chen et al. (1998) reported that the pepper fibrillin expression was extremely low and could only be detected in tobacco and tomato leaf vascular tissue. The pepper fibrillin promoter is active under diverse stress conditions, such as wounding, drought or methyl viologen (Chen et al., 1998) and cold and salt stress, high light intensities, as well as superoxide and single oxygen (Manac'h et al., 1999) or biotic stress (Langenkamper et al., 2001).

The analysis in this work using fibrillin promoter-GUS constructs in transgenic plants, however, demonstrated that the fibrillin promoter is most active in leaves (Fig. 23) and reproductive organs (Fig. 24). The GUS activity was easily detected even after staining for only few hours. Unlike the pepper fibrillin promoter, which was only active under stress conditions (Chen et al., 1998), *Arabidopsis* fibrillin promoter was found to be active in most areas in seedlings and reproductive organs. Quantitative analysis also confirmed that the fibrillin promoter was up-regulated by ABA in *Arabidopsis* (Fig. 26). In addition, the fibrillin promoter was also induced under stress conditions, e.g. salt stress and oxidized glutathione (GSSG) (Fig. 26) in this investigation, in agreement with previous reports (Chen et al., 1998; Manac'h et al., 1999). These analyses, therefore, suggest that fibrillin expression in *Arabidopsis thaliana* is related to plant growth, plant development and ABA signalling or ABA response.

Ectopic expression of fibrillin in *Arabidopsis thaliana*

In transient studies, over-expression of ABI1 and ABI2 in *Arabidopsis* inhibited the activation of ABA-dependent reporter genes by ABA (Hoffmann, 2001). In addition, over-expression of wild-type ABI1 in maize mesophyll protoplasts blocked ABA induction of gene expression (Sheen, 1998), suggesting that ABI1 and ABI2 act as negative regulators of ABA signalling (Merlot et al., 2001). Due to the physical interaction between fibrillin and ABI2, it is also interesting to study fibrillin's role in ABA signalling. Using the protoplast expression system, ectopic expression of fibrillin resulted in the inhibition of expression from two ABA-dependent reporter constructs, i.e. rab18-LUC and Lti65-LUC, by a factor of 2 to 4 in the presence of ABA (Fig. 22). The inhibition of ABA-dependent reporter genes was almost the same as achieved with ABI1 and ABI2 as effectors (Hoffmann, 2001). The results demonstrated that fibrillin repressed ABA-triggered gene expression, indicating that the fibrillin functions as a negative regulator of the ABA signal transduction pathway.

Localization of the fibrillin-GUS fusion protein in plant root cells revealed that the GUS staining was detected in the cytoplasm either as plates or bands (fibrillar-structure) (Figs. 40-41). The fibrillar-structure of fibrillin-GUS fusion proteins are sometimes very long and thick and exist in entire cells, in agreement with the proposed model for fibril architecture (Deruére et al., 1994b). However, the fibrillar-like structures were greatly decreased in number and in length (Fig. 41) after the cell suspensions were subjected to ABA or high osmoticum treatment. The reason for this reduction of fibrillar-structures in response to ABA and osmoticum is still unknown. The explanations could be that fibrillin is imported into plastids such as chloroplasts and chromoplasts or that the components of the fibrils are disassembled after challenge to ABA or osmoticum. In line with this interpretation, it has been shown that accumulation of the CDSP34, fibrillin-like protein in potato required the ABA signal (Gillet et al., 1998).

According to the expression pattern of fibrillin-GUS fusion protein in plant cells in this study, fibrillin might be involved in structural stabilization of cells. It has also been proposed that the potato CDSP34 protein participates in structural stabilization of thylakoids (Eymery and Rey, 1999; Gillet et al., 1998). In bell pepper fruit chromoplasts, fibrils are organized in parallel clusters with cross-links being visible in electron microscopy (Deruére et al., 1994b). A pepper plastid lipid-associated protein (PAP), homologous to fibrillin, is involved in carotenoid storage in fruit chromoplasts and is localized within chloroplasts in both stromal and thylakoid subfractions in tobacco (Rey et al., 2000).

Previous studies show that fibrillin does not exist in green fruits and leave tissues in which carotenoids are known to occur. Fibrillin is considered to be involved in the packaging and organization of excess carotenoids which accumulate in fruits and form fibrils together with lipids and fibrillins (Deruére et al., 1994b). Therefore, no carotenoids are present in root and leave cells, that are required for proper assembly of authentic fibrils. In this work, however, it was shown that fibrillin overexpression resulted in formation of fibrillar-structures in root and green leaf cells of *Arabidopsis* cell culture upon ectopic expression. These structures may be precursors of carotenes, for example, phytoene and phytofluenes could substitute for carotenoids and fibrillin may form complexes with these precursors. Whether fibrillin has enzymatic activity involved in the carotenoid biosynthesis or a structural role in plant cells has to be subject of further studies.

Further analyses using a combination of Real time PCR, RT-PCR and Northern blot demonstrated that the fibrillin mRNA abundance was dramatically increased (factor 6 to 30 or even more) in transgenic *Arabidopsis* sense lines but significantly decreased (factor 2-5) in RNA interference lines (Figs. 35, 36 and 37). Thus, the alteration of fibrillin mRNA abundance provided a basis to analyse the physiological responses of fibrillin transgenic plants to ABA. The ABA-mediated inhibition of germination demonstrated that fibrillin RNA interference plants were hypersensitive to ABA (factor 3) compared to fibrillin overexpression and control plants (Fig. 29-A). However, the hypersensitive response was not reflected during vegetative growth of the roots. Further analysis supported a role of fibrillin in stomatal regulation. According to previous studies, the altered stomatal responses of ABA -insensitive or -hypersensitive mutants to ABA correspond to enhanced or reduced water loss by detached leaves, respectively (Meyer et al., 1994; Pei et al., 1998). In this work, the rate of water loss was increased (17%) in fibrillin overexpression lines and was reduced (20%) in fibrillin RNA interference lines (Fig. 31). This result also supports the notion that fibrillin RNA interference lines are hypersensitive to ABA.

Analysis of stable fibrillin transgenics also revealed that the alteration of ABA- sensitivity of fibrillin RNA interference plants was clearly associated with a delay in flowering time and an increase in number of rosette leaves compared to the fibrillin sense and the control plants (Fig. 27). However, phenotypic alterations of the transgenic plants ectopically expressing fibrillin were not evident. In other studies, over-expression of pepper fibrillin exhibited enhanced growth and accelerated flowering under high light condition, suggesting a higher stress tolerance of fibrillin transgenics during this transition (Rey et al., 2000). In this work, fibrillin sense plant did not provide any evidence for modification of plant development, but the phenotype of fibrillin RNA interference plants is in agreement with a role of fibrillin in stress tolerance.

Model of fibrillin in ABA signal transduction pathway

In this work a new role of fibrillin in ABA signal transduction was discovered. The fibrillin of *Arabidopsis thaliana* (gene accession AF075598) has been systematically characterized *in vivo* and *in vitro*. According to the analysis in this work, a model for the functions of fibrillin in ABA signal transduction pathways is proposed (Figure 69). The physical interactions in yeast and *in vitro* demonstrate that the fibrillin interacts with ABI2 but not with abi2. An interaction between fibrillin and full length ABI1 could not be demonstrated. However, the expression of the fibrillin is dependent on ABI1. Further analyses revealed that ABA induced the expression of the fibrillin gene at the transcriptional level. It is possible that the induction of fibrillin gene expression by ABA is due to the presence of an ABA-responsive element (ABRE) in the upstream of transcription start of fibrillin gene. Stresses and light also induce the expression of the reporter gene under the control of the fibrillin promoter, indicating that some light- and stress-responsive elements probably exist in the promoter region of fibrillin. As a plant hormone, ABA is the most intimately involved in stress signal transduction (Xiong et al., 2002), thus there are extensive connections between stresses, such as cold, drought, salinity, and ABA signal transduction pathways (Ishitani et al., 1997).

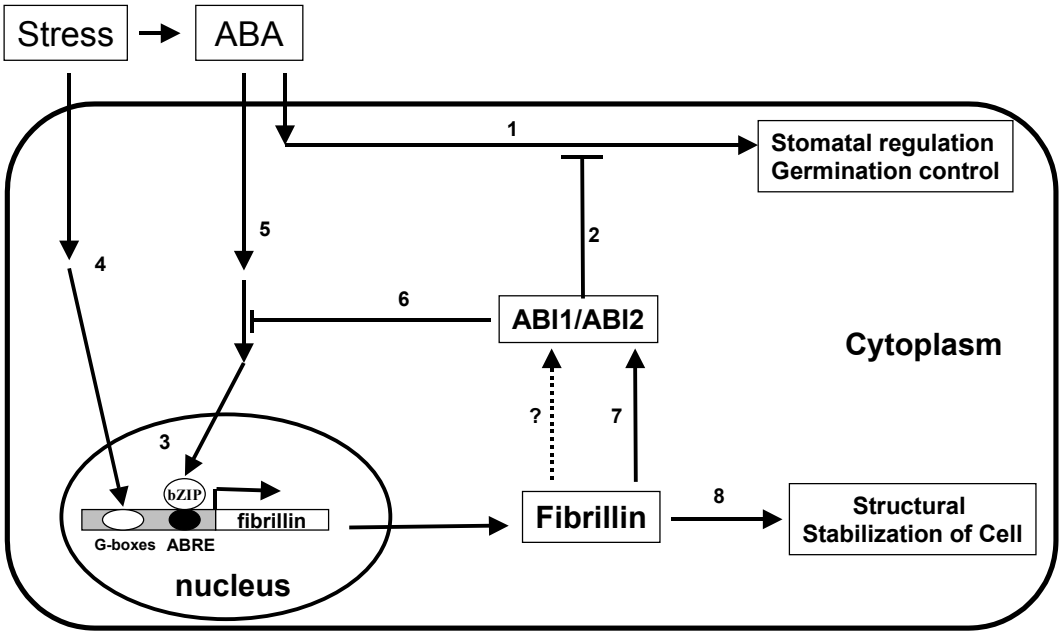


Figure 69. The hypothetical model for the role of fibrillin in ABA signal transduction.

Abscisic acid (ABA) plays a major role in stomatal regulation, stress response and growth regulation etc. (1). ABI1/ABI2 are two central elements in ABA signaling and act as negative regulators (2). The fibrillin promoter has an ABRE and perhaps some stress-inducible elements (3), therefore, the expression of the fibrillin gene is up-regulated by stresses (4) and ABA (5) in an ABI1-dependent manner (6). Fibrillin positively regulates ABI2 (7) (maybe ABI1 as well) thereby supporting the inhibitory action of ABI2 (ABI1?). Decreasing the fibrillin abundance results in ABA hypersensitivity of fibrillin transgenic plants. In addition, fibrillin is also involved in the cellular stabilization (8).

Genetic evidence indicates that ABI2 is a negative regulator of ABA action (Gosti et al., 1999). At the molecular level, it is demonstrated that the ABI1/ABI2 phosphatases act in a negative feedback loop of the ABA signalling pathway (Merlot et al., 2001). As an interacting partner of ABI2, ectopic expression of fibrillin in *Arabidopsis thaliana* inhibits the expression of ABA-responsive reporter. Physiological analysis clearly demonstrated fibrillin-mediated alterations of ABA responses in transgenic *Arabidopsis* plants. Ectopic expression of fibrillin does not alter ABA sensitivity of the plants during germination, but down-expression enhanced the plants sensitivity to ABA. Ectopic expression of fibrillin affected the regulation of the water status of the transgenic plants by increasing the loss of water. In addition, the fibrillin RNA interference plants have an opposite alteration, reducing the loss of water. Phenotypic and physiological evidences support that fibrillin acts as a negative regulator in the ABA signal transduction pathway. Besides, the function of fibrillin may be associated with cellular stabilization according to its fibrillar-like protein-lipid binding capacity as proposed in potato and bell pepper (Deruére et al., 1994b; Eymery and Rey, 1999).

4.3 *Glutamyl tRNA synthetase (AtGluRS) and ABA signalling*

Glutamyl tRNA synthetase gene family

Aminoacyl-tRNA synthetases (aaRSs) are a family of enzymes well-known for their role in protein synthesis (Eriani et al., 1990). Glutamyl tRNA synthetase (GluRS) belongs to class I of aaRSs which have a consensus HIGH amino acid sequence in the putative ATP binding site and an additional highly conserved sequence KMSKS motif at the putative binding site of the 3' end of tRNA (reviewed by Freist et al., 1997).

The first GluRS was isolated from *E. coli* and has 471 amino acids (Russell and Pittard, 1971; Sanfacon et al., 1983). In yeast, the GluRS is found in a set of mitochondrial aaRSs. In *Drosophila*, the N-terminal domain of the largest polypeptide of a multi-synthetase complex has GluRS activity, whereas the C-terminal part has prolyl-tRNA synthetase activity. In the N-terminal domain, it contains the HIGH signature and a VLSKR sequence instead of KMSKS motif (Cerini et al., 1991). In human genome, GluRS and prolyl-tRNA synthetase are encoded by a single human gene and located at opposite ends of the gene. GluRS is found to be active as monomers with molecular weight about 50 kDa in bacteria, yeast or animals (reviewed by Freist et al., 1997).

Figure 70. Protein sequence comparison of eukaryotic glutamyl-tRNA synthetase

Box I: HIGH motif in ATP-binding site; Box II: LSKRK motif at the putative binding site of the 3' end of the tRNA. 1-5: GluRS in (1) *Schizosaccharomyces pombe* (716 a.a), (2) *Saccharomyces cerevisiae* (724 a.a), (3) *Encephalitozoon cuniculi* (495 a.a), (4) *Oryza sativa* (715 a.a) and (5) *Arabidopsis thaliana* (719 a.a). a.a: amino acid.

In plants, several GluRS were isolated as dimers with molecular weight from 110-160 kDa from chloroplasts, mitochondria and the cytoplasm of wheat (Ratinaud et al., 1988; Thomas et al., 1983), and from chloroplasts of barley (Andersen, 1992). In *Arabidopsis thaliana*, cytoplasmic glutamyl tRNA synthetase (AtGluRS) has 719 amino acids containing the highly conserved ATP binding site, the HIGH motif, and another putative binding site of the 3' end of tRNA (Fig. 70). However, this motif is changed from 'KMSKS' in Class I aaRSs to 'LSKRK' in AtGluRS (Day et al., 1998).

Biological functions of GluRS

The most important function of GluRS is the transfer of glutamic acid, a constituent of proteins and a neurotransmitter in the nervous system to $tRNA^{Glu}$ (Seeburg 1993). This reaction is mediated by a two-step process in which there is a formation of an aminoacyl adenylate followed by an ATP/PPi exchange reaction (Deutscher et al., 1967a, b; Lapointe and Söll, 1972). In addition, GluRS also participates in the synthesis of Gln-$tRNA^{Gln}$ by acylating $tRNA^{Gln}$ with glutamic acid to generate Glu-$tRNA^{Gln}$, which is converted by an aminotransferase to Gln-$tRNA^{Gln}$. As the precursor of porphyrins biosynthesis, δ-amino-levulinic acid (ALA) is synthesized from Glu-$tRNA^{Glu}$ in plants, green algae, *cyanobacteria* and some *eubacteria*. As shown in Figure 71, GluRS is the first enzyme in biosynthesis of porphyrins by acylation of $tRNA^{Glu}$ with glutamic acid. Glutamyl tRNA reductase generates then glutamate-1-semi-aldehyde and glutamate-1-semialdehyde amidotransferase converts the semialdehyde to δ-aminolevulinic acid (ALA) (Kumar et al., 1996). GluRS is part of a multienzyme complex in lower and higher eukaryotic organism (Yang, 1996; Harris and Kolanko, 1995).

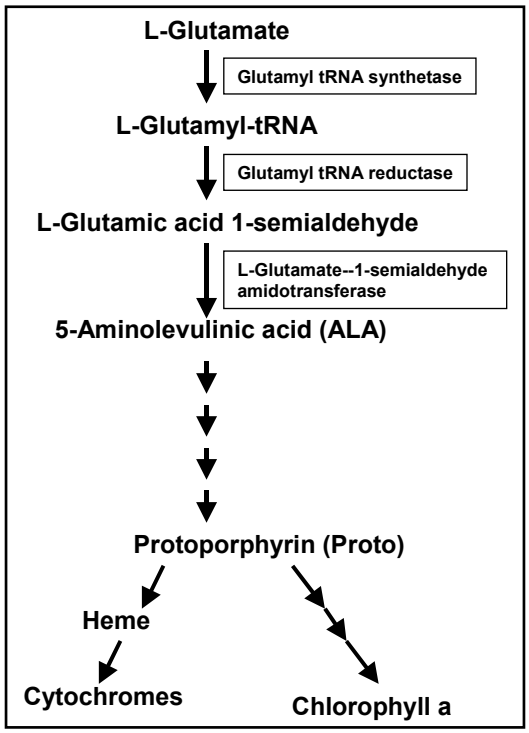


Figure 71. The protoporphyrin biosynthesis pathway in higher plants.

In higher plants, glutamyl tRNA synthetase is the first enzyme in the tetrapyrole biosynthesis pathway.

In yeast, GluRS and methionyl tRNA synthetase (MetRS) interact with Arc1p which functions as a scaffold protein of these synthetases *in vivo* (Simos et al., 1996). The N-terminal domain of Arc1p interacts with GluRS and MetRS and the C-terminal region binds tRNA. Due to binding to Arc1p, the affinity of the synthetases for their cognate tRNAs as well as their catalytic efficiency are enhanced (Galani et al., 2001). In *Arabidopsis thaliana*, it was demonstrated that AtGluRS binds to a 14-3-3 protein (Cotelle et al., 2000). Members of the 14-3-3 protein family are constituents of transcription factor complexes and interact with components of ABA-induced gene expression machinery (Fulgosi et al., 2003).

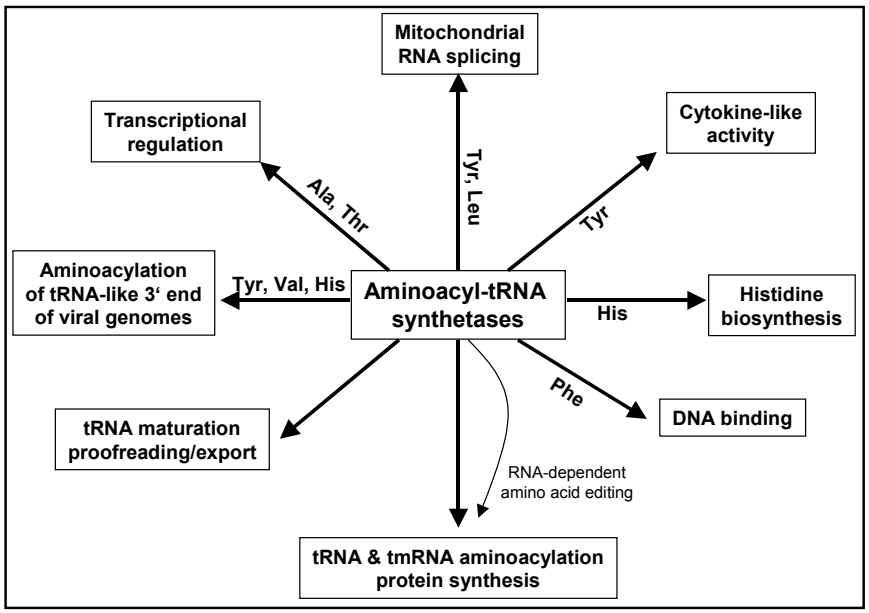


Figure 72. Cellular roles and functions of aminoacyl-tRNA synthetase.

Particular aaRSs involved in each of the represented activities are indicated by the three letter code of the amino acid that they activate. [according to Martinis et al., 1999]

Recent studies suggest that aaRSs are also involved in a number of critical cellular activities besides protein synthesis. Specific aaRSs play roles in tRNA processing, RNA splicing, RNA trafficking, apoptosis, transcriptional and translational regulation (reviewed by Martinis et al., 1999a, b). Figure 72 shows the new appreciation for the role of aaRSs. For example, novel evidence proves that mature tRNAs are charged with their cognate amino acids in the nucleus prior to export into the cytoplasm. Blockage of the tRNA export machinery resulted in accumulation of nuclear charged tRNA and introduction of inhibitors specific to a particular aaRS decreased export of the relevant uncharged tRNA, resulting in the discovery of a nuclear control mechanism for tRNA maturation (Lund et al., 1998).

Physical interaction of AtGluRS with PP2Cs

It was reported that a partial-length gene for AtGluRS was identified as a positive clone in the yeast two-hybrid screening for protein interacting with a cyclin protein (CyclbAt) (Day et al., 1996). However, no evidence of interaction could be found between CyclbAt and AtGluRS (Day et al., 1996). In this study, AtGluRS was identified as an interaction partner of ABI2 in yeast two-hybrid screening. According to the analysis of protein-protein interaction in yeast, the ability of ABI2 to interact with ΔAtGluRS (N-terminus of full length AtGluRS, 1-262 amino acids) is correlated with both PP2C activity and the N-terminal sequence of ABI2. Deleting the N-terminal domain of ABI2 and reducing the enzymatic activity by a single point mutation completely abolished the interaction (Fig. 11-B). It indicates that both PP2C activity and N-terminal domain of ABI2 are required for the interaction between ABI2 and ΔAtGluRS.

However, the ability of ABI1 to interact with ΔAtGluRS in yeast is much stronger. According to the quantitative analysis ΔAtGluRS showed strong interaction also with N-terminal deleted ABI1 and even stronger interference with N-terminal deleted abi1. The N-terminal domain alone and non-active ABI1 (NAP) have a reduced activity with ΔAtGluRS (Fig. 11b-B). The data suggest that neither PP2C activity nor N-terminal part of ABI1 play a key role in the interaction between AtGluRS and ABI1. Thus, the interaction cannot discriminate against the catalytically deficient abi1. Due to weaker interaction between ΔAtGluRS and N-terminal part of ABI1, the C-terminal domain of ABI1 seems to be decisive for the interaction. The analysis of protein-protein interaction between ΔAtGluRS and ABI2 or ABI1 in yeast two-hybrid system suggests that PP2C activity as well as N-terminus of ABI1 seems not critical for binding but minor structural difference between ABI1 and its homologue ABI2.

Effect of AtGluRS on the PP2C enzymatic activity

The analysis of the protein-protein interaction in yeast revealed that AtGluRS interacted with ABI2 and ABI1 which are two protein phosphatases (PP2Cs) (Meyer et al., 1994; Leung et al., 1994; Rodriguez et al., 1998). Therefore, the PP2C enzymatic analysis could be used to determine AtGluRS's role in the interaction with ABI1 *in vitro*.

Protein serine/threonine phosphatases (PPs) are classified into four major groups (PP1, PP2A, PP2B and PP2C) depending on their substrate specificities, bivalent action dependencies and sensitivities to various inhibitor molecules (Cohen, 1989), whereas only three major groups of PPs (PP1, PP2A and PP2C) have been reported in plants (Smith and Walker, 1996). PP2C is distinct from the other three major PPs because the enzymatic

activity of PP2C strictly requires magnesium (Mg^{2+}) or manganese (Mn^{2+}) ions and its amino acid sequence shows little similarity to those of other PPs (Tamura et al., 1989). ABI1 and ABI2, being identified as two protein serine/threonine phosphatases of type 2C (PP2C), act as key regulators in the responses of *Arabidopsis thaliana* to ABA (Meyer et al., 1994; Leung et al., 1994; Rodriguez et al., 1998).

Recent reports provided insight into the regulation of PP2C activities in *Arabidopsis thaliana*. The phosphatase activities of both PP2Cs are activated by increasing [Mg^{2+}] and are highly sensitive to pH *in vitro* (Leube et al., 1998). A pH-driven increase of PP2C activity would result in decreased ABA sensitivity, on the basis of the genetic evidence that ABI1 is a negative regulator of ABA responses (Gosti et al., 1999). Characterisation of the redox sensitivity of ABI1 and ABI2 revealed that hydrogen peroxide (H_2O_2), a secondary messenger of ABA signalling, strongly inactivated the protein phosphatase activity of both PP2Cs (Meinhard et al., 2001; 2002).

According to this study, the enzymatic activity of ABI1 was significantly reduced in the presence of AtGluRS (Figs. 49, 50), suggesting that AtGluRS acted as an inhibitor of PP2C. However, the inhibition of the enzymatic activity of ABI1 by AtGluRS was found only at lower concentration of Mn^{2+} (less than 2 mM). In addition, in the presence of ABA, the activity of PP2C was slightly reduced *in vitro* (Fig. 47-B). However, the inactivation of PP2C by ABA seems not to be significant.

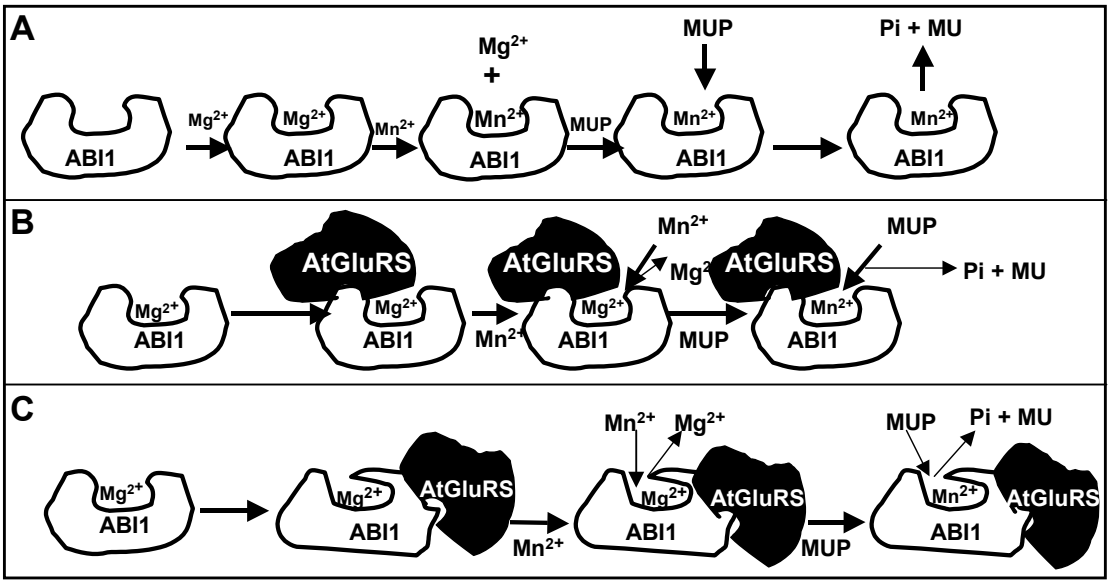


Figure 73. Hypothetical mechanism of interference by AtGluRS in the activation of ABI1.

A: The enzymatic mechanism of dephosphorylation of MUP by ABI1 is based on the exchange of Mg^{2+} by Mn^{2+} in the catalytic site of ABI1; **B:** AtGluRS binds to the catalytic site of ABI1 in which magnesium ions bind so that the magnesium ions could not be easily exchanged by the manganese ions through this complex; **C:** AtGluRS binds to another position of ABI1 thereby changing the physical structure of the catalytic site.

The activation of ABI1 is found to be inhibited by AtGluRS at lower Mn^{2+} concentration (< 2mM) in the assay with MUP as the substrate. In this reaction, the exchange of Mg^{2+} by Mn^{2+} in the catalytic site of ABI1 is necessary to allow dephosphorylation of the substrate MUP (Fig. 73-A). An explanation for the inhibition of ABI1 by AtGluRS is that AtGluRS interferes with the exchange of Mg^{2+} by Mn^{2+}. The mechanism of the interference by AtGluRS could be: (1) AtGluRS binds to the position (e.g. catalytic site) of ABI1 in which Mg^{2+} is bound so that the exchange of Mg^{2+} for Mn^{2+} is inhibited. This competitive effect subsequently leads to the reduction of ABI1 activity; (2) AtGluRS binds to another position in ABI1 so that the physical structure of the catalytic site is changed by an allosteric effect. In consequence, the sterical change reduces the enzymatic activity of PP2C; (3) AtGluRS also competes for binding of Mg^{2+} and Mn^{2+} with ABI1 at the lower Mn^{2+} concentration, leading to the reduced activity of ABI1.

An alternative enzymatic assay also supports the idea of inhibition of ABI1 activation by AtGluRS. With casein as a substrate, the dependence of ABI1 and ABI2 activation on Mg^{2+} yielded a bell-shaped curve, with half-maximal activity in the presence of approximately 1 mM Mg^{2+} and maximal activity in 10 mM Mg^{2+} (Leube et al., 1998). In the present study, the ability of ABI1 to dephosphorylate phosphocasein is inhibited only after pre-incubation with AtGluRS in the absence of Mg^{2+} (Fig. 52). The finding indicates that the activation of ABI1 is inhibited, if AtGluRS is added prior to Mg^{2+} and suggests that there is an interaction between ABI1 and AtGluRS. The consequence of the interaction could be an alteration of the physical structure of ABI1 or reduced accession of ions to the binding sites leading to a diminished ABI1 activity. This is in agreement with the first and second hypothetical mechanism of the interference by AtGluRS in the activation of ABI1, i.e. AtGluRS is likely to bind to the catalytic site or the specific site of ABI1 and inhibits Mg^{2+} binding, leading to the reduced activity of PP2C (Fig. 73-B and C). The inhibition of the activation of ABI1 at low concentrations of Mg^{2+} is the same as at high concentrations. Therefore, AtGluRS seems not to compete with ABI1 for the Mg^{2+} or Mn^{2+} in the reaction.

The AtGluRS mediated inhibition of the activation of the negative regulator ABI1 provides evidence that the AtGluRS may act as a positive regulator in ABA signal transduction pathway.

AtGluRS is likely to be involved in the interaction with ABA *in vitro*.

ABA regulates plasma membrane and tonoplast ion channel activities very rapidly (reviewed by Rock, 2000). Hence, it is possible that ABA interacts directly with transport proteins or other metabolic factors, for example, enzymes. ABI1 or ABI1 protein complexes might have binding sites for ABA.

The *in vitro* analysis of the ABA-binding property of ABA-interacting proteins by equilibrium dialysis revealed that ABA interfered with ABI1 or AtGluRS together but the proteins alone did not result in any alteration of ABA distribution. According to this analysis, the diffusion of ABA was 12% in the side of proteins more than in the side of ABA (Fig. 46). In other words, 6 pmol ABA was shifted to non-equilibrium ABA and bound to 2% of the protein complexes. The distribution of ABA towards in the side of ABI1 and AtGluRS is not so significant. However, it must be considered that ABA is in the status of movement in a container so that the observation can not reflect the actual ABA distribution. Repeats of this experiment showed the same results that the complex of ABI1 and AtGluRS led to an alteration of ABA. Thus, the *in vitro* binding assay provides preliminary evidence that ABI1 together with AtGluRS may interact with ABA. Although some ABA-binding proteins have been reported (Pedron et al., 1998; Wan and Hasenstein, 1996), there is no evidence to link these proteins to the physiological effects of ABA. According to the analyses in this work, however, AtGluRS is an interacting partner of ABI2 and ABI1. Ectopic expression of AtGluRS in *Arabidopsis thaliana* displayed an increased sensitivity to ABA, suggesting a functional role for AtGluRS in ABA signalling. Taken together, it is possible that the protein complex of AtGluRS and ABI1 acts as an intracellular binding site of ABA in plants.

Regulation of AtGluRS in *Arabidopsis thaliana*

Details of the gene regulation of AtGluRS are still unknown. According to this work, analyses of AtGluRS mRNA abundance indicate that there is no change of transcript level after ABA treatment. Neither increasing the ABA concentration to 100 µM nor prolonged duration of treatment with ABA altered the expression of the AtGluRS at the transcriptional level (Fig. 43). RNA gel blot analysis in two *Arabidopsis* accessions, RLD (Fig. 63) and La-*er* (Fig. 44), also demonstrated that ABA did not alter the expression level of the AtGluRS mRNA. However, expression pattern of AtGluRS was greatly decreased in *abi1* and *abi2* plants and could be recovered by applying exogenous ABA (Fig. 43), indicating that its expression was dependent on both ABI1 and ABI2.

At the transcriptional level in wild-type plants, the expression of the AtGluRS gene was not regulated by ABA. However, different insights were obtained from analysis using the protoplast expression system. The expression of the reporter gene under the control of the AtGluRS promoter was induced by a factor 2 to 3 fold either in protoplasts treated with ABA or in protoplasts of plants challenged to ABA prior to protoplast isolation (Fig. 53). The basis of the observed difference is not clear. Probably the protoplast expression system is more sensitive to respond to low ABA levels than plants. Alternatively, overexpression of the reporter gene is required to observe this response. The results also suggest that there are

some unknown ABA responsive element(s) or regulatory *cis* elements which mediated the observed regulation of the AtGluRS gene in *Arabidopsis thaliana*.

A model is proposed (Fig. 74) to explain the expression of AtGluRS gene in *abi1*, *abi2* and wild-type plants. The expression of AtGluRS gene in wild-type plants is not altered after application of exogenous ABA, indicating that endogenous ABA level may be sufficient to allow maximal expression of AtGluRS. This expression depends, however, on ABI1 and ABI2 since the expression levels of AtGluRS are very low in *abi1* or *abi2* mutant plants. In these ABA insensitive mutants, low concentrations of perceiving endogenous ABA signal are unable to mediate proper gene expression of AtGluRS. After application of exogenous ABA, the expression of AtGluRS gene in *abi1* and *abi2* mutant plants is rescued. However, AtGluRS expression levels in *abi1* mutant plants were still lower than that in *abi2* plants. This suggests that AtGluRS gene expression is primarily dependent on ABI1 rather than ABI2.

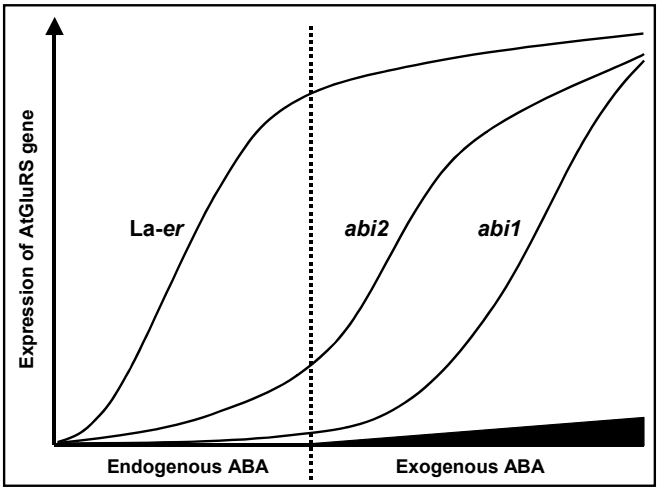


Figure 74. The model for the expression of AtGluRS in *abi1*, *abi2* and the wild-type plants. Details are explained in the text.

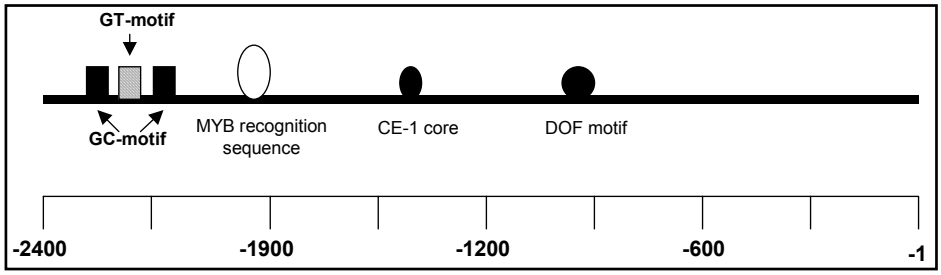


Figure 75. Regulatory *cis*-elements predicted in promoter region of GluRS in *Arabidopsis thaliana*.

The promoter region of AtGluRS has one GT-motif, two GC-motif, one MYB (myeloblast) recognition sequence and one DOF (DNA binding with one finger) motif and one ABA coupling element-1 (CE-1) core. Lower line represents the number of nucleotides upstream of the starting point of AtGluRS.

According to the genomic sequence of AtGluRS accessible *via* the databank, no abscisic acid response element (ABRE) is predicted in the promoter region of AtGluRS. There are

only two GC-motifs, one GT-motif, one DOF motif, one MYB recognition sequence and one CE-1 core in AtGluRS promoter (Fig. 75). Most of these elements are located at a distance (1500 bp) to the transcript starting site of the AtGluRS gene. Thus, the absence of an ABRE element could explain why exogenous ABA does not affect AtGluRS gene expression in *Arabidopsis* in wild-type plants.

Deregulated expression of AtGluRS in *Arabidopsis thaliana*

In order to study the role of AtGluRS in ABA signalling, the expression of the gene was deregulated in transgenic *Arabidopsis* plants. The analyses of the generated transgenic plants indicated that AtGluRS mRNA was dramatically overexpressed (more than factor 7) in AtGluRS sense lines and decreased (factor 2 to 8) in RNA interference lines (Figs. 61, 62 and 63). The analyses of physiological responses demonstrated that the ABA-mediated inhibition of germination and stomatal closure in AtGluRS sense plants had an increased sensitivity to ABA. For example, AtGluRS sense lines were by a factor of 4 more sensitive to ABA in ABA-mediated inhibition of germination and were 12% less in water loss than the control plant (Figs. 58 and 60). In addition, ectopic expression of AtGluRS resulted in delayed onset of reproductive development and increased number of rosette leaves (Fig. 57) that is a late flowering phenotype (Suarez-Lopez et al., 2001). Thus, AtGluRS influences the development of plants by extending the vegetative growth. AtGluRS is likely to act as a positive regulator in ABA signal transduction.

Down-regulation of AtGluRS mRNA by RNA interference led to reduced expression of several ABA-controlled genes (Figs. 64 and 65). The expression of HB6 (Himmelbach et al., 2002), for example, was decreased in AtGluRS RNA interference lines (1 and 6) compared to the control and the AtGluRS sense line. In the presence of ABA, the expression of HB6 was also reduced in the AtGluRS RNA interference lines. Similarly, NCED3 (Iuchi et al., 2001), a key enzyme of ABA biosynthesis, is up-regulated by ABA (Figs. 39 and 64). However, the expression of NCED3 was decreased in the AtGluRS RNA interference lines (1, 2 and 6), indicating that deregulation of AtGluRS expression also interferes with ABA biosynthesis. In addition, the expression levels of ABI1 and ABI2 were also decreased in AtGluRS RNA interference lines (Fig. 65). Thus, the repression of ABA-regulated gene expression in AtGluRS RNA interference lines suggests that AtGluRS is involved in transcript regulation of ABA-controlled genes *via* controlling mRNA maturation.

Previous studies in *Bacillus subtilis* suggested that aminoacyl-tRNA synthetases are involved in transcriptional and translational regulation. Expression of most aaRSs is regulated through a common mechanism involving transcriptional anti-termination where

the principal effector is the uncharged tRNA (Condon et al., 1996). *E. coli* AlaRS binds to a palindromic sequence in its own promoter and represses transcription *in vitro* (Putney et al., 1981).

Some mitochondrial aaRSs, e. g. leucyl tRNA synthetase (LeuRS) and tryptophanyl-tRNA synthetase (TyrRS), assist with group I intron splicing due to some specific domains (Martinis et al., 1999a, b). For example, *Neurospora crassa* TyrRS has a special domain which contributes to splicing, whereas yeast LeuRS-dependent splicing requires a second protein co-factor, a maturase (Martinis et al., 1999a, b). Gene expression is controlled during transcription and translation as well as during the mRNA processing step. Thus, aaRSs can control gene expression during processing step. Overexpression of AtGluRS was found to affect the processing of transcripts of two ABA-regulated genes, ABI1 and ABI2 (Fig. 65).

Table 8. Splice junctions in *ABI1* and *ABI2* of *Arabidopsis thaliana*

	Exon I	Intron I	Exon II	Intron II	Exon III	Intron III	Exon IV	splicing
ABI1	..TTCTC**AG**	**GT**AAAAG... .TGTGT**AG**	**GT**AGCGA. ..CCATA**AA**	**GT**AAGCA... .TGTGT**AG**	**CC**GGATA.. ..TCCATT**G**	**GT**AAGCT... GTGAC**AG**	**GC**GATAG	-
ABI2	..TTCTC**AG**	**GT**AATGA... ..TGTTT**AG**	**GT**AGCGA. ..TCACA**AA**	**GT**AAGTA... .TGTGT**AG**	**CC**GGATA.. ..TCCATT**G**	**GT**AATTA.... CGAAC**AG**	**GC**GATAG	-
Fibrillin	..TCCTC**GC**	**GT**AAGTA... CGTGA**AG**	**CT**ACACG.. ..TGTCC**AG**	**GT**AAATC... .GTTGT**AG**	**AT**TAAGT...			+
HB6	..TCCAC**AG**	**GT**ACTTA... ..ATTAC**AG**	**AT**GAGCA.. .TCAAG**AG**	**GT**ATAAA... GATGC**AG**	**AT**TAGTA... ..			+
Tubulin	..CTTCA**AG**	**GT**ACTAT... .. TTTAC**AG**	**GG**TTTCA.. ..CCTAG**CT**	**GT**AAGTA... .GATGT**AG**	**TT**GGAGA.. .			+
AtGluRS	.CCGAC**GG**	**GT**ACTTT.... .GATGT**AG**	**GA**GGAAA. ..CATGA**AA**	**GT**TCGAA... .TTCTC**AG**	**AT**TGATG.. ..CTTGCTG	**GT**AAGAA... .TTGGT**AG**	**GA**ACTGG	+

The nucleotide boundaries of intron-exon from *ABI1, ABI2,* fibrillin*, HB6*, tubulin and AtGluRS were concluded to compare the differences of splice junctions between *ABI1/ABI2* and other genes. Partial cDNAs of transcripts were amplified using gene specific primers with which different introns in specific genes were included. e.g. intron I of *ABI1* was included in specific primers for *ABI1*, introns II and III of *ABI2* was included in primers for *ABI2*. Intron II of fibrillin was included in primer for fibrillin, introns I and II of AtGluRS were included in AtGluRS and intron I was included in *HB6* and tubulin, respectively. Transcript analysis revealed that the intron I in *ABI1* and introns II and III in *ABI2* were not spliced in AtGluRS overexpression line 3; however, the introns of the other genes were processed properly. Introns analyzed are framed in bold.

The splicing of pre-mRNA to mature mRNA of ABI1 as well ABI2 did not work properly in one AtGluRS overexpression line 3. RT-PCR analyses indicated that the intron I of *ABI1* and intron II and intron III of *ABI2* were included in the gene specific primers and they could be not spliced efficiently after transcription (Table 8). However, mRNAs of other ABA-regulated genes, such as *HB6*, *Rab18*, *Lit65S*, *NCED3*, fibrillin and AtGluRS itself, which have one or two introns between the specific primers (Table 8), appeared to be processed normally (Fig. 65). Analysis of nucleotide sequences of several genes revealed

that the nucleotides of exon-intron boundary between *ABI1/ABI2* and other genes, such as fibrillin, *HB6*, tubulin and AtGluRS (Table. 8) are in consensus with most of the exon-intron junction of the genes in *Arabidospsis* database produced by ACEDB 3.0 software (Durbin and Thierry-Mieg, 1989). This suggests that overexpression of AtGluRS specifically inhibits the proper processing of ABI1 and ABI2 mRNA though a sequence-specific motif for the difference observed could not be deduced.

This phenomenon was found only in one AtGluRS overexpression line 3 in which AtGluRS is expressed to a level much higher than in other AtGluRS sense lines (Fig. 63). Thus, the observation could indicate that proper processing of ABI1 and ABI2 mRNA is impaired when AtGluRS is expressed above a certain threshold level. Interestingly, if this overexpression line is challenged to ABA, the splicing process of ABI1 and ABI2 worked perfectly (Fig. 65). An explanation for this finding could be that addition of exogenous ABA leads to reduced expression level of AtGluRS in transgenic *Arabidopsis* plants under the threshold line or that ABA stimulates mRNA splicing of ABI1/ABI2 transcripts (Figs. 61 and 63), resulting in the recovery of the proper processed mRNA of ABI1 and ABI2. Taken together, these observations suggest that over-expression of AtGluRS inhibits the splicing of ABI1 and ABI2; however, applying exogenous ABA will recover the correct splicing capacity.

The role of AtGluRS in ABA biosynthetic pathway–regulation of NCED3 gene

As discussed above, AtGluRS is involved in the regulation of several ABA-controlled genes encoding, such as *HB6, ABI1, ABI2* and *NCED3* (9-*cis*-epoxycarotenoid dioxygenase 3). NCED3 is an enzyme involved in ABA biosynthetic pathway and catalyzes the oxidative cleavage of 9-*cis*-neoxanthin to generate xanthoxin by a two-step reaction *via* ABA-aldehyde (Tan et al., 1997). The *Arabidopsis aba2* mutant is impaired in the first step of this reaction, and is thus unable to convert xanthoxin into ABA-aldehyde (Léon-Kloosterziel et al., 1996). In addition to NCED, several other enzymes involved in ABA biosynthetic pathway have been characterized recently (Koornneef et al., 1998; Taylor et al., 2000; Milborrow, 2001) as shown in Figure 76. ZEP (zeaxanthin epoxidase) which catalyzes the epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Rock and Zeevaart, 1991) and AAO (ABA-aldehyde oxidase) which catalyzes ABA-aldehyde to ABA, the last step of ABA biosynthesis (Schwartz et al., 1997; Bittner et al., 2001).

In a previous study *NCED3* of *Arabidopsis thaliana* is reported to be induced by drought stress but not by ABA (Iuchi et al., 2001). This work, however, clearly revealed that *NCED3* was up-regulated by ABA more than 8-fold at the transcript analysis (Figs. 39 and 64). This phenomenon was observed in all fibrillin and AtGluRS sense and anti-sense

transgenic lines as well as in the control line. In all three tested AtGluRS anti-sense lines, the expression of *NCED3* gene was reduced by a factor of 2-4 (Fig. 64). The data indicates that AtGluRS is perhaps also involved indirectly in the control of ABA biosynthesis by regulating the expression of *NCED3* gene at the transcriptional level. In addition, ABA also positively feed back the ABA biosynthesis by upregulation of *NCED3* gene.

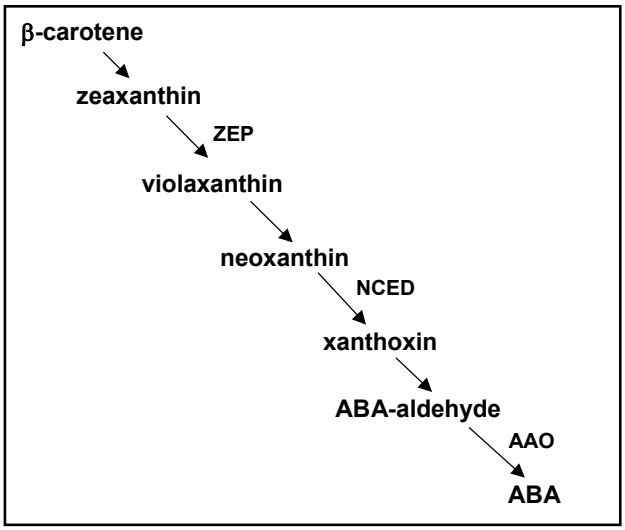


Figure 76. Pathway and regulation of ABA biosynthesis.

ABA is synthesized from a C40 precursor b-carotene via the oxidative cleavage of neoxanthin and a two step conversion of xanthoxin to ABA via ABA-aldehyde. ZEP: zeaxanthin epoxidase; NCED: 9-cis-epoxy-carotenoid dioxygenase; AAO: ABA-aldehyde oxidase. [according to Xiong et al., 2002]

Model for AtGluRS in ABA signal transduction pathway

AtGluRS has been functionally characterized *in vitro* and *in vivo* to elucidate its role in ABA signalling. Based on these results, a model for the role of AtGluRS in ABA signal transduction pathway is proposed (Figure 77).

AtGluRS has been identified as an interacting partner of ABI2 and ABI1. The study demonstrated that the expression of AtGluRS was dependent on ABI1 and ABI2 in *Arabidopsis thaliana*. The analysis of the physiological response of ABA-mediated inhibition of germination demonstrated that AtGluRS overexpression in transgenic *Arabidopsis* plants resulted in hypersensitivity to ABA. Analysis of stomatal regulation of the transgenic plants also supported a role of AtGluRS in vegetative responses to ABA with reduced water loss in AtGluRS overexpression plants. According to the ectopic analysis, AtGluRS is a positive regulator of ABA signalling. The *in vitro* analyses also support that

AtGluRS acts as a positive regulator in ABA signalling by inhibiting the PP2C activity of ABI1 and by the effect on mRNA splicing of ABI1 and ABI2 resulting in a reduced activities of the negative regulators of ABA signal pathway.

The *in vitro* binding assay provides evidence that AtGluRS together with ABI1 may interact with ABA. Combined with ectopic analysis of AtGluRS transgenic *Arabidopsis*, it reveals a functional role of AtGluRS in ABA signal transduction. These *in vitro* binding assay and functional evidences in transgenic plants provide the possibility that the protein complex of AtGluRS and ABI1 may act as an intracellular ABA receptor in plants.

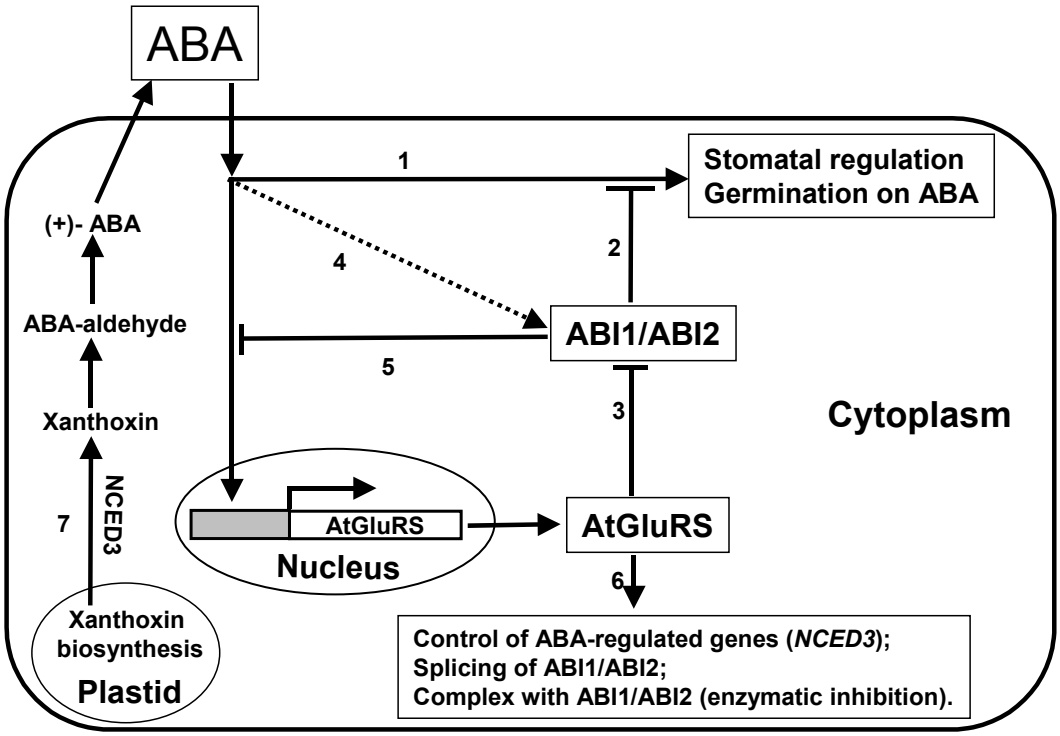


Figure 77. The hypothetical model for AtGluRS action in the ABA signal transduction pathway.

Abscisic acid (ABA) plays a major role (1) in stomatal regulation and germination etc. ABI1/ABI2 are two central elements in ABA signalling and act as negative regulators (2). AtGluRS is an inhibitor of ABI1/ABI2 (3) and the complex of ABI1 may interact with ABA (4). At the transcriptional level, the expression of the AtGluRS gene is dependent on both ABI1 and ABI2 (5). AtGluRS regulates expression of several ABA-regulated genes including the *NCED3* gene and is involved in the correct splicing of ABI1/ABI2 (6). As an enzyme of ABA biosynthetic pathway, *NCED3* is regulated by AtGluRS (7), therefore, AtGluRS may be involved indirectly in ABA biosynthesis.

5 Appendix

5.1 Plasmid constructs

5.1.1 Constructs for the expression of GST fusion proteins

Fibrillin cDNA (945 bp) encoding amino acids 1-315 was amplified from AD/fibrillin identified in this work using two specific primers (FIB for1: 5'-AGATCTGGAATTCGG ATCCTC-3'; FIB rev1: 5'-TTAAGCTTAGATCTCTCGAGGCCCGAAG-3') harboring EcoRI and HindIII sites at its 5' and 3' ends, respectively. After digested with EcoRI and HindIII, fibrillin cDNA was cloned into pSK (Stratagene) which was similarly digested as shown in Figure 78-A.

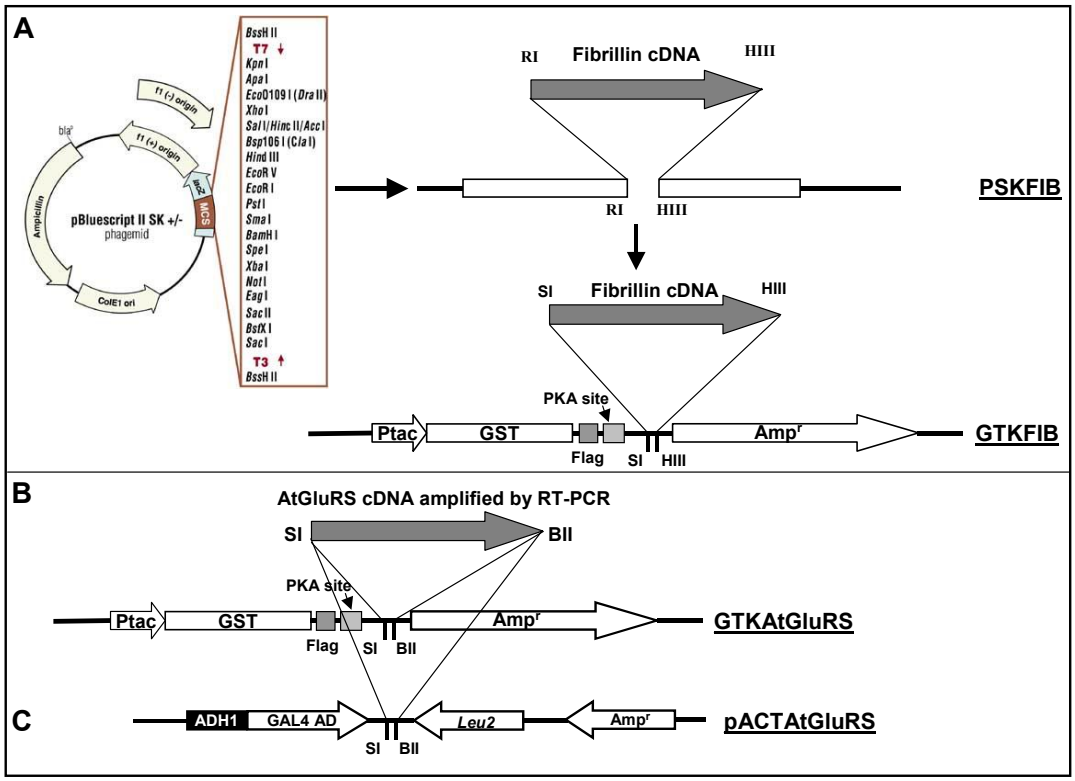


Figure 78. Cloning constructs for overexpression in E. Coli.

A: Fibrillin was amplified from AD/prey with the primers tagged EcoRI and HindIII and cloned into the PSK+, then PSKFIB and GTK were both digested with SmaI and HindIII and ligated. **B:** AtGluRS was amplfied from RT-PCR with SmaI and BglII at the ends of the AtGluRS cDNA. GTK and AtGluRS were both digested With SmaI and BglII then ligated to form GSTAtGluRS; **C**: AtGluRS was cloned into SmaI-BglII sites of pACT10 plasmid for the assay in yeast. RI: EcoRI; HIII: HindIII; SI: SmaI; BII: BglII

For protein overexpression in *E. coli*, GTK (5 kb)--a vector derived from pGEX-2T (Phamacia, USA) tagged with a flag peptide and provided with an N-terminal PKA recognition sequence (RRASV, Blanar and Rutter, 1992) before the polylinker site--was used. PSK-fibrillin was cut with restriction enzymes SmaI and HindIII and ligated into similarly digested GTK as shown in Figure 78-A; the fibrillin cDNA was correctly cloned behind the open reading frame of GST (Glutathione S-transferase) after plasmid was digested with appropriate restriction enzymes.

The full-length AtGluRS cDNA (2.16 kb) was amplified through RT-PCR in *Arabidopsis abi2* mutant seedlings (sequence is shown in Appendix 5.3) with specific primers (Glut f1, 5'-TGGCCCGGGGATGGATGGGATGAAGC-3' and Glut r2, 5'-TCACTTAGCAGAT CTTCCATCTGG-3') harboring SmaI and BglII sites at its 5' and 3' ends, respectively. AtGluRS cDNA and the vector GTK were digested with restriction enzymes SmaI and BglII and then ligated as shown in Figure 78-B. After colonies grew up, the plasmid DNA was isolated and the full-length AtGluRS cDNA sequenced. The AtGluRS cDNA was correctly cloned into the open reading frame of glutathione S-transferase (GST) and the sequence is identical to the AtGluRS provided by Genbank (AF067773) except for 4 nucleotides (see its sequence shown in Appendix 5.3).

5.1.2 Constructs of full length AtGluRS for interaction assay in yeast

Full length AtGluRS amplified from RT-PCR was digested with SmaI and BglII restriction enzymes. The vector pACT10 (Clontech) used for yeast two-hybrid assay was simultaneously digested with the same enzymes. After ligation of the inserts and vectors as shown in Figure 78-C, the full length AtGluRS was correctly cloned in the open reading frame of GAL4 AD according to the result from DNA sequencing.

5.1.3 Constructs of the reporter genes under the control of the fibrillin or AtGluRS promoter

To analyze the expression of fibrillin *in vivo*, several constructs were made to permit transient expression in protoplasts and expression of reporter genes in plants. The reporter genes used in this work were β-glucuronidase (GUS) (Jefferson, 1987) and luciferase (LUC) (Luehrsen et al., 1992). The vector for transient expression was pBI221 (Jefferson, 1987) (Figure 79-A) which has CaMV 35S promoter harboring GUS reporter gene. The binary vector for transgenic plants used in this work was pBI121 (Jefferson, 1987) (Figure 79-B) which has also the same 35S promoter and GUS gene cassette as pBI221. In addition, it has a T-DNA region.

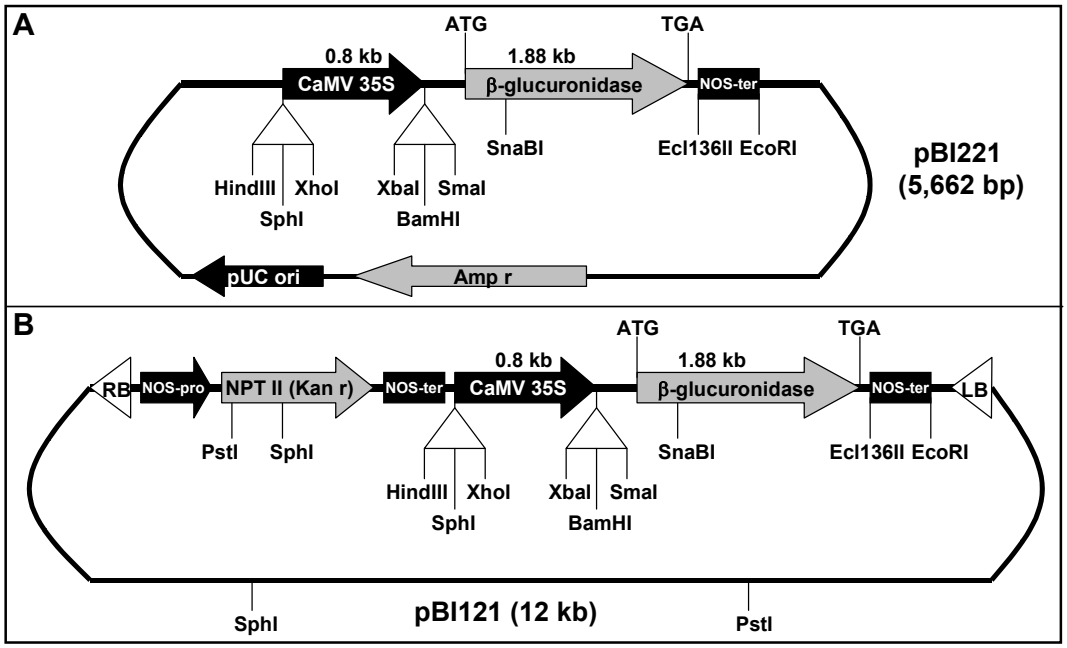


Figure 79. Vector pBI221 (A) and Binary vector pBI121 (B) from Clontech (Jefferson, 1987)

5.1.3.1 Constructs of the reporter genes under the control of the fibrillin promoter

The fibrillin promoter was amplified by PCR (Pwo-polymerase, PeqLab) with the specific primers (FPRf1: 5'-TTAAAGCTTATGAAACATCGTCAGATC-3'; FPRr1: 5'-A ATGGATCCTGTGTTTGTTCTTCAGAGAAACC-3') harbouring HindIII and BamHI sites, respectively. The template DNA was amplified from a BAC T24H24 clone provided by ABRC, Ohio, USA. The BAC T24H24 clone is 88.8 kb in which the fibrillin gene (AF075598) is located. With the primers, one c.a. 1.7 kb fragment from +43 bp to –1700 bp upstream of the fibrillin gene was amplified (sequence is shown in Appendix 5.3). The fibrillin gene and its promoter region in BAC T24H24 in chromosome 4 of *Arabidopsis thaliana* is shown in Figure 80. Fibrillin gene is a 1.4 kb long and interrupted by two introns of 86 and 342 base pairs. There are one abscisic acid response element (ABRE), one CE-1 core, one MYB recognition sequence and one MYC-related DNA binding in its promoter region. Between the fibrillin gene and the next gene upstream in *Arabidopsis* genome there is only a 1.8 kb regulatory region, so the 1.7 kb fragment was selected as the promoter region of fibrillin in this work. After digestion with BamHI and HindIII, the fragment was sub-cloned to PSK digested with the same restrictions enzymes.

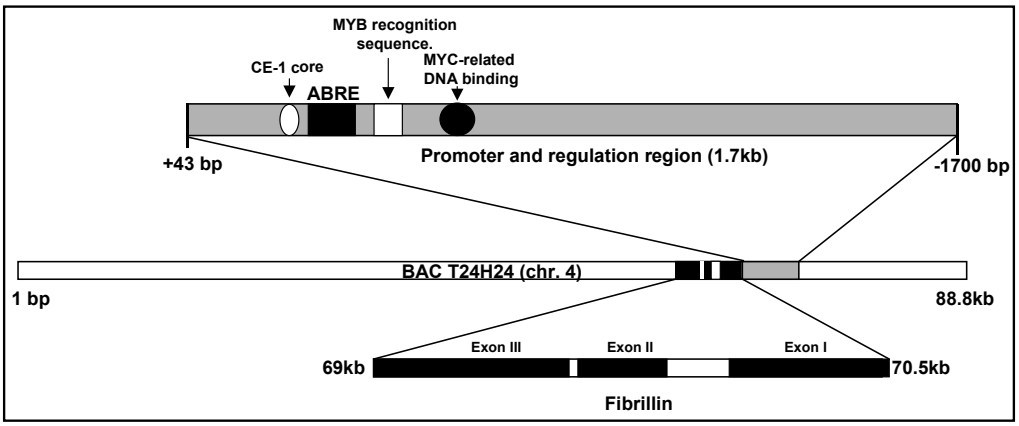


Figure 80. Fibrillin gene and its promoter region

For transient expression studies, the fibrillin promoter sequence was cloned into BamHI-HindIII sites of pBI221 (Jefferson, 1987) in front of the luciferase-nos terminator cassette (Himmelbach et al., 2002) thereby replacing the CaMV 35S promoter. For transgenic *Arabidopsis*, the fibrillin promoter sequence was cloned into BamHI-HindIII sites of the binary vector pBI121 (Jefferson, 1987) in replacement of CaMV 35S promoter. DNA fragments cloned were verified by sequence analysis. Figure 81 illustrated the cloning strategy.

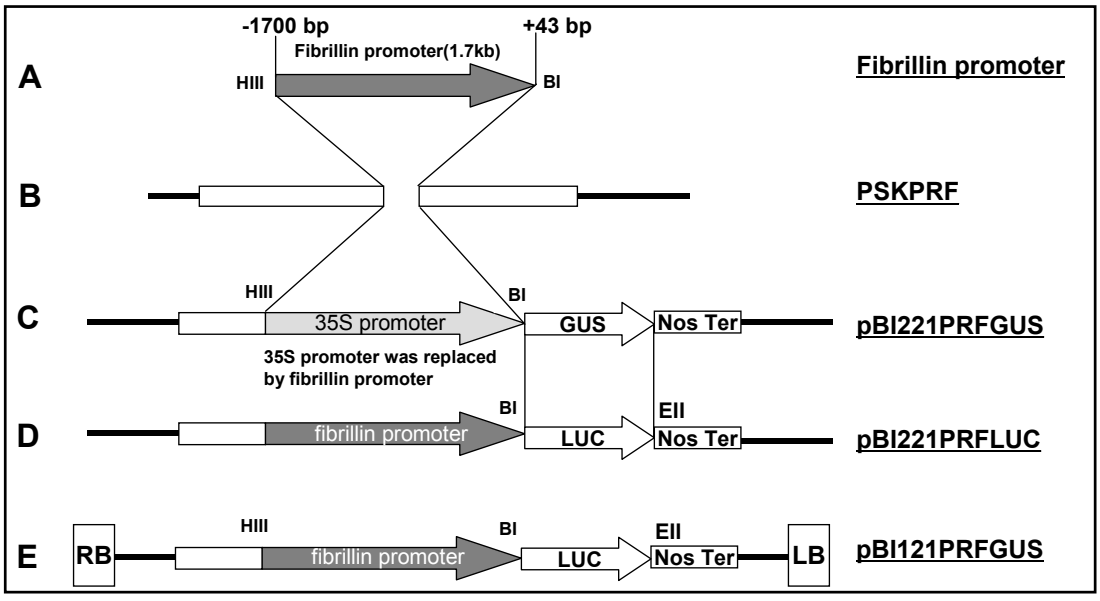


Figure 81. Cloning of fibrillin cDNA and promoter for transient expression in protoplasts.

A: fibrillin promoter (+43 to -1700) was amplified from BAC T24H24 with the primers tagged HindIII and BamHI. B: Subclone fibrillin promoter into pSK in the HindIII and BamHI sites. C: pBI221 which has a GUS gene driven by CaMV 35S promoter and the 35S promoter was replaced by fibrillin promoter After pBI221 and PSKPRF were both digested with HindIII and BamHI and then the vector and insert was ligated. D: GUS reporter gene was replaced by luciferase reportre gene (LUC) after pBI221PRFGUS was digested with BamHI and EcI136II. E: Clone fibrillin promoter into another vector pBI121 was the same like into pBI22. HIII, HindIII; BI, BamHI; EII, EcI136II; XbI, XbaI; PRF, Fibrillin promoter region.

5.1.3.2 Constructs of the reporter genes under the control of the AtGluRS promoter

Isolation of AtGluRS promoter

AtGluRS is located in BAC F21E10 in chromosome 5 of *Arabidopsis thaliana*. The gene is 2.8 kb long with 5 short introns from 77 to 135 base pairs in length (Figure 82).

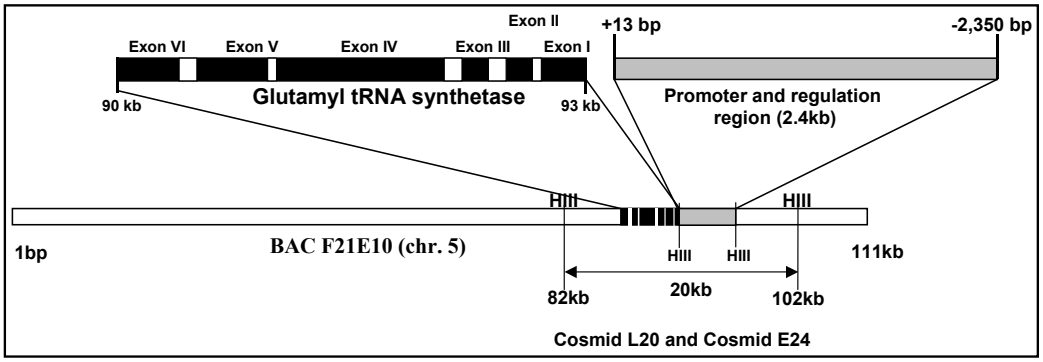


Figure 82. Glutamyl tRNA synthetase and its promoter region in *Arabidopsis thaliana.*

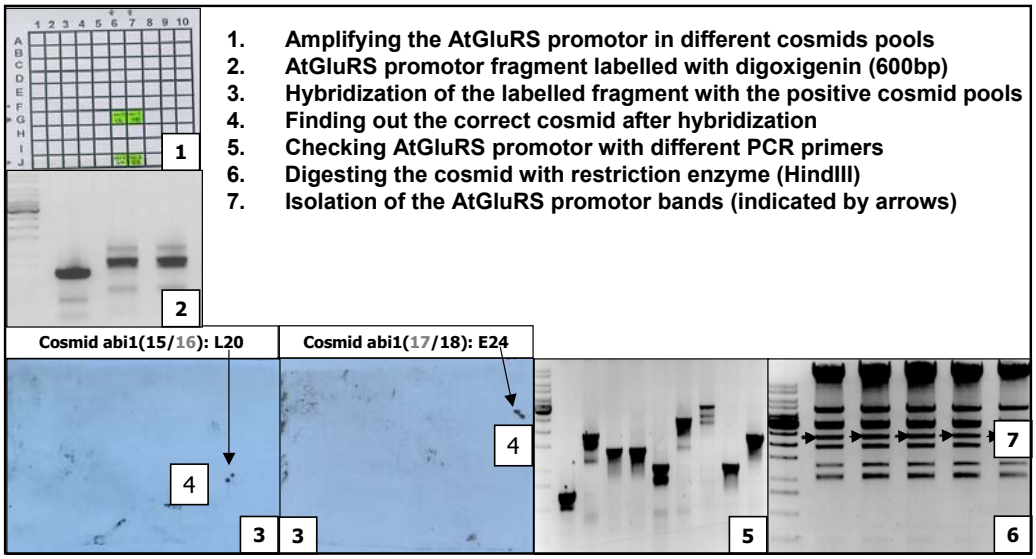


Figure 83: Isolation of glutamyl tRNA synthetase promotor with cosmid screening in *Arabidopsis*.

Cosmid library of *abi1* mutant plant was used to screen glutamyl tRNA synthetase promoter region. 1 to 7 indicate the screening process.

The promoter of AtGluRS studied in this work was digested from the cosmid DNA after screening cosmid DNA pools with *abi1* mutant plants materials (Figure 83). Cosmid L20 and E24 are identical and are 20 kb in length containing AtGluRS. In the upstream region of AtGluRS gene, there is a more than 6 kb regulatory region between AtGluRS and the previous gene. In order to avoid mismatch by PCR, HindIII was selected to digest the cosmid. After cosmid was digested with HindIII, a 2.36 kb fragment with the AtGluRS promoter region from +13 bp to -2350 bp in the upstream of AtGluRS gene was isolated (predicated sequence *in silico* is shown in Appendix 5.3). The sequence was confirmed by PCR with different combinations of primers and digested with different restriction enzymes according to the sequence in databank. All of the PCR products and digestion patterns were the same as expected (Figure 83).

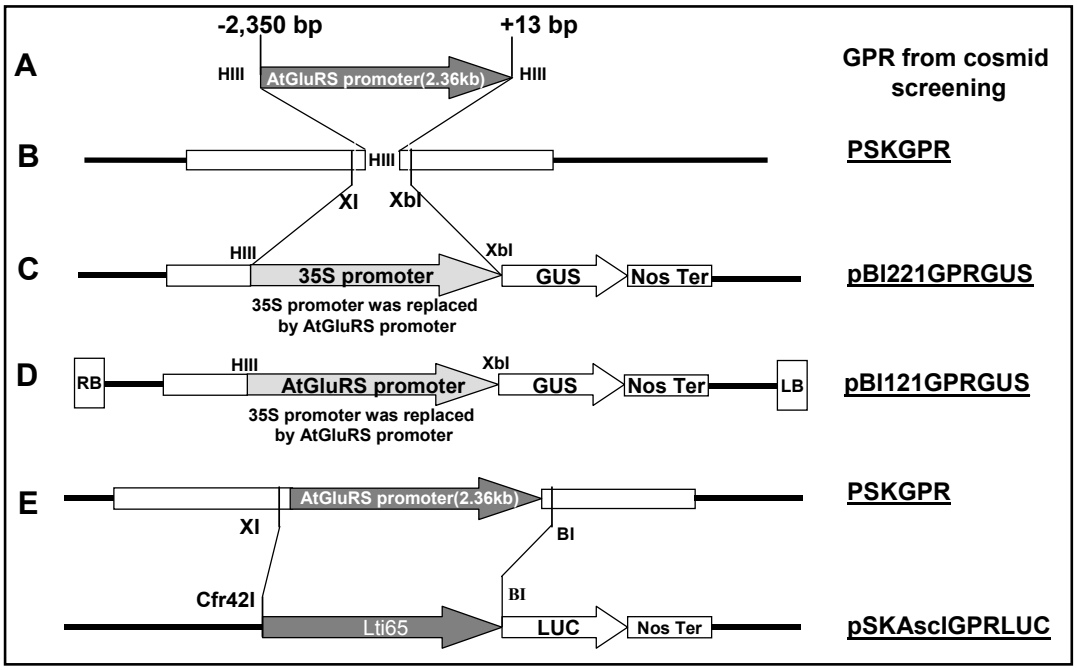


Figure 84. Cloning of AtGluRS promoter with reporter gene.

A: AtGluRS promoter (+13 to -2350) was digested from cosmid E24 with HindIII. **B:** Subclone AtGluRS promoter into pSK in the HindIII site and identify the direction. **C:** pBI221 which has a GUS gene driven by CaMV 35S promoter and the 35S promoter was replaced by AtGluRS promoter after pBI221 was digested with HindIII plus fillin with klenow fragment and XbaI and PSKGPR was digested with XhoI and XbaI, fillin the XhoI site to creat a blunt end and then the vector and insert was ligated. **D:** Clone AtGluRS promoter into another vector pBI121 was the same like into pBI22. **E:** pSKAscILtiLUC was digested with Cfr42I Plus fillin and BamHI and PSKGPR was digested with XhoI plus fillin and BamHI, then insert and vector were ligated, and Lti65 promoter was replaced by AtGluRS promoter. HIII, HindIII; BI, BamHI; XI, XhoI; XbI, XbaI; SI, SmaI; EII, EcI136II; GPR: AtGluRS promoter region.

Constructs of the reporter genes driven by AtGluRS promoter

AtGluRS promoter was subcloned into pSK vector in HindIII site. The PSKGPR (pSK with AtGluRS promoter sequence) plasmids were digested with different restriction enzymes to determine the orientation. The PSKGPR was digested with XhoI then filled in by klenow fragment to create blunt ends and then digested with XbaI. At the same time pBI221 and pBI121 were also digested with HindIII then filled in with klenow fragment to produce blunt end at first, and then digested with XbaI so that the 35S promoter was cut out and replaced by AtGluRS promoter fused with GUS reporter gene. In order to construct AtGluRS promoter with LUC reporter gene, pSKAscILUC was used to clone the construct for transient expression in protoplasts. PSKAscILtiLUC (Hoffmann, 2001) was digested with Cfr42I and filled in using klenow fragment, then digested with BamHI. The PSKGPR was at first digested with XhoI and filled in, then digested with BamHI. After ligation of the vector and inserts, the Lti65 promoter (Nordin et al., 1993) was replaced by the AtGluRS promoter. Sequencing results showed that the reading frame was correct with LUC reporter gene. Figure 84 illustrated the entire AtGluRS promoter constructs used in this work.

5.1.4 Constructs of full length fibrillin and AtGluRS cDNA under the control of 35S promoter

In order to analyze the over-expression of AtGluRS and fibrillin genes in protoplasts and in plants, the constructs of AtGluRS and fibrillin genes under the control of 35S promoter were cloned for this work.

5.1.4.1 Fibrillin cDNA clones

Fibrillin cDNA was amplified from AD/fibrillin screened from the yeast system and was subcloned into pSK (Stratagene). PSKFIB (PSK with fibrillin cDNA sequence) was digested with HindIII and filled in with klenow to produce a blunt end, and digested with XbaI to produce a sticky end. Vectors pBI221 and binary vector pBI121 (Jefferson, 1987) were digested with Ecl136II and XbaI, so the GUS gene was replaced by fibrillin after the ligation of vector and insert. pBI221FIB was used for transient expression in protoplasts and pBI121FIB was a sense construct for overexpression in plants. To construct the anti-sense (RNA interference) orientation of fibrillin, the PSKFIB was digested with SmaI and HindIII to produce blunt ends. The binary vector pBI121 was digested with SmaI and Ecl136II and then dephosphorylated with CIP. After ligation of the vector and insert, the clone with RNA interference orientation was selected after

digestion with the appropriate restriction enzyme. Figure 85 illustrated the constructions for functional analysis of full length fibrillin cDNA in protoplasts and transgenic plants.

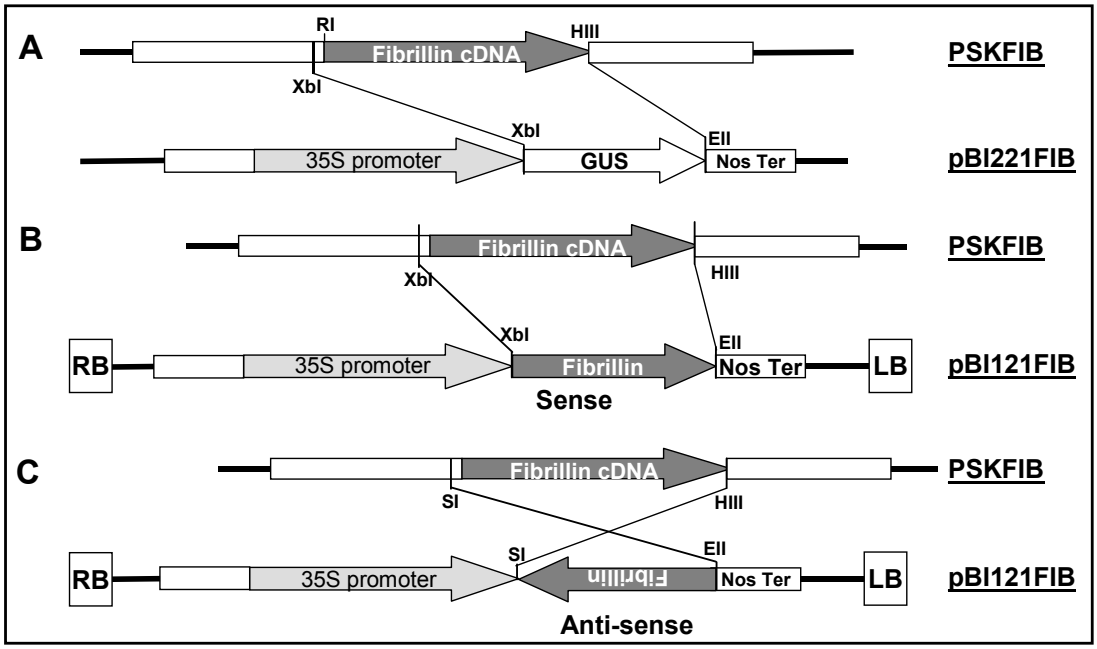


Figure 85. Cloning of Fibrillin cDNA for functional analysis in protoplasts and transgenic plants.

A: Digest fibrillin cDNA fragment from PSKFIB with XbaI and HindIII and fillin the HindIII site with klenow fragment, digest pBI221 with XbaI and EcI136II and then ligate the insert and vector, the GUS gene Was replaced by fibrillin cDNA; B: Sense construct for trasgenic plant was cloned as the same as the cloning into pBI221; C: Anti-sense construct was cloned from the digestion of PSKFIB with SmaI and HindIII and filling the HindIII site to creat blunt end, e vector pBI121 was digested with SmaI and EcI136II, after ligation of vector and insert, select the anti-sense orientation for infection of plant. XbI, XbaI; HIII, HindIII; EII, EcI136II; SI, SmaI; FIB: Fibrillin cDNA.

5.1.4.2 AtGluRS cDNA clones

Full length AtGluRS cDNA was amplified from RT-PCR and directly cloned into the GTK vector. GTKAtGluRS (GTK with AtGluRS cDNA sequence) was digested with SmaI and BglII and then filled in with klenow to produce blunt ends. Fragments of AtGluRS cDNA were isolated with QIAquick gel elute kit. The vectors pBI121 and pBI221 (Jefferson, 1987) were digested with SmaI and EcI136II and dephosphorylated. After ligation of the vector and inserts, the GUS gene was replaced by AtGluRS cDNA. The constructs of pBI221AtGluRS and pBI121AtGluRS were digested with appropriate restriction enzymes to determine the orientation of the clone. The correct orientation of pBI221-AtGluRS was used for transient expression in protoplasts, and both orientations of full length AtGluRS fused with the 35S promoter in binary vector pBI121 were used for overexpression of AtGluRS in plants, one in sense orientation and the other in RNA interference orientation. Figure 86 illustrated the constructions for functional analysis of

full length AtGluRS cDNA in protoplasts and transgenic plants.

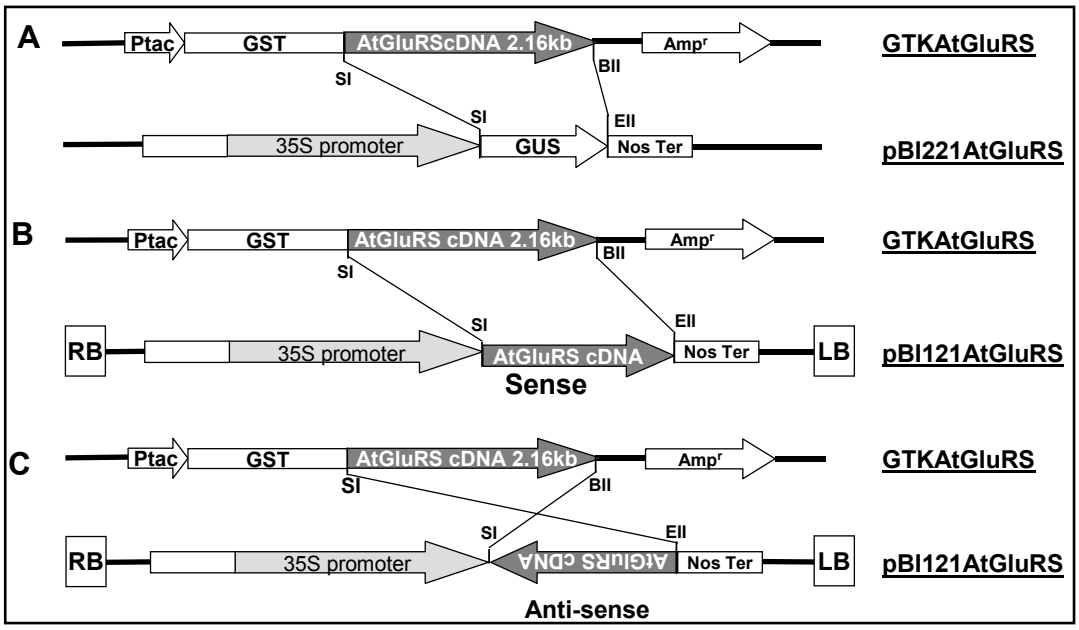


Figure 86. Cloning of AtGluRS cDNA for functional analysis in protoplasts and transgenics.

A: AtGluRS cDNA was digested from GTKAtGluRS with SmaI and BglII, BglII site was fulled with klenow fragment to creat blunt end, pBI221 was digested with SmaI and Ecl136II, after the ligation of vector and insert, the GUS gene was replaced by AtGluRS cDNA. **B-C:** sense and anti-sense constructs for transgenic plants was cloned as the same as the cloning procedures of pBI221-AtGluRS. The both ends of fragment was blunt, so the sense and anti-sense constructs were identified according to the orientation of AtGluRS cDNA. SI, SmaI; BII, BglII; EII, Ecl136II.

5.1.5 Constructs for cellular localization of fibrillin-GUS fusion protein

Plasmid pMESHI (Figure 87-A), obtained from Czaba Koncz, MPI, Köln, was used to establish the constructs for cellular localization of fibrillin-GUS fusion protein. β-glucuronidase cDNA (1.88 kb) digested from pBI221 (Jefferson, 1987) with BamHI and EcoRI was at first cloned into a position behind disrupted c-myc epitope of similarly digested plasmid pMESHI as shown in Figure 87-B. Fibrillin cDNA (about 1 kb), digested from PSKFIB with HindIII and XbaI and filled in with klenow, was cloned into a position ahead of disrupted c-myc epitope of similarly digested pMESHI-GUS. pMESHI-GUS was also used as the control construct. The binary vector pPCV812-MENSHU (constructed by Gabino Rios et al., MPI, Köln, per personal communication; Figure 88) was used to infect the plant cells. Plasmid pMESHI::FIB-GUS was digested with NotI yielding the 35S promoter-fibrillin-GUS expression cassette which was cloned into the binary vector pPCV812-MENSHU, previously digested with NotI (Figure 88), so the cassette from MENSHU was replaced by the cassette from pMESHI with Fib-GUS.

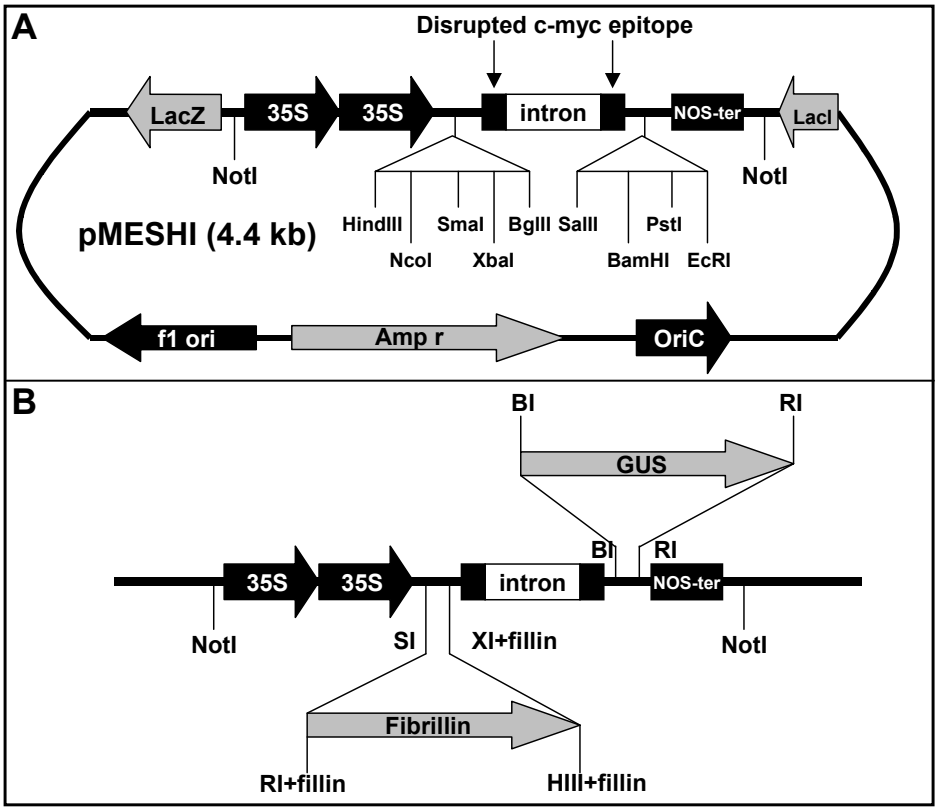


Figure 87. Construct for cellular locatlization of fibrillin::GUS fusion protein.

A: Vector pMESHI (personal communication with Czaba Koncz, MPI, Köln;
B: β-glucuronidase cDNA was cloned behind intron with BamHI and EcoRI, then fibrillin cDNA was cloned behind 35S promoter. RI: EcoRI; SI: SmaI; XI: XbaI; HIII: HindIII; BI: BamHI.

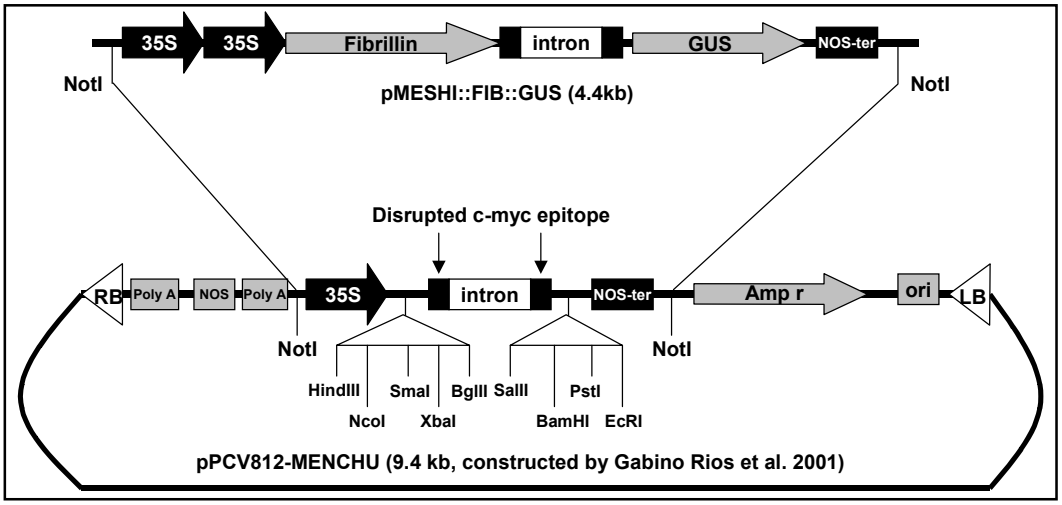


Figure 88. Construct for cellular localization of Fibrillin::GUS fusion protein.

pMESHI::FIB::GUS was digested with NotI and then cloned into binary vector pPCV812-MENSHU (constructed by Gabino Rios et al., MPI, Köln) which was also digested with NotI.

5.2 cDNA sequences of the candidates identified using yeast two-hybrid screening

Twenty AD/preys (12 from Ohio and 8 from Clontech libraries) which showed positive to both reporter genes were sent to be sequenced. The comparison of the insert sequences to databank revealed that glutamyl tRNA synthetase (AF067773) and one unknown protein (AC023628) were found in two independent cDNA libraries with the same sequence. Two of twenty had too short sequences and gave no any identity in databank. Three sequencing did not give the results due to technical problem.

Following are the cDNA sequences of the candidates identified from Ohio and Clontech cDNA libraries as interacting candidates of ABI2 using yeast two-hybrid screening. The cDNA sequences are inserts in AD/prey to be sequenced by MWG with AD forward or AD reverse primer. The sequences belonging to vector are deleted. Most of the candidates are located in the open reading frame of GAL4 AD except for catalase and HB6. The sequences showed here were behind the AD from 5' to 3' with clone identification, encoded protein (accession number in databank) and library detected, respectively.

lc9, catalase (ATU43340) was identified from Ohio library:

ACACGCCGATTTGCGAGATACACACAGTCTCACCTAAAAATCGCAAAAGAAGAAGAATT
CGGATCAAAATTGTCTTCAAGCATC**ATG**GATCCATACAGGGTTCGTCCTTCAAGCGCTC
ATGATTCCCCTTTCTTCACTACAAACTCGGGTGCTCCTGTCTGGAACAACAACTCTTCT
TTGACTGTCGGAACTCGAGGTCCAATCCTTCTGGAGGACTATCATCTGCTTGAGAAACT
CGCTAACTTTGACAGGGAGCGGATTCCTGAGAGGGTTGTTCATGCCAGGGGAGCCAGTG
CTAAGGGTTTCTTTGAAGTCACTCATGACATTACCCAACTTACTTCTGCTGATTTTCTT
CGAGGACCTGGTGTTCAGACTCCTGTTATCGTTCGTTTCTCAACTGTCATCCATGAGCG
TGGCAGTCCCGAGACTCTCAGAGATCCCCGTGGTTTTGCTGTTAAGTTTTACACCAGAG
AGGGGAACTTTGATCTTGTTGGGAACAA

lc1, fibrillin (AF075598) was identified from Ohio library:

CAAAA**ATG**GCGACGGTACCATTGTTCACCCAGTTTCCCTGCAAAACCCTAAATCCAAGC
TCATCAAACACTAAACACCAATCGAAATCTCCGATTCTACTACCGATTAACTCATTAAA
TCGGCGATCGGAGATTGGAGTCTCTGTTCATCGGCCAGATTTCAAAATCCGAGCGACGG
ACATCGACGACGAATGGGGTCAAGATGGAGTGGAGAGAGTATTTGCCTCATCTTCAACC
GTATCGGTAGCAGATAAAGCAATCGAATCCGTGGAGGAGACGGAGAGGCTAAAGAGATC
ACTAGCGGATTCGTTGTACGGAACAGATCGAGGTTTAAGCGCATCGAGTGATACGAGAG
CTGAGATCAGCGAGCTCATCACACAGCTCGAGTCTAAGAACCCTACTCCAGCTCCTAAC
GAAGCTCTGTTTCTCCTCAACGGCAAATGGATCCTCGCCTACAAGTCGTTCGTGGGGTT
GTTCCCATTGCTCTCACGAAGAATTGAACCGTTGGTTAAAGTGGATGAGATCTCACAAA

CCATTGATTCCGATAGCTTCACCGTTCAAAACTCTGTCCGGTTCGCTGGTCCGTTTTCC
ACAACGTCGTTTAGCACCAACGCTAAGTTTGAAATCCGAAGTCCTAAACGTGTCCAGAT
TAAGTTCGAGCAAGGTGTTATAGGGACTCCTCAGCTAACGGATTCGATTGAAATACCGG
AATCCGTGGAGGTTCTTGGTCAGAAAATCGATCTCAATCCCATTAAAGGTTTACTTACA
TCAGTCCAAGACACTGCTTCTTCAGTGGCTAGAACCATTTCAAACCAACCACCATTGAA
GTTTTCTCTGCCTAGTGACAACACGCAGTCGTGGCTGCTCACAACTTATCTCGACAAGG
ACCTTCGGATCTCGAGAGGCGATGGTGGAAGCGTCTATGTGCTCATCAAAGAAGGAAGC
TCTCTCGGCCTTCGGGCCTCGAGAGATCTA

Ilc3, Erd15 (ATHERD15) was identified from Ohio library:

CTAGAGTTTTGAGA**ATG**GCGATGGTATCAGGAAGACGATCTACTCTAAACCCCGACGCA
CCTCTTTTTATTCCGGCAGCTGTACGACAAGTGGAAGATTTCTCACCGGAGTGGTGGCA
ATTGGTGACAACTTCGACTTGGTACCCTGATTACTGGATCAGTCAGCAGCAGCAAGGCG
CGGATGGTTTCTATGACAACGGAGAGAATGAGAATGGTGGAGGTCATATCGATGTAGCT
GATCTTCTTCCAGAATCATTTGATTTTGATGATATGGAAGATTTCTTTGACACTGATGC
TGCTGAGTTTGATCAAGGATTCGATGGAAGAATGTATTACCAAGCACCTTCCGAATTTG
GCTTTGGAAAGAATGGTGAGATGGTTAAGAAATCAAGTGGAAACAGGAGCCCGAGATCG
ATTGTGGAACCAGCTAAGTATGCGGAAAAGCCAGCGAAATGGGGAAACCAGAGGGTTGC
TGCTGCTCCGAGAAACATCCACCAGCCTCGCT

Ilc6, RibA (ATHJ0053) was identified from Ohio library:

CCATCTAACCATCCATTGTCAACCTTTTCCATTAAAACCAATACTGGAAAAGTTAAGGC
TGCAGTGATCTCTAGAGAAGATGATCTGCTCTCATTCACCAACGGAAACACTCCTCTCT
CAAATGGGTCTCTCATTGATGATCGGACCGAAGAGCCATTAGAGGCTGATTCGGTTTCA
CTTGGAACACTTGCTGCTGATTCTGCTCCTGCACCAGCCAATGGTTTTGTTGCTGAAGA
TGATGACTTTGAGTTGGATTTACCAACTCCTGGTTTCTCTTCTATCCCTGAGGCCATTG
AAGATATACGCCAAGGAAAGCTTGTGGTGGTTGTGGATGATGAAGATAGGGAAAATGAA
GGGGATTTGGTGATGGCTGCTCAGTTAGCAACACCTGAAGCTATGGCTTTTATTGTGAG
ACATGGAACTGGGATAGTTTGTGTGAGCATGAAAGAAGATGATCTCGAGAGGTTGCACC
TTCCTCTAATGGTGAATCAGAAGGAAAACGAAGAAAAGCTCTCTACTGCATTTACAGTG
ACTGTGGATGCAAAACATGGTACAACAACGGGAGTATCAGCTCGTGACAGGGCAACAAC
CATATTGTCTCTTGCATCAAGAGATTCAAAGCCTGAGGATTTCAATCGTCCAGGTCATA
TCTTCCCACTGAAGTATCGGGAAGGTGGGGTTCTGAAAAGGGCTGGACACACTGAAGCA
TCTGTTGATCTCACTGTTTTAGCTGGACTGGATCCTGTTGGAGTACTTTGTGAAATTGT
TGATGATGATGGTTCCATGGCTAGATTACCAAAACTTCGTGAATTTGCCGCCGAGAACA
ACCTGAAAGTTGTTTCCATCGCAGATTTGATCAGGTATAGAAGAAAGAGAGATAAATTA
GTGGAACGTGCTTCTGCGGCTCGGATCCCAACAATGTGGGGACCTTTCACTGCTTACTG
CTATAGGTCCATATTAGACGGAATAGAGCACATAGCAATGGTTAAGGGTGAGATTGGTG
ACGGTCAAGACATTCTCGTGAGAGTTCATTCTGAATGTCTAACAGGGG

lc35, glutamyl tRNA synthetase (AF067773) (AtGluRS) was identified from Ohio and Clontech libraries:

CCGTCTCTCTCTCAAACCCACATTGCACAATCCATTTCTCGTTTCTCTGATTTAGATCC
AAAG**ATG**GATGGGATGAAGCTTTCGTTCCCACCGGAAAGTCCACCACTTTCAGTCATCG
TTGCTCTTTCTCTCTCAGCTTCTCCGGTGACGATTGATTCTTCCGCCGCTGCAACAACC
GTCCCTTCTTTTGTCTTCTCCGACGGGAGGAAATTGAATGGAGCCACCGTTCTTCTTCG
CTATGTTGGTCGATCAGCGAAAAAGCTTCCTGATTTCTATGGCAACAATGCTTTTGATT
CTTCTCAGATTGATGAGTGGGTAGATTACGCATCTGTCTTCTCTTCTGGTTCAGAGTTT
GAGAATGCTTGTGGTCGTGTTGATAAGTATCTCGAGAGTAGCACGTTTCTTGTTGGCCA
TTCTCTTTCCATTGCTGATGTCGCTATTTGGTCAGCTCTTGCTGGAACTGGTCAAAGAT
GGGAAAGTTTGAGGAAATCTAAAAAGTATCAGAGTCTTGTTAGATGGTTCAATTCGATA
TTAGACGAGTACAGTGAGGTGCTTAACAAGGTTCTAGCAACTTATGTTAAGAAAGGATC
AGGGAAGCCTGTTGCTGCACCTAAGTCTAAAGATAGCCAACAAGCTGTGAAAGGAGATG
GTCAGGATAAAGGTAAGCCTGAAGTGGACTTGCCGGAAGCGGAGATTGGAAAGGTTAAA
CTCCGGTTTGCTCCAGAGCCAAGTGGTTATCTTCACATAGGACATGCTAAGGCTGCGTT
GCTGAACAAGTATTTCGCTGAGCGTTACCAAGGGGAAGTGATTGTGCGTTTTGATGATA
CTAACCCTGCTAAAGAAAGC

CT134, unknown protein (AB025622) was identified from Clontech library:

GACCGACGCGGAGGTATTGAGAAACCGTGGCTTAAGACGGAGGAAGAAGATGGAAGCTA
ACGGGATTGAGAACTTGACGAATCCGAATCAGGAAAGAGAGTTTATAAGGAGACATCAT
AAGCATGAGCTTGTGGATAATCAGTGTAGCTCTACGCTTGTTAAACATATCAACGCTCC
TGTTCATATTGTGTGGTCACTTGTGAGAAGATTTGATCAGCCACAGAAGTATAAGCCGT
TTATCAGTAGATGTGTGGTGAAAGGAAACATGGAGATTGGTACAGTAAGAGAAGTTGAT
GTGAAATCTGGACTACCAGCAACTAGAAGCACTGAGAGATTGGAGTTACTTGATGACAA
TGAGCATATTCTCAGTATCAGAATCGTTGGTGGTGATCATAGACTTAAGAACTATTCTT
CAATCATCTCTCTTCACCCCGAGACTATAGAAGGAAGAATAGGAACACTCGTGATTGAG
TCATTCGTGGTTGATGTACCAGAAGGAAACACAAAGGATGAGACTTGTT

CT137, AtHB-6 (ATH5J17) was identified from Clontech library:

GCGGCCGCGTCGACCTCTCTCTCTCTCTTTTGGACTTTCTCAATCCATATCCCTCTCTC
TGTTTTTTCTAATTTGAGCCTCTAAAGTCTCCTCGTTTTGTTCATGTTGTTGTTCAAGT
GAAGAAGCTCAATTATGTTTACAACATTGTTGTAATTTCAAAACTTCATAAGAATTTCT
CTGATAATAAAGAAAAAGCTGGAGTAGAACTATTTTAAAGTGTCATCATGAAGAGACTA
AGCAGCTCAGATTCAATGTGTGGTCTAATCTCCACTTCTACAGATGAACAGAGTCCAAG
AGGGTACGGAAGTAATTACCAATCTATGCTTGAAGGTTACGATGAAGATGCTACACTAA
TCGAGGAATATTCCGGCAACCACCACCACATGGGTCTATCGGAGAAGAAGAGAAGATTA
AAAGTTGACCAAGTCAAAGCTCTTGAGAAGAATTTCGAACTTGAGAATAAACTCGAACC
TGAGAGGAAAACTAAATTAGCACAAGAGCTTGGACTTCAACCTCGTCAAGTAGCTGTTT
G

The last 138 bp is identity to HB6 (46 amino acids)

CT266, unknown protein (ATAC006587) was identified from Clontech library:

CAATGACTTCCTTTCATTATTAAATAAAAATACAAATATACAAAACGGTCTCCTTCTCG
TTAGCGATAGGAGAAGATTCTTAGAATCTTAATACAACCTCGCTCTTTTATCTAATATG
ACTACATAATATTAACACGTAACATGTTTGAAAAGTTTGTAGCAGTTTCGTTACGTGCT
TATACATAATTGGTTGACTTAGAGAAATTAAGAAAAAAACAAACTACGCATATATCATA
TCCTAGGTCTTCAATATGCATATGGAAGTAACAACTAAAGCGCTTTAAAGTGAGCTAAT
AATACCATAAGTTATGATCATGTCCAAAGAGGATCCCACCACGACATTGATGAATTATT
GTTGTTGTTGTTGTCCTCAAGGAACCCCAAATCATTGCTCTCTTCGTCCGGGCTACTAA
TGAACTCATCGCCGCTTTCAAATGAACTCTCAAAGGACATCTTCTGTTGTCCTTGCTCT
TGTACTGACAACTTTGTTTGCTCCATATTATATATCAATTCTAGTAGACTTGATCTTTG
GAGTTGCATGCCTACTAACTCTACTTGAGTTTT

IV17, hypothetical protein (AF007271) was identified from Ohio library:

CGATGATGAAGATACCCCACCAAACCCAAAAAAAGAGATCTGGAATTCGGATCCTCGAG
GCCACGAAGGCCCTTCGGTTAAAGGATGGACAACCGGTTCCGGTATGGAAGGACCGTCT
CTACCGGCTAAAACCGATACAGACACGGTTTCCACATTTCCATGGTCATTATTCACTAA
ATCGCCTCGTAGGCGAATGCGTGTTGCTTTCACTTGTAACGTATGTGGGCAAAGAACTA
CAAGAGCTATTAATCCTCATGCTTACACTGATGGCACTGTCTTCGTGCAGTGTTGTGGA
TGTAATGTGTTTCATAAGCTGGTCGATAATCTCAACTTGTTTCATGAGGTTAAGTATTA
TGTGAGCAGCTCGAGCTTCGATTACACCGATGCTAAGTGGGATGTTAGCGGCTTGAATC
TTTTCGATGATGAGGGGCCTTCGTGGCCTCGAGAGATCTATGAATCGT

VII45, Ath Cab binding protein (CAA39534) was identified from Ohio library:

CCGCCGTGTACCCTTCGCTTCTCTCTTCTTCCAAGTCTAAATTCGTATCCGCCGGAGTT
CCACTCCCAAACGCCGGGAATGTTGGTCGTATCAGAATGGCTGCTCACTGGATGCCTGG
CGAGCCACGACCAGCTTACCTTGACGGTTCTGCTCCTGGTGACTTTGGGTTTGACCCAC
TTGGACTTGGAGAAGTTCCAGCGAACCTTGAGAGATACAAAGAGTCAGAGCTCATCCAC
TGTAGATGGGCTATGCTCGCTGTTCCTGGGATTTTGGTACCAGAAGCATTAGGATATGG
AAACTGGGTTAAGGCTCAGGAATGGGCAGCACTACCAGGGGGTCAAGCCACTTACTTGG
GAAACCCAGTCCCGTGGGGTACTTTGCCCACAATCTTGGGGCCTTCGTGSCCTCGAGAG
ATCTATGAATCGTAGATACTGAAAAACCCCGCAAGTTCA

VII48, Ath mitochondrial genome (MIATGENB) was identified from Ohio library:

GGCCGCGGTCATTTAGGGGCCTTAGCTGGTGATCCGGGCTGTTTCCCTCTCGACGATGA
AGCTTATCCCCCACCGTCTCACTGGCCGACCTTGACCCCAGTTATTTTGAGGTCATATC
TAGTATTCAGAGTTTGCCTCGATTTGGTACCGCTCTCGCGGCCCGCACCGAAACAGTGC
TTTACCCCTAGATGTCCAGTCAACTGCTGCGCCTCAACGCATTTCGGGGAGAACCAGCT
AGCTCTGGGTTCGAGTGGCATTTCACCCCTAACCACAACTCATCCGCTGATTTTTCAAC
ATCAGTCGGTTCGGACCTCCACTTAGTTTCACCCAAGCTTCATCCTGGTCATAGATAGA
TCACCCAGGTTCGGGTCCATAAGCAGTGACAATTGCCCTATGAAGACTCGCTTTCGCTA

CGGCTCCGGTGGGTTCCCTTAACCAAGCCACTGCCTATGAGTCGCCGGGGCCTTCGTGS
CTCGAGAGATCTATGAATCGTAGATACTGAAAAACCCCG

XIII6, peroxiredoxin (CAA71503) was identified from Ohio library:

GGCCTGTCAATGGCGTCTATAGCTTCTTCTTCTTCCACCACCCTACTCTCTTCCTCTAG
GGTTCTTCTTCCTTCAAAGTCTTCTCTTTTATCTCCTACCGTCTCTGTCCCCAGAACCC
TACACTCTTCCTCGGCATCATCCTCTTCTCTCTGTTCCGGGTTCTCCAGTCTCGGTTCC
CTCACCACCAGCCGCTCCGCCTCACGCCGGAACTTCGCCGTCAAGGCTCAGGCTGATGA
TTTACCACTGGTCGGTAATAAGGCGCCTGATTTTGAAGCAGAGGCAGTTTTTGATCAAG
AGTTCATAAAGGTGAAGCTCTCTGAGTACATTGGCAAAAAGTATGTTATTCTATTCTTC
TACCCTTTGGACTTCACTTTTGTCTGCCCCACTGAGATTACTGCCTTCAGTGACCGTTA
TGAAGAATTTGAGAAGCTAAACACCGAAGTATTAGGGGTCTCTGTCGACAGTGTGTTCT
CGCATCTCGCGTGGGTCCAAACAGACAGAAAGTCGGGAGGGCTCGGTGATCTGAATTAT
CCTCTTGTTTCGGATATCACTAAATCCATTTCAAAATCGTTTG

CT176, unknown protein (AC023628) was identified from Ohio and Clontech libraries:

GGCCGCGTCGACGGACGGCGTTGAAGGCGGCACGGCGATGTACGGTGGTCTCGAGACGG
TGCAATACGTACGGACGCATCATCAACATCTGTGCAGAGAAAACCAGTGTACCTCTGCT
CTTGTCAAACACATCAAAGCTCCTCTTCATCTCGTTTGGTCACTTGTACGGAGATTTGA
TCAGCCGCAGAAATACAAACCGTTTGTGAGCAGATGTACAGTAATCGGTGATCCTGAAA
TCGGCAGTCTTAGAGAAGTCAATGTTAAATCTGGTCTTCCTGCAACAACATCTACTGAG
AGATTAGAACTTCTTGATGATGAAGAACACATCCTCGGTATCAAAATCATCGGTGGTGA
TCACAGACTTAAGAATTACTCGTCGATTTTGACGGTTCATCCGGAGATAATCGAGGGAA
GAGCAGGAACGATGGTGATTGAATCGTTTGTAGTTGATGTTCCTCAAGGTAACACAAAG
GATGAGACTTGCTACTTTGTTGAAGCACTTATCAGATGTAATCTCAAGTCACTAGCAGA
TGTTTCTGAAAGATGGCTTCTCAGGACATTACTCAGTGAACTACATAATCAGTCGACGC
GGCCGCGAATTCCAGATCTATG

5.3 Sequences of fibrillin promoter region, AtGluRS gene and its predicted promoter region used in this work

Fibrillin promoter region:

A 1.7 kb fragment in the upstream region of fibrillin gene was amplified from BAC T24H24 (DNA Stock Center, ABRC, Ohio, USA) with primers (forward: 5'-TTAAAGC TTATGAAACATCGTCAGATC-3'; reverse: 5'-AATGGATCCTGTGTTTGTTCTTCA GAGAAACC-3') harbouring HindIII and BamHI sites, respectively. TATA-box is as indicated in box.

(+43 bp)

tgtgtttgttcttcagagaaacctcacgaattaggat**tttata**accgttgagctggact
gtaaatgacgtatataccccttatctggtacactttcttatgaacatataagtcaagatc
caagggatagaataggtatttggaatagaacaaatgaggtaagctaaggtgggattttg
acacgtggatggttaaagatcattgttttaagaatcgacctggactcgtgtgaagttgc
cgactttatgaaaaattcataaaatttgtggaagattttatttcttcaatagggaaac
aatttggtttaatttggtttttgtcccattatttgtgtgggggagaatttgtttgggtt
ttctacgagagttaggccatgcaatgcaaatggcatgatggatctagaatcttcggacg
tgtactatacttttttggccggttttggtgatcttcgatcttcagttttctgaacggtga
agattctctccaatgcaaaatatctaaacggcgattaatcaacagtgtaataagtagtt
ggactaaatgggcctggatagtatatttaatgggcctttattaacaaagaagacccatt
aaaaatttaaaatgaatacaatagttttgtatccaatagaccaataggtccaaaacgtt
cttaaagggagaaaaattagctggtaaaataatagtaccggtgtttgttcaaaaaaaat
aataataataactgggcaggtgtagatattcgggtactcatatctgttcggttcttt
ttttttttgagtatcggatctatttaggtatgaagttaaaaaccattcatgtattctaa
aattttggttcggttttggtttgagtttggatcgatttagttattttagtaagaattga
aaaataatcaaaaatactcaaaatatttttgggttcaggtcgagttcgagtttttact
catctatacctaaatctaaccgagatgcctgattagtttatccaaaaatatattagtta
tattttaccaaaatataaccaaatgtaaccaaaatacttaaattacttagaaaactata
gggtgagttaaaaatatatacatggataaacagaatattaagaatttatacataaaaat
atgttatttacgtaaataggaaagagaaataaaatataaaccaaaaaatatccttccgg
tttagattaattacaaaaatgccattgattcaaaatactttaatatttaattgggagat
ttgcaaaactgcccttattctaataacctttttgtaaaaatgtcctttcctgaaggccat
ttttaaaactacccttttctaatatggcaaatgactgtcttacccttactgaatgattag
gtttcacattcgtttcacaatacaattcgttttggcacaaacttcttcgttttggccaa
cgaattttcctatccgatttcactcaaaatcgccggcgaatttcctctccgatttcact
caaaattgccgacgaatttcctctccgatttcactcaaaatcgccaacgaatttcctct
ccgatttcactcaaaatcgccgacaaattttctctccgagaaaccccaaaatcgctgac
gaatttcctctccgataaatctgttcttcgctgacggattcagatcatttctctacgac
gcaaacaatccccagagatatcgatagatccgacggtaaccgtttttataatcgatttt

atagtcgattttatagtagatctgacgatgtttcataacatgttttgcag (-1704 bp)

AtGluRS gene:

AtGluRS used in this work was amplified by RT-PCR with specific primers (Glut f1, 5'-TGGCCCGGGGATGGATGGGATGAAGC-3' and Glut r2, 5'-TCACTTAGCAGAT CTTCCATCTGG-3'). Total RNA was isolated from *abi2* mutant plant. Then, the full length AtGluRS cDNA was cloned into GTK vector (Fig. 78-B) and to be sequenced (MWG, Germany). The DNA sequences between glutamyl tRNA synthetase amplified by RT-PCR in this work and presented in databank are almost complete identical except for four nucleotides.

The changed nucleotides are presented as following:

1. In the position of +1680 bp, adenine (A) in the sequence presented in databank is changed to guanine (G). Corresponding amino acid encoded does not changed;
2. In the position of +1738 bp, guanine (G) is changed to adenine (A). And, the encoded amino acid in the position of 580 is changed from valine (V) to isoleucine (I) in the protein sequence;
3. In the position of +1776 bp, thymine (T) is changed to cytosine (C) and the encoded amino acid does not changed;
4. In the position of +1962 bp, thymine (T) is changed to guanine (G). The amino acid in the position of 654 is changed from aspartic acid (D) to glutamic acid (E).

Analysis of sequencing for three times displayed the same results. Four nucleotides were written in capital letter with boxes as shown in the following cDNA sequence.

(+1 bp)
atggatgggatgaagctttcgttcccaccggaaagtccaccactttcagtcatcgttgc
tctttctctctcagcttctccggtgacgattgattcttccgccgctgcaacaaccgtcc
cttcttttgtcttctccgacgggaggaaattgaatggagccaccgttcttcttcgctat
gttggtcgatcagcgaaaaagcttcctgatttctatggcaacaatgcttttgattcttc
tcagattgatgagtgggtagattacgcatctgtcttctcttctggttcagagtttgaga
atgcttgtggtcgtgttgataagtatctcgagagtagcacgtttcttgttggccattct
ctttccattgctgatgtcgctatttggtcagctcttgctggaactggtcaaagatggga
aagtttgaggaaatctaaaaagtatcagagtcttgttagatggttcaattcgatattag
acgagtacagtgaggtgcttaacaaggttctagcaacttatgttaagaaaggatcaggg
aagcctgttgctgcacctaagtctaaagatagccaacaagctgtgaaaggagatggtca
ggataaaggtaagcctgaagtggacttgccggaagcggagattggaaaggttaaactcc
ggtttgctccagagccaagtggttatcttcacataggacatgctaaggctgcgttgctg
aacaagtatttcgctgagcgttaccaaggggaagtgattgtgcgttttgatgatactaa
ccctgctaaagaaagcaatgagtttgtggataatcttgtgaaggatattgggaccttgg
ggatcaagtatgagaaagtgacatacacttcggactattttcctgaattgatggatatg

gcggaaaaactgatgcgtgagggtaaggcatatgttgatgacacaccgagggagcagat
gcagaaagagaggatggatgggattgattcgaaatgtaggaatcatagcgtcgaggaga
atttgaagctatggaaggaaatgattgcaggaagtgagagaggattacagtgctgtgtt
cgtgggaaattcaacatgcaagatcccaacaaagccatgcgtgacccggtttattaccg
atgcaatcctatgtctcaccaccgtatcggggataagtataagatatatccaacatatg
actttgcttgcccgtttgttgattcccttgaaggtataacgcatgctcttcggtctagt
gagtatcatgaccgaaatgctcagtactttaaagttctggaggatatgggactgcgaca
ggttcagctttacgaattcagccggttaaacctagtttttacacttctcagtaagcgca
agcttctctggtttgtccaaactggattggttgacgggtgggatgatccacgtttcccg
acagtccaaggaattgttcgtagaggtttgaaaatcgaggctctgattcaattcattct
cgagcaggggcttcgaagaatctaaatttgatggaatgggacaaactttggtctataa
ataagagaataattgatcctgtgtgccctagacacactgctgtggttgcagaacgtcgt
gtactatttaccttaacggatggtcctgatgagccgtttgttcgcatgataccaaagca
caagaaattcgaaggtgctggagaaaaGgcgaccactttcactaagagcatttggctcg
aggaagctgatgcgagtgccatatccAttggtgaggaagtaactttgatggattgggga
aatgcCatcgtaaaggaaatcacaaaggacgaggagggtcgtgtcactgccttatctgg
tgtcttgaatctccaaggttctgtaaagactacaaagctgaagctgacatggcttcctg
atactaatgaattggtcaatctcacattaacagagtttgattatctaatcaccaagaag
aagctggaagatgaGgatgaagttgctgattttgtgaatcctaacacaaagaaggaaac
attggcacttggtgattcgaatatgaggaatctgaaatgtggagatgtgattcagcttg
agaggaaaggctatttcagatgtgatgtgccttttgtcaaatcttcaaagcccattgtc
ttattctccattccagatggaagagccgctaagtga (+2160 bp)

Promoter sequence of AtGluRS (predicted according to databank after digested with HindIII):

Promoter region of AtGluRS was screened from *abi1* cosmid library and the fragment containing promoter region of AtGluRS was digested by HindIII and purified (See Appendix 5.1.3.2). The sequence was confirmed by PCR with different combinations of primers and digestion with different restriction enzymes according to the sequence in databank. All of the PCR products and digestion patterns were the same as expected.

(+13 bp)
tcatcccatccatctttggatctaaatcagagaaacgagaaatggattgtgcaatgtgg
gtttgagagagagacgagaaacagaaccctagtttttggttgagtggtttcgggtctt
aggattaggtttttacttttttaactgtgggcggctccaagttgtcaacttttcaatt
tttagtccataaagtatcaatctttacatagtgtttttttttttaactcaacttggatt
agagtaataagtagttaaattggtatgtacacatcatttggagaatcatactatactaa
aactatccttggttatatgagttatgtgactggtttctgtatcagtgtctctgcagttc
tcttttcaagagagaatacttcatgggagctccaatgcagaggcaattcagaaaagcta
aaaggcaatacaaatctttgaactaaaatctctcatagactcatataggaaaaaatcga
aacagataaacactgtgccaatagaactaaggaatgggaagtaaccagatactcattta

gcaataccactgaaatccaatgctaaatggtccctcttctttggacaaggtcattgctt
aacctgctttttgttcgattcacgaaaccagaaagactatcaatcaactctcaaccaaa
tgtgcacaccacacatcctaaacaatctaccgagacatcattataaacctagccatcac
taaaatctctgtaaaccaagtcacaacaggctatattcagtcctaggctagttttagta
gaccaatagactaagtcttgagcacctaaacttcttcgaatacacacattatctgtctt
gaactatcatcttacacatttggcacattagagagacaaaaaaatcatctaaatttact
ctgcactgaagccaatcttaatctaaatacattgaacttactataagtttgatcatctc
ccacaaagctaactcaactaaatgaaatctaccgataacaaatctgattcaatacacaa
ttgccctttaaagaatcatcactggtctaaaacataagaaacaaaacactcatagcca
tttgatggagagattctatacagattgatccaaagaaagaaacccaaaaccaaaaaatc
tataaatataagaatcaagcaaccccaaatcccaataacgaaacatacaaaatattcac
cgatgtggattacactcatcagagcagaaatcattagtctctaacataactctcaacat
ctcatgactaaacctttcatcattaggatcagaaactggagacgaaaccgaagaatcat
catcacctccttctctccttcgaggtctcatcacacgaaagcacccacctttacgcgac
ccagaccatcgttttggcgatttgggtttcttacgaaacttacctaaaaatcgattctt
actctttaccaattgattggtggtatcgaattgattatcgtcatcatcaacgattgtta
catctttcttcttcttcttccttgtcgtcgtcttcgtcttggtatcgttatcatcg
actctgttttgttcattcaatgaaatctcactgtgattcccaggcattaaccaaacgag
aaaaagaaagattccgaatttctcaaaacccagatcagaattgtgaagaaattgaggtc
tgcttttgtgttgttgttgttgttgttgttgttgtattgtctctcttcttcactta
ggtttttccccaatcagaaaaaatcttcgctggctctcacaaattaattgtgtgtttaa
cgacttttattaatttagtattattttattagattcgggtatatcgctgtttttatatg
gaaattttattattattattactgtttttttttcttattattattgttggttgactgaaa
aataaaatcttattactggttttgatgaagaacacgttttatacttgccattgttgttt
tgtttgtcccaagcaaaagttaccaaaattgaaacggatctcgataaaggagtagttac
gccggctttattctctatatttggtttcagtttgtgattctttagcatttttataatat
tctattagaggggtgtacattcttgtttacggtttggtttttgtttttcaacttcaaaa
caaaaatagaaaatagtttcttcatttcttcggcatctaagtttcagtgttagccattc
ttaataaagtgaatttgcaccgtttagtatattttggtttgtgagattctatttagtga
tatagttcatggaattgtccgataaaccgtgttcacatgacttgggaatagtatgtgat
ccggggcttatcggtctttgctgcggacggccaatctcgggcctcatgttaaa
(-2354 bp)

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Publications

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