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RESEARCH PAPERS

# The ABA-Binding Protein AA1 of *Lupinus luteus* Is Involved in ABA-Mediated Responses[1]

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**Abstract**—We characterized AA1 (Abscisic acid Activated 1), a protein from *Lupinus luteus* L. predicted to be located in the apoplastic space who's mRNA and protein levels are strongly regulated by ABA, salt stress, and hypothermia. A fragment from the recombinant AA1 protein binds ABA as shown by the spectrofluorimetric titration assay of the protein by ABA. The BLAST software of the DFCI database identified more than 200 ESTs from 46 dicots and monocots, including three genes with unknown function from *Arabidopsis thaliana*, which are closely related to the lupine *AA1*. The central part of the proteins encoded by these genes contains the TolB motif from *Escherichia coli* and shares conserved WD40-like repeats, which form the basis for the tertiary beta-propeller structure and provide a potential platform for the assembly of protein complexes. Our data suggest that the highly conserved AA1 proteins from *L. luteus* and other higher plants are involved in ABA-mediated responses.

*Keywords: Lupinus luteus, Arabidopsis thaliana*, abiotic stress, ABA-binding protein, phytohormones, differential display, gene expression

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## INTRODUCTION

Phytohormones, in particular ABA, are important for adaptation mechanisms of plants to stressful environmental conditions. ABA is involved in the protection of plants against a wide range of environmental stressors, such as drought, salinity, cold, as well as pathogen attack. The hormone plays an important role in the colonization of ecological niches where water availability is limited or unstable [1]. Understanding the mechanisms of ABA perception and signaling also attracts attention for improving drought tolerance of cereals and other crops [2].

An important area of ABA signaling is the identification of ABA-binding proteins or receptors. The existence of receptor sites for ABA binding was postulated about two decades ago [3], and many proteins with ABA-binding features were identified in the last decade [4]. The best characterized and most favored candidates for ABA receptors are members of the PYR/PYL/RCAR protein family. A variety of methods, including Röntgen structural analyses of ABA-receptor complexes [5], mutants inactivated in *PYR/PYL/RCAR* genes and the analyses of a minimal ABA signal transduction pathway in protoplasts [6] demonstrated that members of the PYR/PYL/RCAR family are ABA receptors characterized by specific hormone recognition and signal transduction [7].

However, at least 10 more potential ABA receptors have been proposed: ABAP1 from barley seed aleurone, the receptor-like protein kinases RPK1 from *Arabidopsis thaliana*, ABAR/GUN5/CHLH identified as H subunit of the heterotrimeric $Mg^{+}$-chelatase complex from *A. thaliana* and its homologue from *Vicia faba*, which have been characterized as ABA-binding membrane proteins, the putative GPCR-protein GCR2 and its homologues GCL1 and GCL2 [4], as well as the GTG1 and GTG2 receptors bound to heterotrimeric G-proteins [8].

The description of other ABA-binding proteins, the complexity of ABA signaling [9], and the integration of ABA in numerous signaling networks, which include second messengers such as $Ca^{2+}$, phosphory-

[1] This text was submitted by authors in English.

*Abbreviations*: *AA1* – *Abscisic acid Activated 1*; Ab – antibodies; BA – benzyladenine; DPP IV – IV dipeptidyl peptidases; FITC – fluorescein isothiocyanate; IgG – immunoglobulins; *154N-AA1* – coding sequence of *AA1* gene in vector pQE-30 including the sequence encoding the 6xHis and additional 5 amino acids from the pQE-30 vector.

*Electronic supplementary material*: The online version of this article contains supplementary material, which is available to authorized users.

lation cascades, phosphoinositides, phosphatidic acid, and reactive oxygen species [1, 10] suggest that additional components involved in ABA perception and signaling may exist.

We identified a novel gene (*Abscisic acid Activated 1*, *AA1*) from *Lupinus luteus* L., which was activated by ABA and inhibited by cytokinin. The objective of this work was to study the physicochemical properties and biological role of AA1 from *L. luteus*.

## MATERIALS AND METHODS

**Growth of yellow lupine seedlings.** Seeds of yellow lupine (*Lupinus luteus* L., cv. Akademicheskii I) were sterilized with concentrated sulfuric acid for 10 min, scarified, and germinated in the climatic chamber at 23°C in darkness for 3 days on moist tissue paper. The cotyledons were cut in dim green light and kept in darkness for further 24 h on water to decrease endogenous cytokinin and ABA levels. They were then placed in Petri dishes on tissue paper soaked with water or solutions containing either ABA ($7.6 \times 10^{-5}$ M) or cytokinin (BA, $2.2 \times 10^{-5}$ M) in darkness or high-intensity white light (120 μmol/($m^2$ s). To study the effects of abiotic stressors, lupine was grown in trays on moist tissue paper in darkness until the 9th day; then seedlings were transferred to 150 mM NaCl solution (the concentration was optimized in pilot experiments) or cooled to 4°C. The plant material was fixed in liquid nitrogen 1, 2 or 3 days after the start of the experiment.

**DNA isolation and Southern analysis.** Genomic DNA was isolated from lupine cotyledons as described by Sambrook et al. [11]. The DNA was digested with the indicated restriction enzymes, and DNA fragments were separated on a 1% agarose gel (10 μg DNA per line) before transfer to nylon membranes. Filters were hybridized to radiolabelled DNA fragments [11].

**RNA isolation and northern analysis.** Total RNA was extracted from lupine cotyledons using TRiozol reagent (Gibco/BRL, United States) according to the manufacturer's protocol. RNA was electrophoresed on a 1.2% agarose–formaldehyde gel and blotted onto Hybond-$N^+$ membranes (Amersham Pharmacia Biotech, England) by capillary transfer [11].

Radioactive probes for hybridization were produced by PCR in the presence of [α-$^{32}$P]-dCTP. PCR-generated and purified fragments of the corresponding genes served as templates. RNA gel-blot hybridization with [$^{32}$P]-labeled probes and subsequent membrane washing were carried out as described [11]. Radioactive signals were detected and quantified using a Phosphorimager (Typhoon Trio$^+$, GE Healthcare, United States) or by autoradiography.

**Differential display.** The differential display method was mainly performed according to Liang et al. [12], as specified in the protocol of the GenHunter mRNA Differential Display Kit (GenHunter Corporation, United Kingdom). The differential display method was performed as described in the Supplementary Method 1.

Bioinformatic methods used in the work are described in the Supplementary Method 2.

***AA1* constructs.** A full-length cDNA of *AA1* (2137 bp) from the phage library (λgt11) of yellow lupine was cloned into the *Eco*RI site of the pBlueScript II KS+ (Stratagene) vector. A *Bam*HI restriction site was created by the replacement of $A_{241}$ of the cDNA by a C. After amplification, the modified cDNA fragment was restricted with *Bam*HI and *Hin*dIII and cloned into the same sites of the pQE-30 vector. Thereafter, the 3'-region of the cloned sequence was removed by restriction at the *Cla*I site, an internal restriction site in *AA1*, and *Hin*dIII. As a result, a 429 bp-long *AA1* fragment remained in the pQE-30 vector; it corresponds to the region 236 to 664 bp of the initial cDNA. The size of coding sequence of *154N-AA1* in vector pQE-30 is 462 bp (including the sequence encoding the 6xHis and additional 5 amino acids from the pQE-30 vector).

**Overexpression and purification of the recombinant protein using affinity chromatography on Ni-NTA Sepharose.** The cDNA fragment (429 bp of *AA1* plus additions) was expressed in the M15 strain of *E. coli*. The expressed protein represented the fragment of AA1 comprising 143 amino acid residues, the 6xHis tag, and additional 5 amino acids from the pQE-30 vector (mol wt of expressed protein was 17.5 kD). The produced recombinant protein was purified by affinity chromatography on the Gravity Flow column packed with Ni-NTA-Sepharose (Qiagen) as recommended by the manufacturer.

**Production of antibodies (Ab) against the recombinant protein.** The recombinant protein purified on Ni-NTA-Sepharose was used for rabbit immunization to obtain polyclonal antibodies (Ab) [13]. To increase primary Ab specificity to the AA1 fragment, the serum was exhausted on a membrane coated with the recombinant protein. The IgG fraction was purified by affinity chromatography on ProteinG-Sepharose (Sigma-Aldrich, United States) as recommended by the manufacturer. The IgG preparations were mixed with 50% glycerol, frozen in liquid nitrogen, and stored at –70°C.

**Protein extraction and western analysis.** Protein extraction from lupine was performed as described by Conlon and Salter [14] with some modifications. The protein concentration was determined by the bicinchoninic acid assay [15]. Immunoblotting was performed as described in [16]. The proteins were transferred from the gel to the nitrocellulose membrane with the pore size of 0.45 μm (GE Healthcare, United Kingdom). To visualize the signal on the membrane with the peroxidase-conjugated Ab, membranes were stained with 0.05% chloronaphthol and 0.15% hydrogen peroxide, or the chemoluminescent ECL method was used. When second Ab with FITC (fluorescein isothiocyanate) was used, fluorescent signals were

obtained after scanning of the filters using a Typhoon Trio+ Phosphorimager (GE Healthcare).

**Fluorescence studies.** Fluorescence emission spectra were measured using a Cary Eclipse spectrofluorimeter (Varian Inc., United States), equipped with a Peltier-controlled cell holder. Quartz cells with the pathlength of 10 mm were used. Protein concentration was 1.7 μM. Tryptophan fluorescence of 154N-AA1 was excited at 295 nm; the emission band width was 2.5 nm. The absence of inner filter effects in the presence of ABA was confirmed.

**Measurements of protein affinity to ABA.** The fluorescence technique is widely used for the characterization of protein affinity to low-molecular-weight ligands (reviewed in [17]), including plant hormones [18]. The use of fluorescence is advantageous in many cases due to the very low sample requirements of the method. The fluorescence titrations were established to provide protein affinity estimates very close to those measured by alternative techniques (including ITC) [19].

The affinity of 154N-AA1 to (+)ABA was measured by automated spectrofluorimetric titration of the 1.7 μM protein solution with 270 μM (+)ABA stock solution at pH 5.9 (50 mM Mes) and 25°C. The system was equilibrated for 90 s after each ABA addition. Calculation of the equilibrium ABA association constant $K$ of the protein (P) from the experimental data was based on the one-site binding scheme:

$$P + ABA \overset{K}{\rightleftharpoons} P \cdot ABA. \quad (1)$$

The experimental data were fitted using the FluoTitr v. 1.4 software (Institute for Biological Instrumentation, Russian Academy of Sciences, Pushchino, Russia), implementing nonlinear regression algorithm by Marquardt [20]. The fit was achieved by variation of $K$ parameter.

**Affinity chromatography on ABA-Sepharose and immunoenzyme analysis.** Detailed information is given in Supplementary Method 3.

**Statistics.** All experiments were performed at least three times with three replications each. Figures present the mean values and their standard errors.

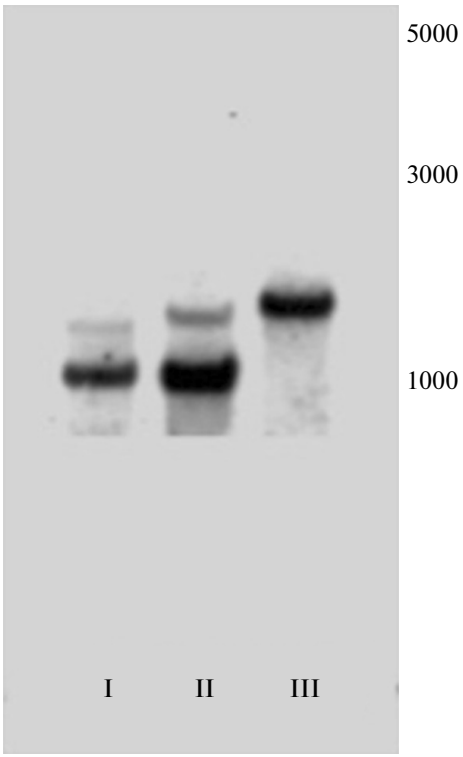


**Fig. 1.** Southern blot analysis of *AA1*.
DNA from lupine cotyledons was digested with *Hind*III (I), *Xba*I (II), or *Eco*RI (III). The DNA fragments were separated on an agarose gel, transferred on the membrane, and hybridized to the radiolabeled *AA1* probe. Numbers show DNA sizes in bp.

## RESULTS

### *Identification of ABA-Regulated Genes*

Yellow lupine (*Lupinus luteus* L.) cotyledons are known for their high sensitivity to treatments with ABA and cytokinin [21]. To identify hormone-regulated mRNAs, cotyledons were detached from 3-day-old etiolated seedlings of lupine, incubated in darkness on water for 24 h, and then exposed to either water, ABA ($7.6 \times 10^{-5}$ M,) or cytokinin (BA, $2.2 \times 10^{-5}$ M) for additional 10 h. Differential display analysis uncovered one mRNA, which was markedly enhanced by ABA and – to a lesser extent suppressed by cytokinin. The corresponding full-length cDNA of 2137 bp (the open reading frame contains 1941 bp) was isolated from the lupine cDNA library and named *AA1* (*Abscisic acid Activated 1*). Southern blot analysis with lupine DNA digested with different restriction enzymes demonstrated that *AA1* is a single-copy gene (Fig. 1). The genomic sequence was isolated, cloned, and sequenced with primer pairs designed from the 5'- and 3'-ends of the full-length cDNA; it demonstrated that the genomic *AA1* did not contain introns (Supplementary Fig. S1).

### *ABA, Cytokinin, and Light Effects on AA1 Gene Expression*

Hormone regulation of the *AA1* mRNA abundance was confirmed over a period of 24 h in ABA-, BA- and $H_2O$-treated (control) etiolated and light-exposed lupine cotyledons (Fig. 2).

ABA strongly increased and BA slightly inhibited *AA1* expression, especially after 5 h of hormone treatments (Fig. 2). The level of the *AA1* mRNA in control cotyledons was stable during the experimental period. The expression was relatively high in the cotyledons of dark-grown seedling, much less in the hypocotyls and

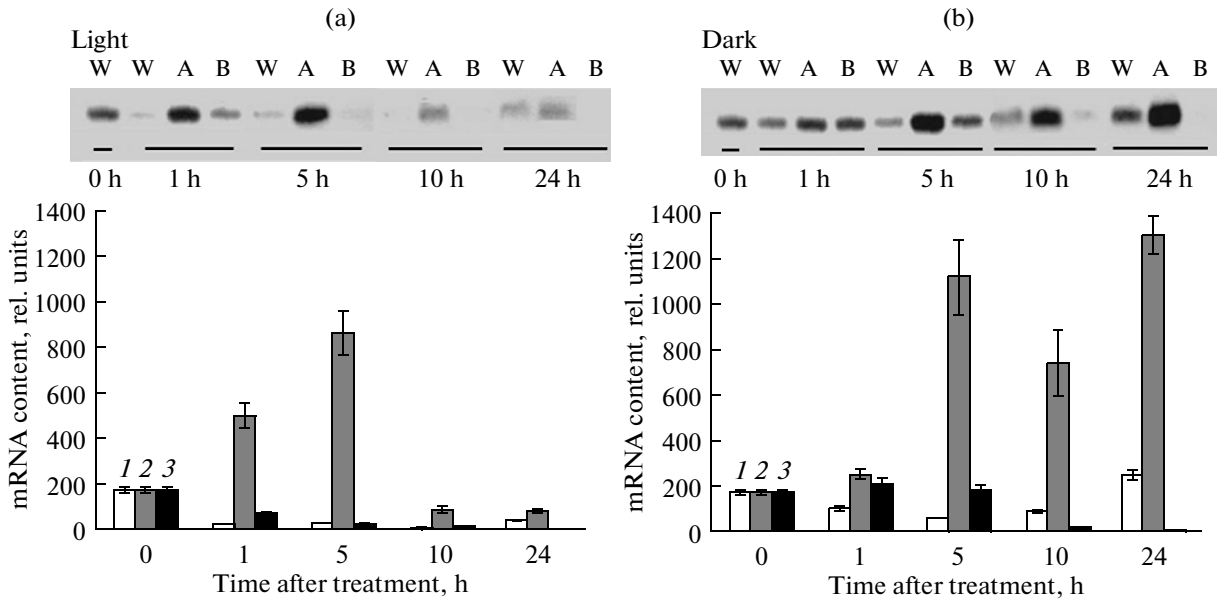


**Fig. 2.** Steady-state *AA1* mRNA levels in *L. luteus* cotyledons after treatments with hormones in the light (a) or in darkness (b). RNA was isolated from cotyledons detached from 3-day-old lupine seedlings, incubated for 24 h on water, and then transferred to water (W), 76 μM ABA (A), or 22 μM BA (B) in the light (a) or in darkness (b). The results of northern hybridization were assessed using ImageQuant TL (GE Helthcare) on the basis of band density. Northern blots are presented above the diagrams. The *tubulin* gene was used as a loading control (not shown). (*1*) Water; (*2*) ABA; (*3*) BA.

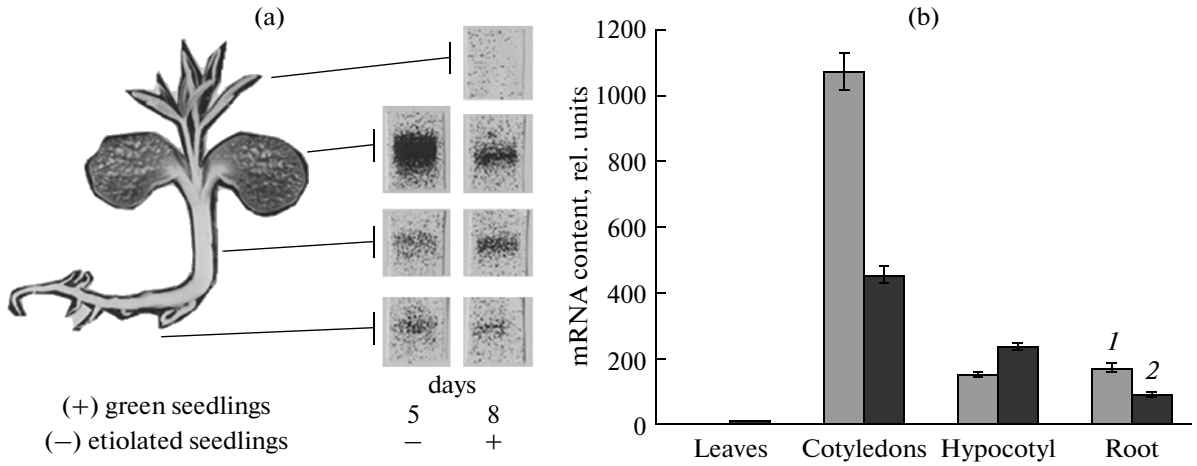


**Fig. 3.** The amount of *AA1* transcripts in various organs of *L. luteus* seedlings as determined by northern hybridization. (a) Northern hybridization with RNA from various organs of the seedling; (b) relative amount of mRNA in various organs determined with the ImageQuant TL (GE Helthcare) software on the basis of band density. (*1*) 5 days (darkness); (*2*) 8 days (light).

the roots and barely detectable in the first emerging leaves of the plants (Fig. 3).

Furthermore, Fig. 2a demonstrates also light effects on the *AA1* transcript level in the presence of ABA. The peak of expression in the presence of ABA in the light was obtained after 5 h, followed by a strong decline. In darkness, the amount of mRNA increased in the ABA-treated samples until 24 h (Fig. 2b).

### *ABA Increases the AA1 Protein Level in Detached Lupine Cotyledons*

To elucidate whether ABA treatments also stimulate the accumulation of the AA1 protein, an antiserum against an 144 amino acid residue-long protein fragment (corresponding to 432 bp of the *AA1* gene from 236 (208) to 667 (639) bp of the initial cDNA, (the positions relative to the A of the ATG codon are

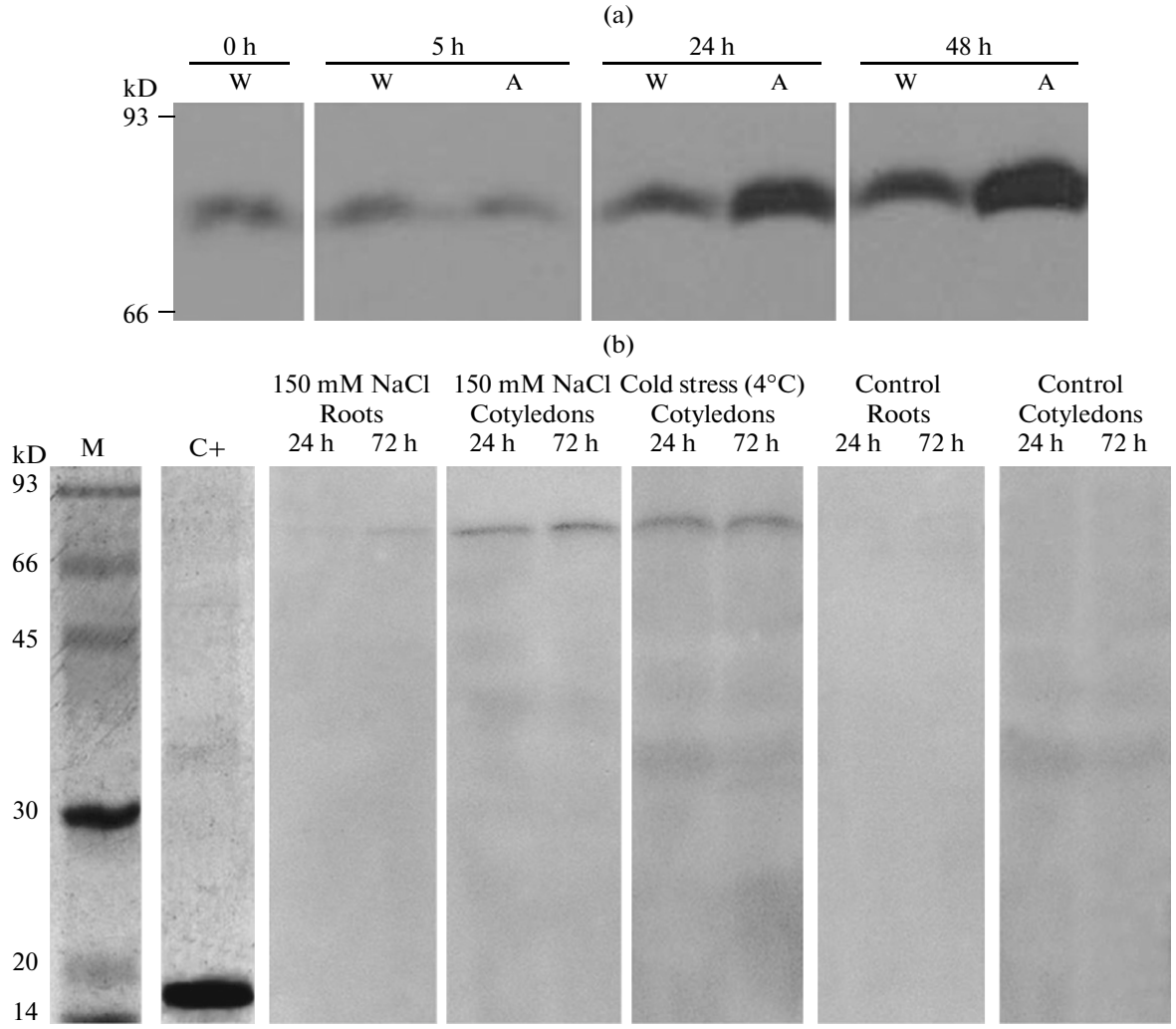


**Fig. 4.** Effect of ABA, hypothermia, and salinity on the AA1 protein levels in lupine seedlings.

(a) Effect of ABA on the accumulation of AA1 in cotyledons detached from 3-day-old seedlings of *L. luteus* grown in darkness. For experimental details, see Materials and Methods section. The results of western analysis are presented. W—water; A—ABA.

(b) Effects of hypothermia and salinity on the AA1 protein levels in 10-day-old lupine seedlings. Prior to harvest, the seedlings were exposed to 150 mM NaCl or 4°C for either 24 or 72 h (control roots and cotyledons: no treatment). For experimental details, see Materials and Methods section. M—molecular weight marker (Amersham); C+—positive control of specific Ab binding (interaction with 154N-AA1).

given in brackets) was raised (see Materials and Methods section).

The AA1 protein levels were studied by western-blots in extracts from cotyledons detached from 3-day-old lupine seedlings treated with ABA, because the *AA1* mRNA level showed a strong transient up-regulation within the first 5 h followed by a decline thereafter (Fig. 2a). Fig. 4a shows that the AA1 protein continues to accumulate over a period of 48 h in ABA-treated dark-grown cotyledons and the protein level is higher than in the water-treated cotyledons. This indicates that ABA controls *AA1* mRNA (Fig. 2) and protein (Fig. 4a) accumulation. Furthermore, ABA-induced *AA1* mRNA accumulation occurs earlier than ABA-induced AA1 protein accumulation.

### *AA1 of L. luteus Is Stimulated by Abiotic Stresses*

It has long been known that ABA and ABA-regulated genes participate in stress responses. Therefore, we checked whether *AA1* is regulated by salinity and hypothermia. Up to 10 days, lupine seedlings grown in darkness on moist tissue contained barely any *AA1* mRNA and AA1 protein (data not shown) in the cotyledons, hypocotyls, and roots. However, in the presence of 150 mM NaCl in the medium or under hypothermia (4°C) the AA1 protein level increased up to 48 h. It is worth noting that salinity resulted in protein accumulation in both cotyledons and roots, whereas under hypothermia stress, the protein accumulated only in the cotyledons (Fig. 4b, data on AA1 protein accumulation in roots under hypothermia stress are

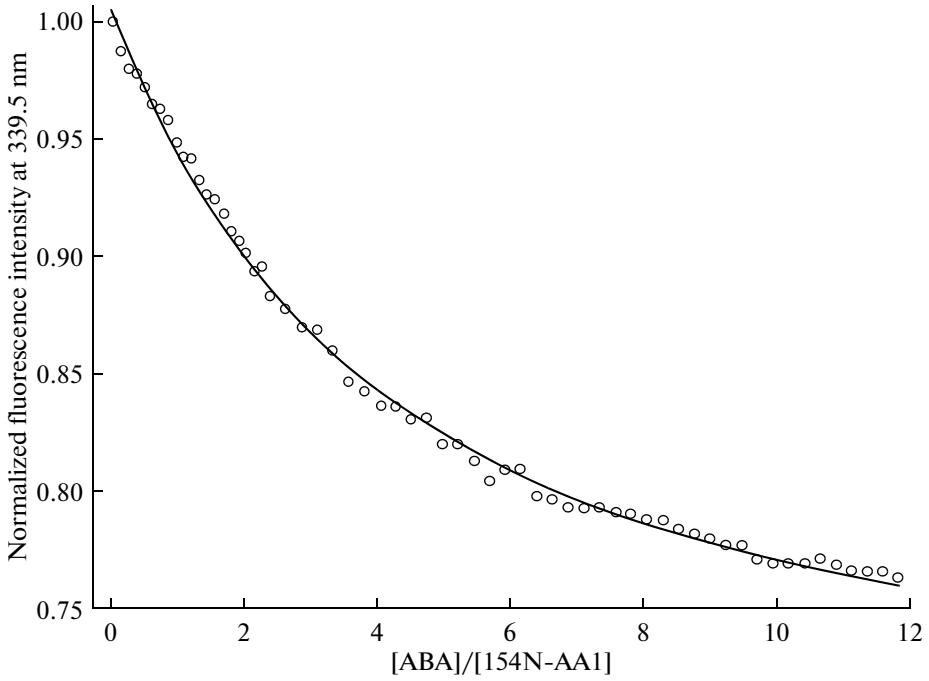


**Fig. 5.** ABA binding to recombinant 154N-AA1.
Fluorimetric titration of 154N-AA1 by (+)ABA at 25°C. Buffer conditions: 50 mM Mes, pH 5.9. Protein concentration was 1.7 μM. Excitation wavelength was 295 nm. Points are experimental, solid line is a theoretical fit computed according to the one-site binding scheme (formula [1] in Materials and Methods section).

not shown). This demonstrates that AA1 is regulated in response to ABA-mediated stress reactions.

### *ABA-Binding Properties of the AA1 Protein Fragment*

To study ABA-binding properties of the AA1 protein fragment, the recombinant 154N-AA1 protein was purified by affinity chromatography on Ni-NTA-Sepharose to about 99% (estimated using ImageQuant TL, GE Helthcare). The affinity of 154N-AA1 to (+)ABA was studied by spectrofluorimetric titration of the protein with a concentrated (+)ABA stock solution at 25°C (Fig. 5).

The intrinsic tryptophan fluorescence intensity and quantum yield of 154N-AA1 decreased with ligand concentration, while the fluorescence spectrum maximum position of 154N-AA1 remained unaffected (data not shown). The fluorescent data are best described by the one-site binding scheme (formula [1], see Materials and Methods section) (Fig. 5); the apparent equilibrium ABA dissociation constant, $K_d$, was 6 μM. One ABA molecule per protein molecule was also shown to be bound to the PYL family receptors [22, 23].

Application of affinity chromatography on ABA-Sepharose and immunoenzyme analysis proved ABA-binding properties of the AA1 protein fragment (Supplementary Fig. S2). Jointly, these data imply that the AA1 protein comprises an ABA-binding site located on the 154N-AA1 fragment.

### *AA1 and Its Homologues Contain Structurally Conserved Domains*

The BLAST software of the DFCI database identified more than 200 ESTs from 46 dicots and monocots (Supplementary Fig. S3), including three genes with unknown function from *A. thaliana* (*At4g01870*, *At1g21670*, and *At1g21680*), which are closely related to the lupine *AA1* (Fig. 6).

Most *AA1* homologues do not contain introns. The central part of these proteins contains the TolB motif from *E. coli* (Supplementary Fig. S4), which is a part of the periplasm Tol-Pal system in bacterial cells and involved in colicin defense, water-salt balance, and membrane stabilization. Furthermore, the higher plant proteins share conserved WD40-like repeats (PD40 repeats, ID: IPR011659), which form the basis for the tertiary beta-propeller structure [24] and provide a potential platform for the assembly of protein complexes.

### *Expression of the L. luteus AA1 Gene Is Similar to Those of Arabidopsis Atg21670 and At4g01870*

Phylogenetic analyses demonstrate that the Arabidopsis genes *Atg21670* and *At4g01870* are in close relationship to lupine *AA1* (Supplementary Fig. S5). Comparison of the lupine expression data with those available for Arabidopsis (http://www.arabidopsis.org) demonstrates similar organ-specific expression profiles during early developmental stages. The highest

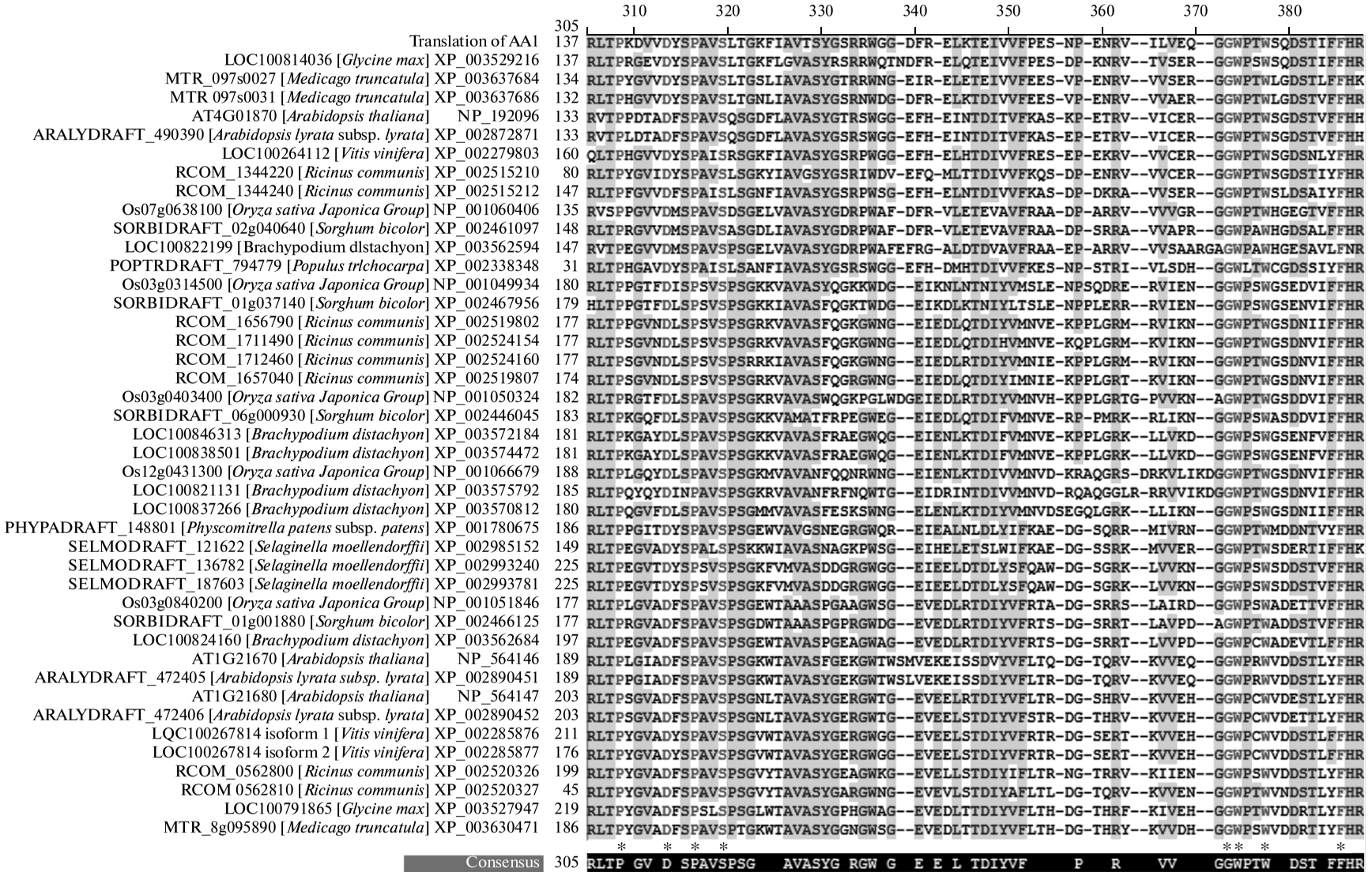


**Fig. 6.** Comparison of yellow lupine AA1 sequence and AA1 sequences deduced from various plant genomes using AlignX.

Conservation level of residues: Marked by stars on the bottom – completely conserved (identical) residues; gray background – conservative residues. All sequence designations, except for AA1, are presented in following formats: NCBI Gene name [species name], NCBI protein sequence ID. Homologous sequences were found by NCBI BLAST search against non-redundant database of protein sequences (restricted by taxid33090 – green plants) using the sequence of the overexpressed fragment of AA1.

expression was observed during early stages of greening of the cotyledons (Supplementary Fig. S6). At later stages, the lupine *AA1* and *At1g21670* mRNAs accumulated mainly in the aboveground organs (leaves, cotyledons), while those of *At4g01870* accumulated mainly in the roots (Supplementary Fig. S7). During the reproductive phase, the expression profile of the two genes become similar again in the aboveground organs: in inflorescences *At4g01870* was even higher expressed than *At1g21670*. The overall expression of *At1g21670* in 21-day-old Arabidopsis plants was ~ fivefold higher than that of *At4g01870*. Since the expression profile of *AA1* from lupine (Fig. 3) resembles more that of *At1g21670* than that of *At4g01870*, in particular when the expression in aerial parts and roots are compared, *At1g21670* was investigated in more details (see below).

Lupine *AA1* mRNA and AA1 protein levels were stimulated by ABA in cotyledons (Figs. 2, 4a), and the protein level was also up-regulated by salinity and cold stress (Fig. 4b). Analysis of the expression data on the TAIR website revealed that the two Arabidopsis genes respond to the same stimuli. Three hours after ABA application, the mRNA levels of both Arabidopsis genes started to increase (Supplementary Fig. S8), similarly to the exposure of seedlings to cold stress where more than a tenfold increase was observed after 24 h of cold (Supplementary Fig. S9). Furthermore, also osmotic stress (150 mM mannitol) stimulated *At1g21670* expression in the aboveground organs (Supplementary Fig. S10).

## DISCUSSION

Here, we characterized the ABA-inducible gene *AA1* from *L. luteus*. Expression of this gene is regulated by light (Fig. 2) and activated by salinity and cold stress (Fig. 4). Furthermore, the gene is preferentially expressed in the aerial parts of the plant and during flower development. We also observed a strong up-regulation of the AA1 protein level after ABA application. The protein is characterized by its central TolB motifs and several conserved WD40-like repeats (IPR011659) (Supplementary Fig. S4). The deduced amino acid sequences encoded by the lupine cDNA *AA1* contain an N-terminal extension, which directs the protein into the endoplasmatic reticulum.

The *AA1* mRNA level was higher in etiolated lupine cotyledons compared to light-adapted cotyledons, and we observed a strong stimulatory effect of ABA in etiolated lupine seedlings (Fig. 2). Comparable regulations by light and ABA have been described for other genes [21, 25]. For instance, Xu et al. [26] proposed an involvement of proteins of the light-harvesting complex in ABA signaling and the regulation of stomata aperture. The authors suggested that these proteins might be components of the ABA signal transduction pathway functioning via the regulation of ROS generation. ROS are known to be important regulators of ABA signaling [1, 10]. Since the AA1 protein level does not increase during the first 5 h after ABA application in detached lupine cotyledons (Fig. 4a), we anticipate that AA1 is not involved in early phases of ABA signaling.

More than 10 proteins have been identified as ABA-binding proteins and potential receptors for the phytohormone [3, 4, 27]; among them, PYR/PYL/RCAR family members are the best characterized [5–7]. Considering the diversity of ABA-regulated processes [1, 10], it is conceivable that several ABA-binding proteins participate in quite different developmental processes or may even initiate independent organ- or development-specific signaling events (see. [1]). The affinity of 154N-AA1 to ABA was studied by spectrofluorimetric titration of the protein with a concentrated ABA stock solution (Fig. 5) and affinity chromatography and solid-phase immunoenzyme analyses (Supplemental Fig. S2). The estimated apparent ABA dissociation constant for 154N-AA1 (6 μM) closely approaches the value previously reported for the PYL5 ABA receptor (1.1 μM), but is significantly lower than that for VvPYL1 receptor (78 μM) [22, 23]. These values are comparable to in vivo ABA concentrations, which are in the range of 7 to 13 μM in mesophyll and guard cells [28], but they can be about 7 times higher under stress conditions [29]. Hence, the observed micromolar level determined for the affinity of 154N-AA1 to ABA is of physiological significance. ABA-binding features of 154N-AA1 are consistent with the observed involvement of the protein in ABA-regulated processes.

AA1 from lupine as well as its close homologues from *A. thaliana* comprise two structural domains: a TolB-like beta-propeller domain (which is built on the basis of PD40 repeats) and a C-terminal domain with homology to type IV dipeptidyl peptidases (DPP IV). The bacterial TolB protein participates in the structural and functional organization of the bacterial Tol-Pal system defending cells against penetration of peptide toxins (colicins), thereby maintaining water–salt balance due to the stabilization of the outer membrane. The structural rearrangement of the bacterial protein is regulated allosterically, which serves as a specific switch for the Tol-Pal system. Many proteins with a beta-propeller domain function as a platform for protein–protein interactions [24]. The ABA-binding properties of AA1 and its Arabidopsis homologues, their conserved structures and their predicted extracellular location suggest that they might be involved in ABA-regulated post-translational protein modifications. The role of the peptidase epitope requires further investigations.

According to microarray data available on http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) several Arabidopsis genes exhibit striking similarities to *AA1* from lupine. Like *AA1*, *At1g21670* and *At4g01870* respond to ABA treatment (Supplementary Fig. S8), cold (Supplementary Fig. S9), and osmotic stress

(Supplementary Fig. S10). Furthermore, the genes are highly expressed at early developmental stages and during early phases of greening (compare Supplementary Figs. S6 and. 3). Besides, like *AA1* from lupine, *At1g21670* and *At4g01870* participate in ABA-dependent abiotic stress responses (Fig. 4, Supplementary Figs. S9 and S10). Since their protein sequences also share substantial sequence similarities, we propose that they might fulfill similar functions in both plant species.

The experiments on the expression of the *L. luteus AA1* under hormone treatment and stress conditions as well as the expression profiles of Arabidopsis homologues presented in data bases clearly show that the genes modulate the plant response to ABA treatment and probably act as negative regulators of ABA-stimulated processes. However, further experiments are required to fully understand their role in the hormonal scenario.

A possible explanation could be that the lupine protein AA1 binds ABA, thereby reducing the available amount of physiologically active ABA. Another explanation could be that AA1 does not participate directly and linearly in the ABA signaling events, but balances ABA responses. The proteinase-like domain on the C-termini of AA1 and its homologues as well as the apoplast location of the proteins [30] allow them to act as signal interrupters due to protein–protein interactions and thus modification of their interaction partners. Such inactivation mechanisms are well known for many receptor proteins of animal cells, called shedding. After secretion into the extracellular space, these proteins perform post-translational modifications of membrane receptors, thereby preventing receptor activation by the appropriate signals [31, 32]. Proteins involved in shedding may also affect the activity of membrane channels. It would be interesting to know if such a signaling mechanism exists also in plants. Members of the investigated gene family are good candidates for such a role.

In conclusion, the data obtained clearly demonstrate that AA1 and its homologous proteins play important and novel roles in stress-related environmental responses related to ABA.

## ACKNOWLEDGMENTS

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