**Klarifikasi: (lampiran bukti kehadiran)**

Poster yang berjudul: “Lebensfahige Mutante des Auxin-Bildungs-Proteins 1 (ABP1) zeigen morphologische merkmale ung langsamere regulation von IAA-genen“ dipresentasikan dalam bentuk poster presentation pada konferensi tahunan tentang 22nd Molecular Biology of The Plant (22. Tagung Molekularebiologie der Planzen), 17-20 Februari 2009. Penulis sebagai first author pada presentasi poster ini. Konferensi tersebut diselenggarakan secara rutin di kota Dabringhausen Jerman dengan sponsor utama Deutsche Botanische Gesellschaft (DBG). Pada 22 Tagung (Conference 22nd) koordinator untuk kegiatan saat itu adalah Universitaet Rostock – Jerman (Prof. Dr. Birgit Piechulla sebagai Koordinator konferensi). Konferensi ini tidak menyediakan adanya proseding sehingga publikasi ini **tergolong tidak dimuat dalam prosiding**. Informasi tentang konferensi (penyelenggara, peserta dan poster/makalah) terinfokan pada abstract book (dilampirkan). Sebagai bukti keikutsertaan kami dilampirkan

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- Artikel terkait poster yang dipresentasikan
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UNIVERSITÄT ROSTOCK
MATHEMATISCH-NATURWISSENSCHAFTLICHE FAKULTÄT

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# Bescheinigung

Hiermit wird bescheinigt, dass

***Herr Yunus Effendi***

an der Tagung

‚22. Pflanzenmolekularbiologie der Pflanze'

vom 17.2. bis 20.2. 2009
in Dabringhausen teilgenommen hat.

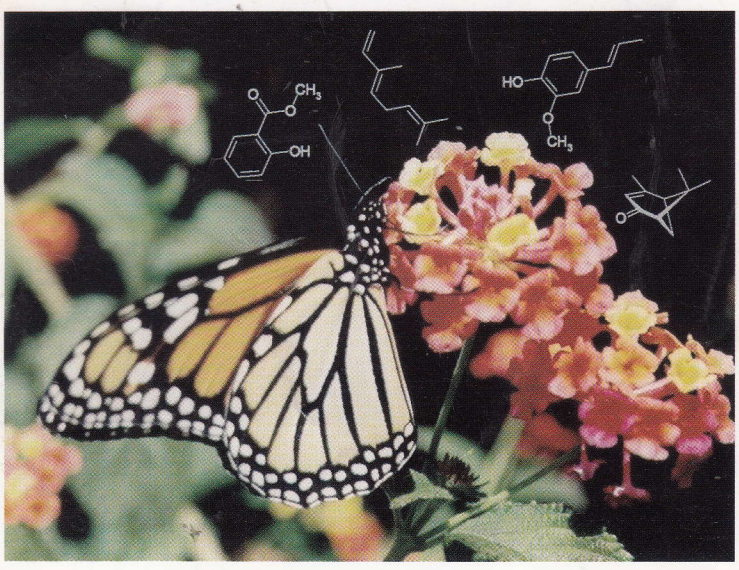
Dabringhausen, 20.2.2009 ----------

Prof. Dr. Birgit Piechulla

22. Tagung

# Pflanzenmolekularbiologie

17.2. – 20.2. 2009, Dabringhausen

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**Funktionen in Auxin-Siganltransduktion, Lichtwirkung und Phos**

77. **Kerstin Schumacher**, Susan Pusunc, Udo Wienand, René Lorbiecke Hamburg)
**Pti1-Kinasen in Mais und *Arabidopsis* zeigen unterschiedliche subzelluläre Lokalisationen und sind vermutlich an unterschiedlichen aber evolutionär konservierten Prozessen beteiligt**

78. **Christian Löfke**, Jiri Friml, Anne Vieten, Thomas Teichmann (Universität Göttingen)
**Interaktion zwischen Gibberellin-Signaltransduktion und Auxintransport**

79. **Alexander Maier**, Sascha Laubinger, Ute Höcker (Universität Köln)
**Biochemische Charakterisierung von SPA-COP1 Proteinkomplexen**

80. **Yunus Effendi**, Günther Scherer (Universität Hannover)
**Lebensfähige Mutanten des Auxin-Bindungs-Proteins 1 (ABP1) zeigen morphologische Merkmale und langsamere Regulation von IAA-Genen**

81. **Anja Liese**, Sandra Franz, Stefanie Macke, Tina Romeis (Freie Universität Berlin)
**Funktion und Calcium-abhängige Regulation von CPK21 und CPK23 in der abiotischen Stressantwort**

82. **Till Ischebeck**, Irene Stenzel, Ingo Heilmann (Universität Göttingen)
**Der Einfluss von Phosphoinositiden auf die Funktion von Spaltöffnungen**

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**FHY1 und Kerntransport von Phytochrom A**

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**Die Rolle von MAP-Kinasen beim Priming für verbesserte Transduktion des Flagellin-Signals in *Arabidopsis***

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# Lebensfahige Mutante des Auxin-Bildungs-Proteins 1 (ABP1) zeigen morphologische merkmale ung langsamere regulation von IAA-genen

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## Abstract

Auxin signaling in plant is regulated by TIR1 as ist major receptor through ubiquitination and gene transcription activities. However, there is remain question about a number of auxin-regulated enzymatic activities or processes within short time after auxin application, such as activation of phospholipase A activity (Scherer and Andre, 1989; Paul *et al*, 1998) after 2 min, regulation of channel activity within 3 min (Martin *et al*, 1991; Rueck *et al*, 1993), and activity of MAP kinase within 5 min (Mockaitis and Howell, 2000) after auxin application. Those processes can not be explained via gene transcription activities of TIR1 complex. Auxin Binding Protein 1 (ABP1) is a candidate for a second auxin receptor, even though ABP1 does not show any similarity with common receptor and it is located dominantly in the lumen of ER where the pH is to high for auxin binding (Tien *et al*, 1995). No pre-report described auxin stimulated gene regulation through ABP1 action. Moreover, since loss-of-function in ABP1 leads to lethality in an early stage of embryo development (Chen *et al*, 2002), it is difficult to obtain evidence the involvement of ABP1 in auxin-dependent development. Because of the embryo lethality from *abp1/ABP1* plants only 2:1 mixtures of *abp1/ABP1* and wt plants can be obtained.

We analyzed first kanamycin resistant viable heterozygous *abp1/ABP1* plants which produce a progeny mixture 2:1 *abp1/ABP1* plants. For investigation on gravitropism, phototropism and flowering in SD, we used such mixed populations. Despite this drawback, it is obvious that gravitropism of root and shoot and phototropism of the shoot is defect and early flowering in SD is clearly indicated. Short day plants also do not show obvious morphological differences to wt plants. We show differences in expression level of IAA gene family *(IAA2, IAA11, IAA13, IAA14, IAA19* and *IAA20*) in heterozygous *abp1/ABP1* 2:1 (selected as kanamycin resistant) and wt *Arabidopsis thaliana* (ecotype Wassilewskija). Heterozygous abp1/ABP1 show less fold expression of those IAA genes in comparison to wt after 0.1 µM auxin treatment (30 min and 60 min). After 30 min auxin application, *IAA2, IAA11, IAA13, IAA14, IAA19* and *IAA20* genes are expressed 2,3,2.4, 12, 30, and 5 times more in wt than in heterozygous *abp1/ABP1* plants, respectively ($P<0.05$). Phenotypic data show that heterozygous *abp1/ABP1* plants produce inflorescence earlier (70 days, SE= 0.46) in comparison to wt (75 days, SE=0.63), fewer rosette leaves and fewer cauline leaves. Moreover, in comparison to wt, the roots of heterozygous *abp1/ABP1* plants show less responsivity to gravity and the hypocotyls are less responsivity to gravity and lateral light.

We also are in the process of making plants were transformed with different vector constructs containing mutated amino acids. By selection and segregation, eventually stable double homozygous plants can be obtained. Currently, plants still may contain wild type protein and over

express the mutated gene. These plants seem to flower early also in LD as indicated by the number of rosette and cauline leaves. Early flowering may be the cause of the dwarfed appearance of mutant 6. A low number of stems may indicate a change in apical dominance. Curly leaves on almost all mutants are reminiscent of the phenotype of *phot1 phot2* double mutant.

Keyword: heterozygous abp1/ABP1, auxin receptor, mutants

## INTRODUCTION

Our knowledge of auxin signaling made considerable progress in the last few years. The identification of the receptor TIR1 and its mechanism of action have provided the crucial missing component in a pathway that can now be seen to be sufficient to account for how auxin can turn a gene on. Following decades of research on auxin molecular biology, several elements of the auxin signaling pathway have been elucidated. These include two or even three types of apparent receptors that perceive the auxin signal. Functions influenced by these receptors are gene expression and also possibly cell wall modifications important for cell expansion (Napier et al., 2001). The apparently two major types of receptors are Auxin Binding Protein 1 (ABP1), a membrane-bound protein thought to initiate cell wall expansion, and more recently Transport Inhibitor Response 1 (TIR1) and related auxin signaling F-box (AFB) proteins, soluble proteins that initiate the regulation of gene expression (Dharmasiri et al., 2005). Recently, intensive investigation about auxin receptor and its function on mediating the expression of auxin induced genes is focused on TIR1 and the related auxin signaling F-Box proteins. Therefore, it is more accepted and explainable that TIR1 is a major auxin receptor. However, some early and rapid physiological processes which appear after auxin application within less than 30 minutes remain unexplained by function TIR1 to initiate the regulation via proteolysis of IAA transcription factors and transcriptional activation of early auxin-induced genes. Because it takes time to generate new proteins by gene regulation which is inevitably slower than signaling, for example, by membrane depolarization or ion fluxes, rapid responses to auxin might not be TIR1-mediated. Some are linked to ABP1 (Badescu and Napier, 2006).

Although currently ABP1 as an auxin receptor is still subject to debate, it has been well known that some physiological processes in plant are mediated by ABP1. Predominantly, ABP1 is found in lumen of endoplasmic reticulum where it is retained by a KDEL sequence (Jones and Herman, 1993). The possible functional role of the protein inside the ER is nothing known but there is an experiment evidence for a function of ABP1 at the outer surface of the plasma membrane (LeBlanc et al., 1999). The pH estimate of the ER lumen is based on an indirect assay, which indicates that the pH is closer to pH 7 than to the binding optimum of pH 5.5. These results indicate that ABP1 does not bind auxin within the ER and point to a site of action that is post-ER (Tian et al., 1995). Klämbt (1990) proposed a plasma membrane docking protein as a model for ABP1 action. He postulated that a small amount of ABP1 manages to escape KDEL retention and is excreted to the apoplasmic space. In fact, there is some experimental evidence supporting this idea (Jones and Herman, 1993; Diekmann et al., 1995). After binding extracellular auxin, ABP1 was postulated to interact with a hypothetical trans-membrane docking protein which transduces the signal into the cell. ABP1 has been shown to be located at the plasma membrane using immunocytochemisty in conjunction with electron microscopy (Jones and Herman, 1993) and silver-enhanced fluorescence microscopy (Diekmann et al., 1995).

Another type of auxin binding protein, $ABP_{57}$ was found in rice (Kim et al., 2000). It was has shown that after IAA application $ABP_{57}$ also binds directly to plasma membrane $H^+$-ATPases and stimulates proton extrusion in rice. However, auxin binding proteins (ABPs) appears to be able to activate the plasma membrane $H^+$-ATPases in the presence of auxin (Steffens et al., 2001). Taken together, these data indicate that ABP1 binds auxins in a specific and physiological meaningful manner at the plasma membrane to bring about rapid hormone responses. However, no data exist to show that ABP1 may trigger gene regulation.

The slower but important receptor-triggered functions in auxin physiology required gene regulation. Such functions are: adventitious and lateral root development and differentiation, sustained elongation, frit development, ethylene induction, xylem and phloem differentiation. Only cell division and, perhaps in part, all elongation have been shown require ABP1 but not these other functions (Campanoni and Nick, 2005). Gene regulation by an auxin receptor is well explained by TIR1 and homologous *AFB* genes and regulation of *IAA* genes and the heterodimeric IAA/ARF complexes acting as transcription factors (Parry and Estelle, 2006). Therefore, TIR1 and the homologous AFB's are regarded as the functionally major receptors. Especially, because the influence of ABP1 on gene regulation is unknown it is still an open question how real functional importance of ABP1 is to be evaluated. Therefore, approaches using mutants of the *ABP1* gene and protein are needed

To elucidate this subject, some efforts have been done by many groups. ABP1 as an auxin receptor remains mysterious. Nevertheless, recently two different workgroups, who work with *ABP1* gene, described a knockout *abp1* mutant (Chen et al., 2001) and the crystallographic structure of ABP1 (Woo et al., 2002), which opened a new perspective for investigating ABP1 as a receptor. Chen et al. (2001) found that a homozygous *abp1* T-DNA-mutated *Arabidopsis thaliana* is lethal at embryonic stage. The embryos are arrested at globular stage and form a clump of cells but no plant. This loss of function which leads to a lethal condition in the plant indicates that *ABP1* must be an essential gene in the plant. Moreover, according Chen et al. (2001) plants with one copy (heterozygous *abp1*) can grow as a normal plant. This information shows another difficult thing to deal with *ABP1* gene. Generally, understanding of gene function can be performed by altering or abolishing the expression of the gene. It can be done by mutating the essential part of the gene by T-DNA insertion which leads to lost of function of the gene, so then the downstream processes which relate with this gene can be observed. Unfortunately, in case of *ABP1* gene, this technique cannot be easily performed, because null mutation of *ABP1* gene will confer to lethality to the plant. Making a 'heterozygous' T-DNA insertional mutant does not confer lethality on plant. A solution to the dilemma for getting mutants of the *ABP1* gene is to use the viable heterozygous insertional abp1 mutants to transform them with a mutated cDNA *ABP1*. Transformed plants then can be selected which are homozygous for the insertion so that no wild type ABP1 protein is expressed but only the mutated ABP1 protein. During the course of the work it also became apparent that plants having an insertion in the *ABP1* gene in heterozygous constellation already have a clear mutant phenotype, so that this was also investigated.

## MATERIAL AND METHODS

### Plant material

*Arabidopsis thaliana* wild type and heterozygous *abp1* mutant ecotype Wassilewskija (Ws-2) were used as object plant. Heterozygous *abp1* mutant seeds were obtained from Nottingham *Arabidopsis* Stock Center (NASC) England. *Arabidopsis* seeds were grown on compost soil

containing silica sand. Aplants were grown in climate chamber or in greenhouse under long day condition (16 h light/8 h dark). Pre-sterilized seeds were sowed on 0.5X Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose and containing 100µg/ml Kanamycin antibiotic. Plants were grown under 18h white light 22ºC condition and transferred to soil after four primary leaf appear then they were grown in green house under long day condition (16 h day light/8 h dark cycle).

**Phenotypic characterization of the heterozygous *abp1* T-DNA mutant**

Phenotypical characters were measured from 11 days old plants. The seedlings were grown as described above. Scanned of the seedlings were then the roots length and waving pattern were scanned with CanonScan 8800F (resolution 600 dot per inchi). Root length and waving pattern were measured from ten of both wild type and heterozygous *abp1* plants by using AxioVisio LE Ver.4.6 software (Zeiss-Germany).

**Auxin treatment**

Three different concentrations of Indole-3-acetic acid (0µM, 0.03µM and 0.1µM) were used and applied for 60 min, 30 min and 0 min to the plants. Due to the handling of the samples, t = 0 min in this experiment referred to about 10 second. Dimethylsulfoxid (DMSO) treatments with equal concentration as much as auxin treatments were used as control. Both of these treatments were applied to heterozygous *abp1* and wild type plants. Two auxin concentrations (0µM and 0.1µM) were used for quantitative real time PCR experiment with the similar time course as semi-quantitative experiment. After the seeds were surface sterilized, seeds were grown on LB agar medium containing 100µg/ml kanamycin. Two days after stratification at 4ºC in dark condition, plates were placed under 18h white light 22ºC condition until seedling has 4-6 primary leaf. Then growth further on 1 ml of 0.5X MS medium containing appropriate IAA concentration was added to the plates. Plates were incubated at room temperature at given time condition (short time, 30 min and 60 min) with gently shaking. Rapidly plants were dried by placing on clean tissue papers, stored in 2 ml sterile eppendorf tubes and frozen as soon as possible by dipping on liquid nitrogen. Samples were placed on -80ºC until RNA was extracted.

**RNA Isolation and cDNA synthesis**

Total RNA was isolated from fresh samples using NucleoSpin® RNA Plant kit (Macherey-Nagel). Isolation of RNA was performed according the manufacture's protocol. A 95µl DNase was applied to each samples reaction mix which was made by mixing 90µl reaction buffer for rDNase and 10µl rDNase. The RNA were eluted by adding 60µl RNase-free water to the center of the membranes and then centrifuged 1 min at 11.000x g. Purity and concentration of RNAs were measured using spectophotometer U 3500 (Hitachi-Japan). RNAs then were stored on -20ºC. Complementary DNA (cDNA) were synthesized by using RevertAid$^{tm}$ H Minus First Strand cDNA Synthesis Kit (Fermentas). For each samples, a 4-5 µg of total RNA was mixed with 1µl random hexamer primer (0.5µg/µl) in a 200µl sterilized PCR tube and added deionized-nuclease free water to final volume 12µl (4µl 5X reaction buffer, 1µl Ribonuclease inhibitor (20U/µl) and 2µl 10mM dNTP mix, 1µl RevertAid$^{TM}$ H Minus M-MuLV RT (200U/µl)). The mixtures then were incubated at 42ºC for 60 min and the reaction was stopped by heating at 70ºC for 10 min.

**Quantitative Real Time PCR and data analysis**

The relative amount of gene expression of auxin-induced early genes in wild type and heterozygous *abp1* mutant plants were measured by performing quantitative real time PCR. Four IAA family genes (*IAA2, IAA11, IAA13* and *IAA20*) were analyzed their expression after auxin treatment on different time courses application. Quantitative PCR reactions were performed using 1 ll of sixfold diluted cDNA, 200 nM primers and 0.2· Power SYBR Green PCR master mix (Invitrogen, http://www.invitrogen.com/) in a StepOnePlusTM system (Applied Biosystems, http://www.appliedbiosystems.com/). For each pair of primers, the threshold value and PCR efficiency value were determined using cDNA diluted tenfold each time in five dilution steps. For all primer pairs, including the internal standard gene, 18S rRNA, the PCR efficiency was >99%. The specificity of PCR amplification was examined by monitoring the presence of a single peak in the melting curves for quantitative PCR. Amplicons were checked for fragment length on 4% agarose gels. For each determination, two to three biological repeats and three technical replicates for each determination were performed for the subsequent PCR reaction. Relative expression was calculated according to the DDCt method using the equation: relative expression = 2)[DC tsample ) DC tcontrol] , where DCt = Ct(sample gene) ) Ct(reference gene) and Ct refers to the threshold cycle determined for each gene in the early exponential amplification phase (Livak and Schmittgen, 2001). The expression level for the control treatment was set as 1-fold. For statistical analysis, REST 2008 software (Pfaffl et al., 2002) was used.

**Gene Construction for mutagenesis**

**Preparation of *ABP1* cDNA**

The cDNA of Auxin Binding Protein 1 for this experiment was obtained from Dr. Thomas Reinard (Institute Plant genetic – Leibniz Universität Hannover). The *ABP1* cDNA was consisted of 168 amino acids with Strep - Flag tags on its C terminus and KDEL motif in the end of the cDNA sequence (figure 6). Before the cDNA was cloned into Entry vector pENTR-D TOPO (Invitrogen), minor modification was performed by adding stop codon AUG in the end of cDNA sequence. Amplification of cDNA was carried out with *ABP1* specific forward primer (5'-CACCATGGATGATCGTACTTTC-3') and reverse primer (5'-CCTGAGATC TCAAGTAGGAAGCGTC-3') with PCR conditions 94ºC 4 min, 34 cycles (94ºC 30 sec, 54ºC 30 sec, 72ºC 40sec). The vector was transformed into competent cell *Escherichia coli* strain TOP 10 (Invitrogen) and cultured in Luria Bertani (LB) medium (see appendices Table 5) supplemented with 100µg/ml kanamycin under 37ºC for 16 hour. Ten colonies were picked up and cultured further on LB medium containing 100µg/ml kanamycin. Plasmid was isolated after cell cultures were incubated 37ºC with rigorous shaking for 16 hour using SV Miniprep kit (Promega) according the manufacture's protocol and was used as template for mutagenesis step.

**Site-directed mutagenesis**

Mutagenesis in this experiment was carried out by using site-directed mutagenesis kits (Invitrogen) according to the manufacture's protocol. Only one primer for each mutation was applied for each mutagenesis. Primers for mutagenesis were shown in tabel 1. Mutagenesis was also carried out according to Zheng et al. (2004) with minor modification. The mutated-vectors were then transformed into competent *E. coli* strain TOP 10 or BMH 71S (Promega) using heat shock method. From each transformation plates (from different mutation constructs), ten colonies were

picked up and grown on LB medium supplemented with 100µg/ml kanamycin on 37°C for 16 hours. For checking insertion on the recombinant plasmid, colony PCR was also performed to the same colonies by smoothly touching the colonies with sterilized-toothpick and dipped on PCR cocktail that has been prepared before. Recombinant plasmid was isolated using SV Miniprep kit (Promega) and concentration of those plasmids was measured using spectrophotometry (Hitachi U 3000, Japan). Plasmids were sequenced (MWG Esenberg -Germany) and were analyzed further by aligning all sequencing results compared with non-mutated cDNA sequence (software CLC Free Workbench Ver. 4.6.).

| Nr | Residue(s) | Expected mutation residue(s) | Mutation product | Primer 5' -> 3' |
|---|---|---|---|---|
| 1 | W135 | Ala135 | Ala135 | GTGGCATAAAAGCGTCTTCGTAG |
| 2 | D134; W135 | Ala134; ala135 | Ala135 | GTGGCATAAAAGCAGCTTCGTAGATG |
| 3 | His106 | Ala106 | Asn135 | GACCTGAGCAGCATCATTGATCGG |
| 4 | W151; D152; E153 | Ala-ala-ala | Trp-ala-ala | CTTGAATGCATTGAGCAGCAGCATAGTAAGGG |
| 5 | W151; D152; E153; C155 | Ala-ala-ala-ala | Ala-ala-ala-ala | GATTCTTGAATAGCTTGAGCAGCAGCATAGTAAGGG |
| 6 | F147, W151 | Ala147, ala151 | Ala147, ala151 | GCTCATCAGCATAGTAAGGAGCCTTCAGCC |
| 7 | T54 | I54 | I54 | CAGGTTCAGAGATTCCAATTCAC |
| 8 | L25 | Y25 | Y25 | GACCAGGTTATTCCCACATGAC |
| 9 | W151 | A151 | A151 | GCATTGCTCATCAGCATAGTAAGGG |

**GATEWAY cloning.**

The *Arabidopsis* seed of heterozygous *abp1* plants which were used in this experiment is donated originally by Alan Jones groups who worked for the first time with *ABP1* knock-out *Arabidopsis* to NASC England. Knocking out was performed by inserting T-DNA into first exon of *ABP1* gene. For detecting T-DNA insertion, they used kanamycin resistance genes ($Kan^R$) as the marker. Due to this reason, a gateway vector, plasmid pB2GW7 (Karimi et al., 2002) which contains a basta resistance gene ($Bar^R$), was chosen as destination vector for all GATEWAY cloning in this experiment in order to distinguishes transformed-plants which contain only T-DNA insertion/$Kan^R$

(heterozygous *abp1* plant) and heterozygous *abp1* plants with mutated-*ABP1* gene insertion ($Kan^R$, $Bar^R$). Recombinant entry vector pENT-D TOPO was ligated with pBGW7 by using Gateway LR clonase II enzyme mix (Invitrogen). LR reactions were performed by mixing 1-7µl recombinant entry vector with 150 ng destination vectors and added 2 µl LR clonase II enzyme mixes. Reactions were incubated overnight at 25ºC. One microliter of LR reaction products were transformed into TOP 10 competent cells and cultured on LB medium containing 100µg/ml spectinomycin and incubated for 16 hour at 37ºC. Ten positive colonies of each constructs were cultured on LB liquid medium supplemented with 100µg/ml spectinomycin and recombinant plasmids (expression vector) were isolated for further transformation into *Agrobacterium.*

## RESULTS

### Characterization of heterozygous *abp1* plants

Since the *abp1* mutant seeds are used in these experiments are heterozygous, the first step which is carried out is the selection of the heterozygous *abp1* plants by eliminating the wild type *abp1* plants from the progenies. The *abp1* mutant seeds (stock number 6498) were obtained from Nottingham Arabidopsis Stock Center (NASC-London). Screening was performed by growing the sterilized-seeds on 0.5X MS agar containing 100mg/ml kanamycin. The plants which successfully grow on this agar medium then are transferred to the soil and maintained further for next experiments or generating seeds. To check the plants which are able to grow in kanamycin agar medium are heterozygous *abp1* plants, analysis by PCR with specific primers for the *abp1* gene and the T-DNA insertion were carried out. All plants which grow on kanamycin agar plates produced the right fragment sizes as expected from amplification.

By using the reverse genomic primer (5'-TGAGATCTC AAGTAGGAAGCGTC-3') and right border primer for T-DNA (5'-TCCCAACAGTTGCGC ACCTGAATG-3'), each selected plants produced a 1500 bp amplification fragment. When using *ABP1*-specific primers (forward, 5'-ACGAGAAAATCATACCAATTCGGACTAACC-3 and reverse, 5'-GTATCTACGTAGTGTCACAA AACCTCAAC-3'), the same plants produced also a 2650 bp amplification fragment (Figure 8). This indicated that all plants which are able to grow on kanamycin agar medium are heterozygous *abp1* plants.

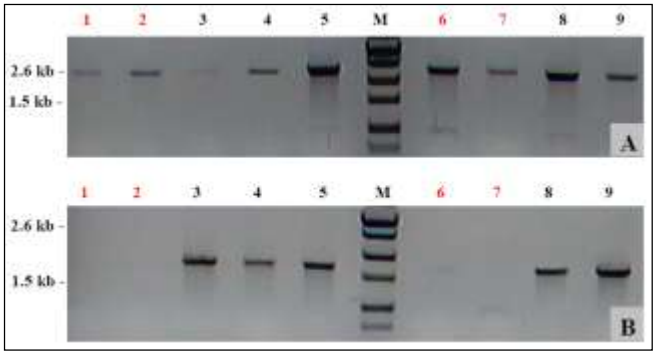


Fig.1. PCR screening for *abp1* T-DNA insertion mutant plants. (A) By using *ABP1* reverse and forward genomic primers fragments for wild type allele of *ABP1* with 2.6 kb length were be obtained. (B) By using *ABP1* reverse genomic primers and right border primers for T-DNA (RB-TDNA) a 1.5 kb fragment was obtained. The samples number 1, 2, 6, and 7 are wild type *ABP1* plants. The samples with number 3, 4, 5, 8 and 9 are heterozygous *abp1* mutant plants. The *ABP1* fragment of plant 3 was weak but distinct.

**Waving and skewing of the roots**

Moreover, we found also differences on root appearance of the seedlings. When grown up right at 90$^{o}$ angle, heterozygous *abp1* seedlings produce clear waving-like pattern and skewing angle, deviating from 90$^{o}$ down ward, in comparison with wild type plants.

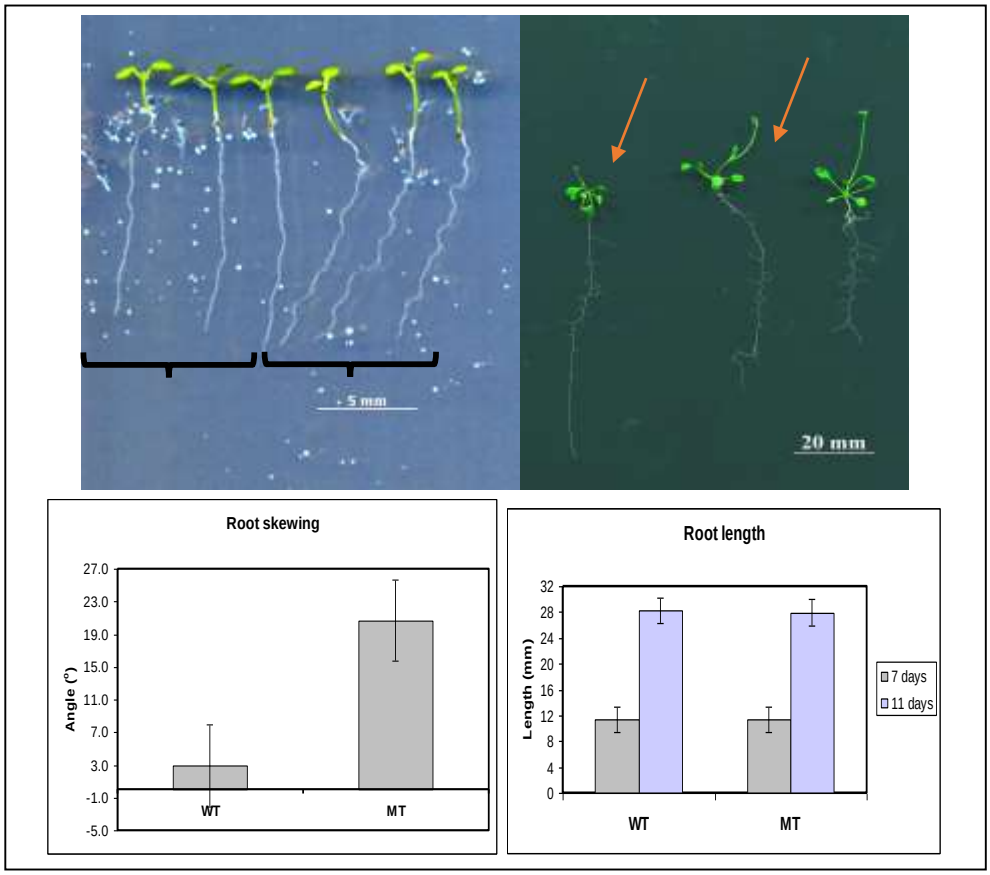


Fig.2. Root skewing on 7 days old plants (A) and inflorescence on 14 days old plants (B). Figure C and D show quantification analysis of root phenotype on wild type and heterozygous *abp1* mutant plants. No difference in root length between wild type and heterozygous *abp1* mutant is shown on graphic (C). Graphic D shows significant difference on root skewness on both plants. Red arrows show flower stalk; Scale bar = 5 mm; * significant with p-value ≤ 0.01; $^{\otimes}$ not significant

In general, the root of wild type produced a very weak waving pattern or almost none. The skewing angle of the mutant was 20.3 ± 3.6$^{o}$ (n = 10, SD) whereas the wild type showed almost no skewing angle (2.9 ± 0.2.4$^{o}$, n = 10, SD) on vertical agar plates. Root length showed no obvious difference between mutant and wild type (Figure 11D). We noticed also that heterozygous *abp1* mutant produce flowers earlier than wild type plant (Figure 2). It was indicated by earlier producing the inflorescence stalk on *abp1* mutant than in wild type. However, during this study, we did not make any specific experiment for analyzing the flowering time of heterozygous *abp1* mutant plants. Taken together, the analysis of morphology data showed that heterozygous *abp1* mutant *Arabidopsis* is phenotypically different from wild type ABP1 plants.

**Evaluation of expression levels of auxin-induced early genes**

From semi-quantitative data we found that a concentration 0.03μM of IAA seemed to give no significant response in the expression auxin-induced genes. Therefore, we decided to use only 0.1 μM IAA in auxin treatment in quantitative RT-PCR. For quantitative real time PCR experiments, four auxin-induced early genes were tested and analyzed. All primers for PCR amplification were obtained from Paponov et al. (2008). Quantifying the relative changes in gene expression was calculated based on the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). By normalizing cycle threshold value (Ct) of gene target with Ct value of specific reference gene, generally housekeeping genes, and Ct value of calibrator sample (ie. without treatment or control sample), relative value expression of target genes can be generated. The common equation for $\Delta\Delta C_T$ is [(Ct gene of interest – Ct reference gene) $_{\text{time } x}$ – (Ct gene of interest – Ct reference gene) $_{\text{time } 0}$]. The $2^{-\Delta\Delta C_T}$ method is a convenient way to analyze the relative changes in gene expression from real time quantitative PCR experiment. This method makes several assumptions, including that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is similar to the internal control gene (Livak and Schmittgen, 2001). For investigating the physiological changes in gene expression, the relative expression ratio is adequate for most purposes (Pfafft, 2001). In this experiment, 18S rRNA gene was used as endogenous standard gene.

In this experiment, the expression of control samples (water treated samples) in wild type and heterozygous *abp1* samples are shown in figure 13. As shown on figure 13, the starting point of expression of *IAA2* gene in wild type was statistically significantly different from wild type expression (p-value ≤ 0.01) in heterozygous *abp1* samples, whereas the others gene were not different. Interestingly, by comparing the expression of those genes with water treated samples from microarray data, it was found that the expression of *IAA2, IAA11*, and *IAA20* genes of wild type control samples from quantitative RT PCR have a similar relative expression pattern in microarray data. *IAA2* was expressed about 5 times higher than *IAA11*, whereas *IAA20* was very low expressed. However, *IAA13* expression differed from quantitative RT PCR data. The reason remains unexplained.

As shown on Figure 3, at t = 0 min, the expression of three tested genes was higher in wild type samples than in heterozygous *abp1* samples. The *IAA20* gene in heterozygous *abp1* sample was expressed higher than in wild type at t = 0 min. A t = 0 min the expression of *IAA11* and *IAA13* genes in wild type were 1.5 times fold higher than in heterozygous *abp1* mutant samples (not statistically significant), and the *IAA2* gene in wild type was expressed 2.8 fold higher than on heterozygous *abp1* (statistically significant). After 30 min of auxin treatment, all genes were found to be expressed higher in wild type samples than in heterozygous *abp1* samples, especially the *IAA20* gene. The statistical significance for the 30 min values was clear. However, after 60 minute of auxin treatment, only *IAA11* and *IAA13* expression was weakly higher (1.2 -1.7 fold) in wild type samples in comparison to heterozygous *abp1* samples. Those expression data indicate that wild type plant expressed *Aux/IAA* genes (*IAA3, IAA5, IAA14*, and *IAA20*) higher than in heterozygous *abp1* mutant plants when treated by 0.1 μM auxin.

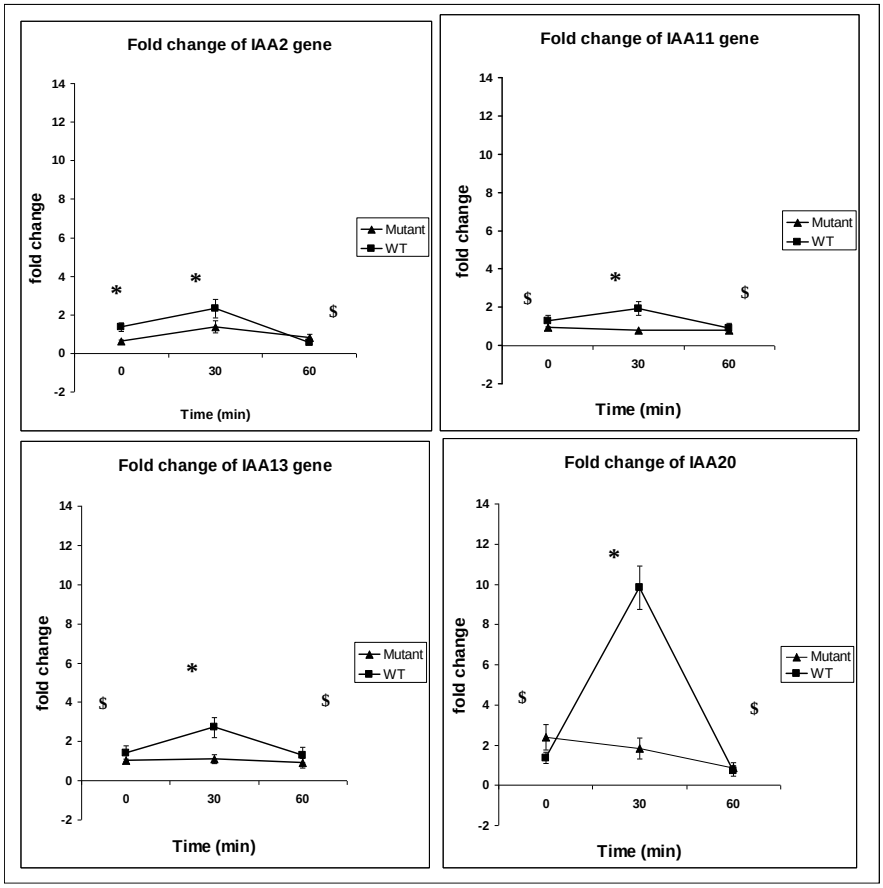


Fig.3. Quantification of gene expression in wild type (wt) and heterozygous *abp1* mutant plants of several *IAA* genes by quantitative Real Time PCR. Data are the average of 9 values obtained as each 3 technical replicates of 3 biological replicates (S.E; n = 9). Statistical significance between wild type and heterozygous *abp1* is indicated by * (p-value ≤ 0.04) or $ (not significant). A value t = 0 represents 10 sec of IAA treatment. A third control with no treatment was set as reference point equaling 1-fold (100%) transcription at t = 0 for each wild type and *abp1* heterozygous plants. The fold change against the internal standart 18S rRNA is indicated *IAA2* gene (A), *IAA11* gene (B), *IAA13* gene (C) and *IAA20* gene (D) of wt (square) and *abp1* (diamond)

## Description of T1 mutant plants

Each entry vector containing a gene construct then was recombined in to the destination vector plasmid pB2GW7 (Karimi et al., 2002) through LR reaction using clonase enzyme (Invitrogen) and transformed into *E. coli* TOP 10 in order to prepare the cassette for transformation into *Agrobacterium*. After each gene construct had been transformed into *Agrobacterium* GV 3101, they were transformed further into heterozygous *abp1* plants which had been grown before.

Plant transformation was carried out by using the floral dip method (Clough and Bent, 1998). The T1 seeds were selected by growing on agar medium containing 100µg/ml kanamycin and 25µg/ml

basta. Different mutation vector constructs resulted in different numbers of plants which can survive on the double selection agar medium. In general, mutant number 3 gave a large number of plants which could survive, whereas of mutant number 1, 2 and 5 no seeds were found which could grow (Figure 3). Re-transformation for these mutants (1, 2, 5) were performed, however, no seeds capable to grow on double selection medium agar were obtained. Some T1 plants after they were transferred on soil are shown in figure 4 and 5. In comparison with wild type plants, the T1 plants showed different characters. Some typical characters such as thickness of shoot, branching, inflorescence, size of plant and leaf were obvious in T1 plants and are shown on figures 4-6 as samples.

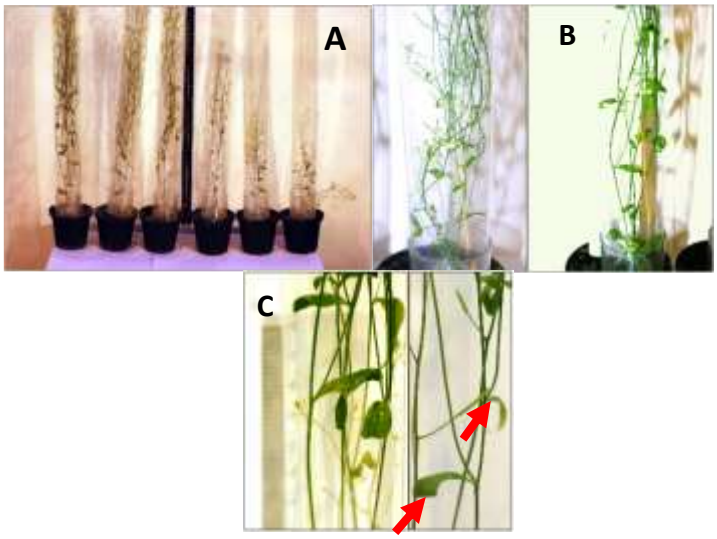


Fig. 4. Morphology of T1 plants of mutant number 3. (A) Variance in plants size of different lines (47 days old); (B) Branching type of shoots; plant have more branches but thinner (left) or plants have few and thicker branches (right); (C) normal and curly leaves. Red arrow point out curly leaves

Each mutation site seemed give rise to different effects on the plant phenotype. From all T1 mutant plants, mutant number 3 showed the widest range of phenotype differences. There were plants with small size and large size such as in wild type plant (Figure 4). Some plants with smaller size showed fewer shoots (3 shoots) and branches (Figure 5B). The taller lines (Figure 6A) also seemed to have only few shoots (3-5) and fewer leaves than wild type (5-7 shoots, not shown). Many plants had curly or thickly leaves (Figure 4C).

Fig. 5. Morphology of T1 plants of mutant number 6 and 7 (40 days old). (A) Plant size differences in mutant 6; (B) enlarged picture of a dwarfish plant of mutant number 6 (left-most plant in (A)) with fewer leaves and irregular inflorescence phenotype; (C) Morphology of T1 plants of mutant number 7 plants; (D) Close up shows fewer leaves and branches.

Mutant number 6 and 7 had also a smaller size in comparison to wild type (Figure 5). In comparison to other mutants, T1 plant lines of mutant number 6 had smaller sizes or were even dwarfish. Moreover, those small plants produced very few leaves and irregularly branches inflorescences (Figure 5). The overall morphology of T1 plant lines of mutant number 7 was similar to wild type plants, only those plants had a smaller size.

Mutant number 9 produced T1 plants having a large size similar to wild type plants (Figure 6). Those plants appeared to have more branches than wild type and produced tall inflorescences such as in wild type plants. We found also some lines which had smaller size, fewer shoots and branches (Figure 6B).

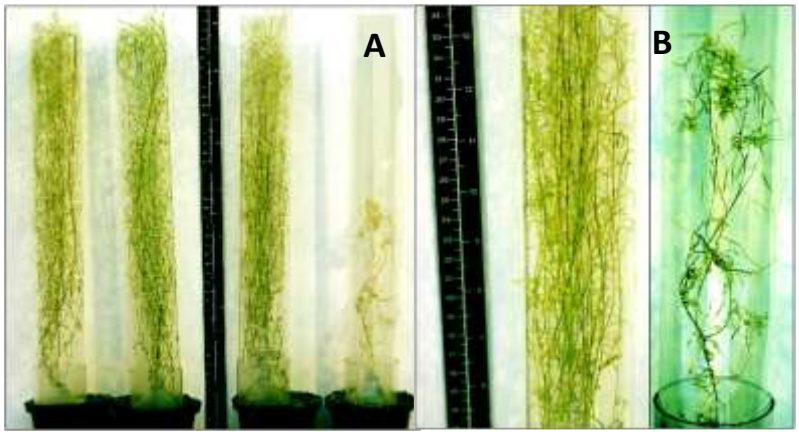


Fig.6. Morphology of mutant number 9. (A) Variance in plant size. Dominantly, the plant size of mutant 9 is similar with wild type and has more branches (B-left). However, there was also plant with smaller size and fewer branches (B-right).

Heterozygous plants grown under long-day conditions not only had reduced apical dominance but often had fewer rosette leaves. The photograph (Figure 7a) taken shortly before the wildtype plants started flowering shows that the population of seeds grown from a kanamycin-resistant abp1/ABP1 plant segregated into approximately one-third that were not as yet flowering and two-thirds that were flowering. When plants of the segregating population were sorted by PCR genotyping, the early-flowering plants had an abp1/ABP1 genotype, whereas the late-flowering plants were homozygous for the wild-type allele. According our hypothesis, defect in auxin receptor, abp1/ABP1, it may cause defect in auxin-related phenotype, such as responses to gravitropism and phototropism in roots as well as in shoots. Data showed that all those auxin-related phenotypes in heterozygous abp1/ABP1 are significantly differ in comparison to WT (Fig7 b-e)

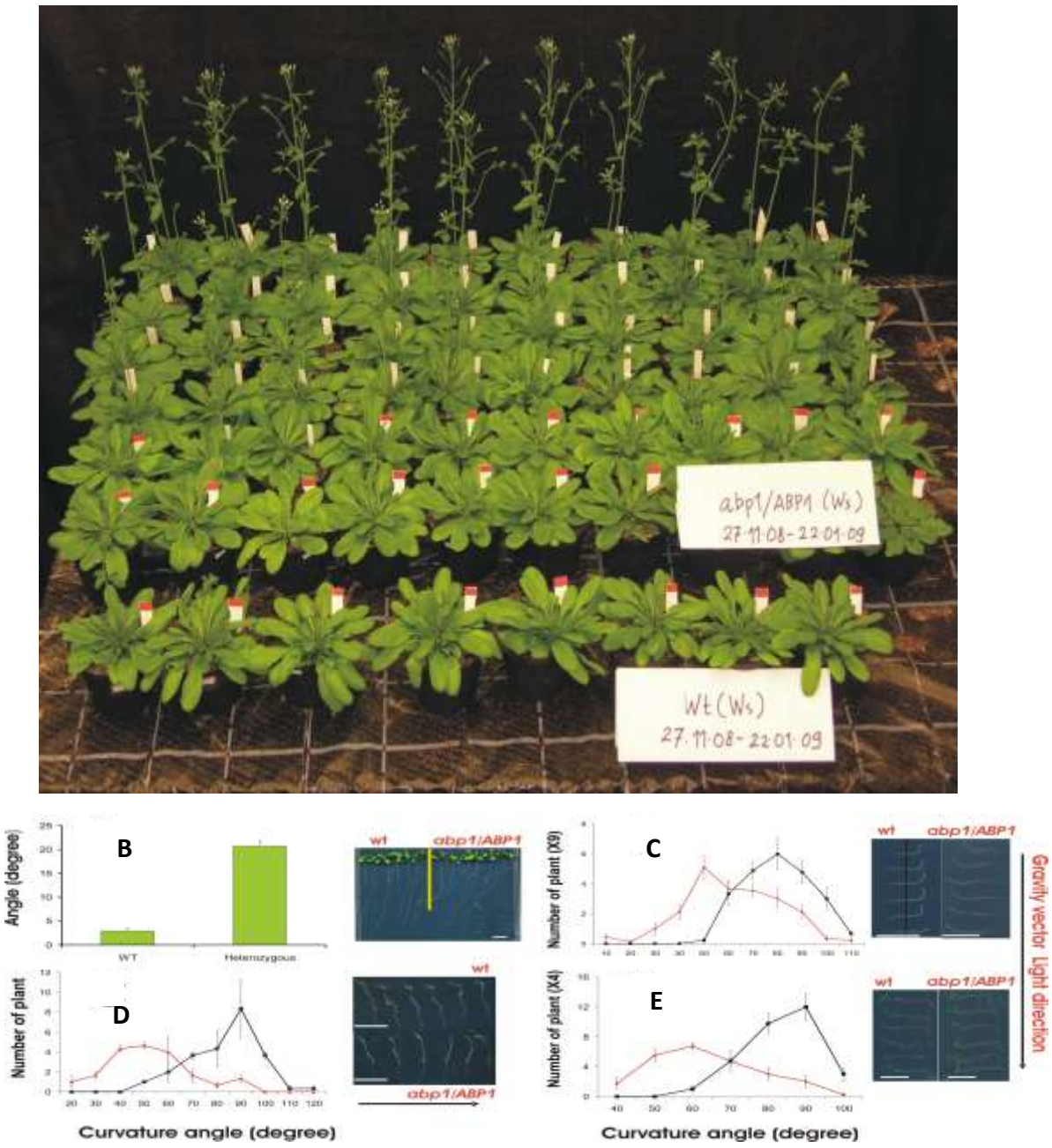


Fig.7. Early-flowering phenotype of wildtype Ws and heterozygous abp1/ABP1 plants grown under short-day conditions (8 h/16 h light/dark) (a–c) or long-day conditions (16 h/8 h light/dark) (d, e). (a) Plants from seeds of a kanamycin-resistant heterozygous abp1/ABP1 plant and Ws wild-type plants, as indicated, grown under short-day conditions. (b) slanting angle in hypocotyl; (c) hypocotyl gravitropic responses; (d) Hypocotyl phototropism responses (Blue light); (e) Root gravitropic responses.

## DISCUSSION

### Characterization of heterozygous *abp1* as a mutant

Before all gene constructs containing mutated *ABP1* cDNA were transformed into heterozygous *abp1* plants, characterization the heterozygous *abp1* mutant plants was performed. Our primary observation was that heterozygous *abp1 A. thaliana* (Ws-2), in general, had similar characteristic such as described by Chen et al. (2001). Analysis of the *ABP1* gene in heterozygous *abp1* plants and wild type plant showed that heterozygous *abp1* mutant plants produced a 1.5 kb fragment and 2.6 kb fragment in a PCR test technique, whereas using the same primers combination, wild type plants produced only 2.6 kb fragment. This verified that the heterozygous *abp1* mutant has two different *ABP1* alleles, one with and without insertion. The *abp1* mutant seeds which were used in this experiments and in Chen's experiments originated from the same seed sources, both are from Alan Jones group who donated *abp1* mutant seeds to The *Arabidopsis* Biological Resource Center – Ohio University (ABRC). Heterozygous *abp1* mutant plants produced brown seed (wild type and heterozygous *abp1* seeds) and white seeds (homozygous *abp1* seeds) in ratio 3:1 as predicted by Mendel's law in self mating of heterozygous organisms with one mutant allele. This is both consistent with Chen et al. (2001).

However, further observations on seedlings and mature heterozygous *abp1* T-DNA mutant plants showed that the plants have a mutant morphological phenotype. Data were observed on seedling stage, immature plants and mature plants have shown clearly differences between heterozygous *abp1* plants and wild type, which in Chen et al. (2001) did not mentioned. Interestingly, we found that two white seeds could grow and produced a complete seedling and one mature green plant, although after further growing for 3 weeks on soil this plant also died for unknown reasons. According Chen et al. (2001), white seeds from heterozygous *abp1* progenies cannot grow to produce viable plant, because the embryo in all white seeds cannot develop to produce normal embryo. Moreover, the second observation was that four weeks old heterozygous *abp1* plants showed clearly plants having a smaller size in comparison to wild type plants of equal age. Thirdly, *abp1* heterozygous plants also had a clear seedling phenotype showing a waving pattern and a skewing angle when growing on vertical agar plate. The fourth observation was that heterozygous *abp1* plants flowered earlier than wild type although this was not quantified. Experiments for evaluating the expression level of appropriate auxin-induced genes were also performed. Even though, due to time restrictions, only few genes could be investigated and properly quantified by qRT-PCR, clear differences in the expression of early auxin-induced genes could also be found in the mutant. It is obvious that the heterozygous state of the *ABP1* gene already is sufficient to make the heterozygous *abp1* plant a mutant. ABP1 very clearly is an excellent auxin binding protein (Napier et al., 2002; Woo et al., 2002) so that it is difficult to assume something else but auxin functions to be associated with it. The lack of chromatid expressing *ABP1* mRNA may result in lower mRNA and, subsequently, lower protein amounts. For reasons of the short time for this project, mRNA of ABP1 was not tried to quantified but reduced amounts of ABP1 protein seem to be most adequate explanation for the observed phenotype. If ABP1 is an auxin receptor this should result in lower signal strength because of a lower number of active receptor molecules, provided the same hormone concentration is kept. Lower signal strength should result in partial losses of functions.

Can reduce steam size, early flowering, root waving, and altered regulation of auxin-induced genes be interpreted as partial loss functions?. The reduced size of the *abp1* heterozygous plants can indeed be explained as a partial loss of elongation. Other mutants related to auxin signaling showing reduced size are, for instance, *axr1* (Lincoln et al., 1990), *axr2-1* (Timpte, 1994), *sax1* (Ephritikhine et al., 1999), *acl5* (Imai et al., 2006), *mya2* (Holweg and Nick, 2004), etc. Thus, in

this study, it is quite obvious that the reduced size phenotype is entirely consisted with a function of ABP1 as an auxin receptor.

Early flowering was indicated by our data although it was not quantified. This rather points out to some link to light signaling because *Arabidopsis thaliana* is a facultative long day plant. Facultative long day plants flower earlier, for instance, if red light signaling by phytochrome B is impeded (Fankhauser and Staiger, 2004). Other factors may also induce such a phenotype (Taiz and Zeiger, 2006). It should be pointed out in this context, that activity of the ABP1 protein is decreased by red light (Jones et al., 1991). This aspect seems worth investigating in further studies on the heterozygous *abp1* mutant.

The root waving phenotype of the young *abp1* seedlings could also be related to auxin functions. Waving mutant are associated with several gene groups most of which are related to a loss of gravity sensing (Simmons et al., 1995; Simmons et al., 2006) and/or auxin transport defects (*agr1/eir1/pin2*) or phototrophism *(wav2)* (Chen et al., 1999; Mochizukia et al., 2005). Other gene defects leading to a waving mutant are also described which, presently, cannot be related to auxin functions (Sedbrook et al., 1999). Lack of gravisensing or genetic defects can contribute to a mutation in the waving pattern, circumnutation and negative thigmotropism an obstacle avoiding response (Migliaccio and Piconese, 2001). Circumnutation also requires auxin transport and gravisensing (Simmons et al., 1995) so that auxin transport and gravisensing disturbances are major contributions to waving phenotypes, other gene defects cannot be excluded (Chen et al., 1998). However, most waving mutants seem to be associated with auxin transport which is integrated into gravitropism and phototropism. Recently, it was found that auxin itself inhibits auxin transport by inhibiting the endocytosis of PIN protein (Paciorek et al., 2005). This inhibition requires an auxin receptor other than TIR1 and several authors suggested this (Tomasz and Friml, 2006; Merks et al., 2007) and ABP1 could be a receptor for auxin inhibition of auxin efflux transport which is faster regulated then gene regulation the only know function of TIR1 (Paciorek et al., 2005; Calderon-Villalobos et al., 2006; Merks et al., 2007). So, recent publication point out that there could be a link of ABP1 to PIN1 endocytosis recycling and auxin transport regulation which might be a basis for a hypothesis stimulating further experiments on the waving phenotype of *abp1* heterozygous mutant. Such experiments could be investigation of gravitropic or phototropic sensitivity or direct measurement of auxin transport.

It is obvious that the data suggest that ABP1 protein is an auxin receptor: *abp1* heterozygous plants have a phenotype affected in several auxin-regulated features, like stem elongation, root waving and regulation of know auxin-regulated genes.

**Analysis of auxin-induced early gene expression**

**Gene expression measured by quantitative real time PCR data.**

Currently, there is no doubt that PCR can be made quantitative. In a number of publications, the quantitative capability of PCR has been demonstrated by comparing it to classical means of nucleic acids quantifying, such as Northern blot or Southern blot analysis (Ferre, 1992). Introduction of quantitative real time PCR has shown that this new technique can be used to quantify more precisely the amplification product. However, this technique require some condition in order to get precision data, such as better primer performance (no dimer), precision on pipetting, the same amount of DNA/cDNA template etc. Optimizing of the PCR reaction includes the cycle number

and right melting temperature also are essential conditions for this technique. Another important factor in quantitative real time PCR experiments is the reference gene. Because the measurement accuracy of gene expression in real time PCR generally relies on normalization of the expression of target gene to specific reference gene, therefore the reference gene is a crucial factor for obtaining a good result. Housekeeping genes are generally used as reference genes such as 18S rRNA, *UBIQUITIN (UBQ), ACTIN (ACT), b-TUBULIN (TUB),* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) because they were supposed to have a uniform expression (Jain et al., 2006). Failure to use an appropriate control gene may result in biased gene expression profiles, as well as low precision. The consequences may be that only gross changes in expression level are declared statistically significant, or that patterns of expression are erroneously characterized (Brunner et al., 2004).

We compared the expression of *IAA2, IAA11, IAA13*, and *IAA20*. Based on Ct values which have been normalized by using Ct value of 18S rRNA gene, fold change of tested-genes were obtained. Predominantly, the expression Aux/IAA genes in wild type samples differ significantly in comparison with heterozygous *abp1* samples, especially after 30 min auxin exposure (significant with a p-value $< 0.03$). Only *IAA2* did not show a significant difference after 30 min auxin application. After zero time auxin exposure, the expression of *IAA2* and *IAA11* showed a significant difference (significant with p-value $\leq 0.02$) between wild type samples and heterozygous *abp1* samples. There are no statistically significant differences between wild type samples and heterozygous *abp1* samples on the expression of all tested genes in this experiment at 60 min after auxin exposure. Those data show that all tested genes were varying their expression at different times after auxin application and the changes were transient. Currently, there is no information on whether ABP1 is involved in rapid transcriptional responses to auxin (Kepinski and Leyser, 2005) but the data here clearly indicate this. However, several phenotypic differences in wild type and heterozygous *abp1* plants in this study and other publications about auxin-induced cellular processes such as swelling of the protoplast (Steffens et al., 2001), stomatal closure (Blatt and Thiel, 1994; Gehring et al., 1998), etc, indicated that it is probable any expression genes are modulated via ABP1 perception.

Regarding differences of *Aux/IAA* genes expression data on wild type and heterozygous *abp1* samples, there are some possibility reasons that those genes did not show clearly differences. Several publications have reported that auxin induces the expression of many, but not all, Aux/IAA gene family members. For example the *Arabidopsis IAA1* through *IAA14* and *IAA19* genes are auxin inducible with varying induction kinetics, and their mRNA accumulation varies in different parts of the plant (Abel et al., 1995; Tatematsu et al., 2004). Several Aux/IAA genes, including *IAA17* and *IAA28*, show little or no response to exogenous auxin. The diversity in auxin responsiveness and tissue-specific expression among the various gene family members suggests that each member may have a distinct or overlapping function(s) during normal auxin responses required for plant development (Overvoorde et al., 2005).

Paponov et al. (2008) demonstrated that a 0.5 h application of 1μM 1-NAA was enough to alter *IAA11* and *IAA13* transcription. After 1 h exposure with 1μM 1-NAA both *IAA11* and *IAA13* genes and also *IAA2* gene elevated to a two fold change of transcription. However, *IAA20* gene does not show any alteration in transcription on the same condition. A high heterogeneity is observed in the transcription of the Aux/IAA gene family in response to auxin in different microarray experiments by using different auxins and plant material (Paponov et al., 2008). However, in comparison with Paponov et al. (2008), there are two factors which differ compared to our experiment. First, we

used IAA instead of 1-NAA, and second, we used a very low IAA concentration 0.1μM for induction gene expression, whereas Paponov et al. (2008) used 1μM 1-NAA. Furthermore, they used *Arabidopsis* cell culture as plant material. It may be possibly that those factors give a significant contribution to the differences. ABP1 has higher affinity to 1-NAA than to IAA (Löbler and Klämbt, 1985; Yamagami et al., 2004). The affinity of ABP1 for 1-NAA is reported variously as being between 50 and 200 nM, whereas its affinity for IAA is much lower between 5–10 mM (Badescu and Napier, 2006) and it is affected by pH (Brown and Jones, 1994). The effect of auxin also thought to depend on its concentration, with high and low doses eliciting different responses (Teale et al, 2006). Nevertheless, the existing evidence suggests that there are multiple auxin receptors, and hence the work on ABP1 is expected to answer only part of the question of how the auxin signal is perceived.

**Mutagenesis of some conserved amino acid residues of ABP1**

Mutagenesis has been carried out in this project and all gene constructs have been successfully obtained (supplement 1, 2). Nine gene constructs containing different mutations were transformed into heterozygous *abp1* mutant plants and T1 generation has been collected. Clear differences in phenotypes of these T1 plants were observed and different mutations seemed to give different effects on the phenotypes of the heterozygous *abp1* transformed plants. Some characters which have been collected concerning with T1 mutants are different size of plant, less branching, the number of shoot, leaf appearance, etc (Figure 4-7, supplement 2).

The long objective of this experiment is actually to design clear homozygous *abp1* mutant plants which are not lethal so then further experiments with plants having mutant ABP1 protein can be performed. Although selecting by using double marker (kanamycin and basta resistances) has been carried out and T1 progenies containing both these gene markers were obtained, homozygous *ABP1* progeny was not yet obtained. By self mating, selecting using both markers, and PCR assays of the further T generations (T2, T3, T4, T5), we believe that homozygous mutated-*ABP1* T-DNA insertion progenies with no expression of wild type ABP1 can be collected. However, at the moment the work reached T1 selection for preparing T2 generations.

From T1 generation of different mutant lines, differences of plant phenotype were noticed. Although we cannot prove that those phenotypic differences are due to mutation of given amino acid residue(s), the reason for different phenotypes could be that the mutated protein is expressed from the strong promoter whereas the wild type *ABP1* is expressed from a weak wild type promoter. Conceivably, mutant denies of *ABP1* may dominate the phenotype. Currently, properly experiments for testing this hypothesis cannot easily Currently, properly experiments for testing this hypothesis cannot easily be performed, because the genetic background of those mutated-*ABP1* plants are not yet homozygous and the original wild type ABP1 protein remains in those plants.

We also noticed on those T1 generations that some mutant lines are not able to grow on selective medium (containing kanamycin and basta markers). Mutant lines contain mutations on tryptophan 135 (mutant number 1 and 2) and on tryptophan 151 (mutant number 5) showed those inability to grow on selective agar medium. We noticed also that even though mutant number 4 and 6 which contain mutation on tryptophan 135 were able to grow on selective medium, only few seedlings could grow properly. Currently, we also encountered problems in growing mutant number 4 for

producing the next T generations. This could indicate that the mutant proteins are lethal to seedlings.

Multi-alignment analysis showed that those amino acid residues used for mutation in this project are all conserved. It suggests that those amino acids are likely to have important functions in ABP1 protein. All amino acid targets for mutagenesis this experiment actually have been described in previous publications in which those amino acid residues were predicted to be involved in the binding of auxin by ABP1 protein or in the folding of ABP1 protein. Warwickers et al. (2001) in their publication proposed that there are two tripeptide motifs on ABP1, DDW136 and WDE153, which either one of these tripeptides could occupy the auxin-binding site in the absence of ligand. Displacement of this tripeptide by free auxin would induce a conformation change to initiate signaling. Similar result was also reported by David et al. (2001) working with *Nicotiana tabaccum*. They concluded that site-directed mutagenesis on WDE175, corresponding to WDE153 on *Arabidopsis*, have a critical role in protein folding and functional activity of ABP1 at the plasma membrane. Moreover, Woo et al. (2002) described that the binding pocket of ABP1 is predominantly hydrophobic with a metal ion deep inside the pocket coordinated by three histidines and a glutamate. Auxin binds within this pocket with its carboxylate group binding the zinc and its aromatic ring binding hydrophobic residues including Trp151. All together suggests that tryptophan 135 and 151 have crucial functions in ABP1 protein. According to crystal study of ABP1 protein of maize, Woo et al. (2002) also revealed that Cys155 has a crucial function on ABP1 protein. Together with Cys2, Cys155 form a single disulfide bridge which important for stabilizing the protein both in the presence and absence of bound auxin. No mutation of cysteines was attempted.

However, much works remain need to be done in order to get a better understanding of the function of amino acid residue(s) of ABP1. By obtaining clear homozygous mutated *ABP1* plant lines, we expect that better experiments to study ABP1 functions can be performed. Nevertheless, currently we have proven that introduction of new mutated *ABP1* gene constructs into *Arabidopsis* was successfully carried out. For future works, the next T generations will be collected in order to obtaining homozygous *abp1* mutant plants.

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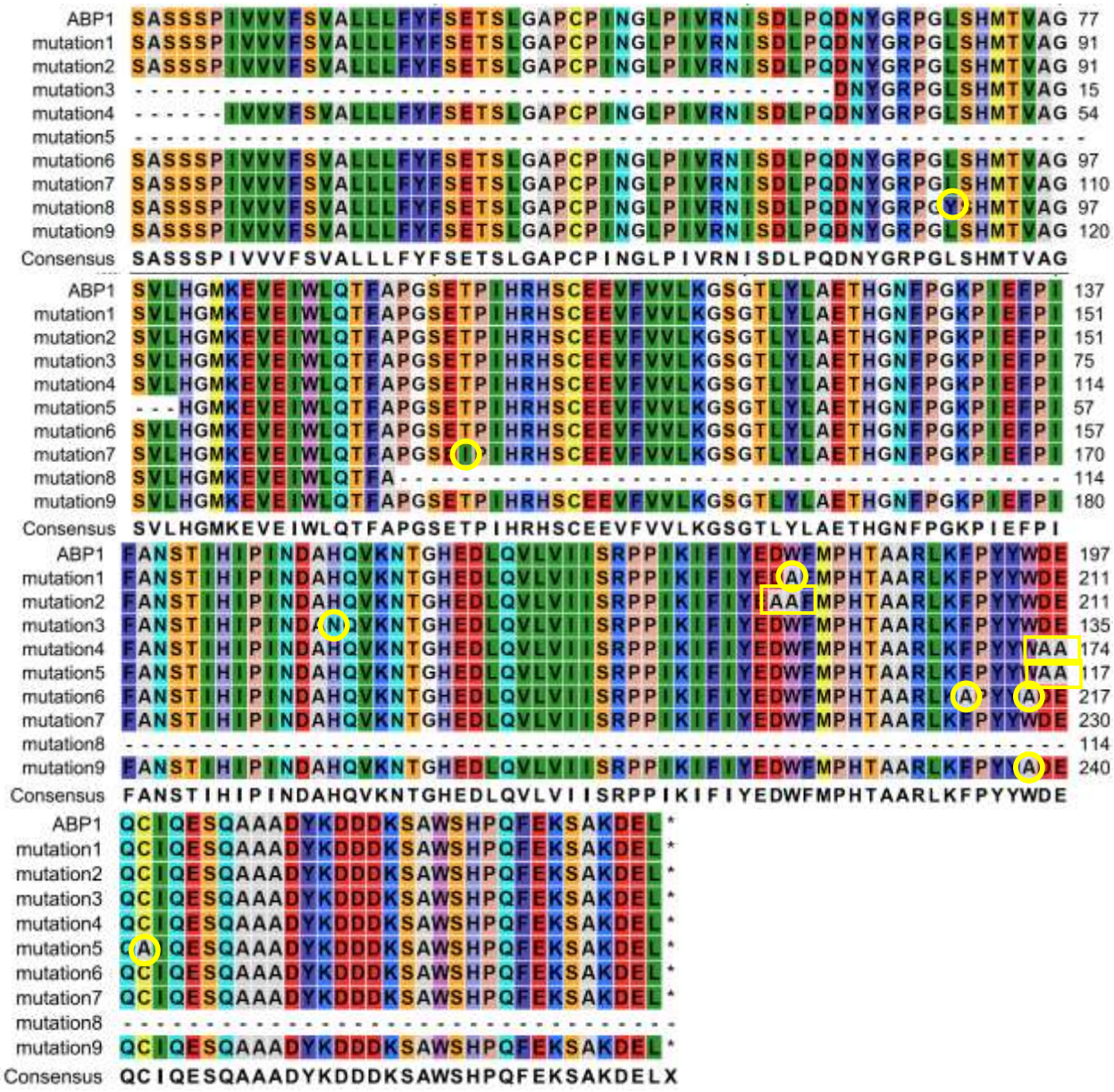
Supplement 1 – Multiple sequence alignment of ABP1 gene in mutants


ABP1 SASSSPIVVVFSVALLLFYFSETSLGAPCPINGLPIVRNISDLPQDNYGRPGLSHMTVAG 77
mutation1 SASSSPIVVVFSVALLLFYFSETSLGAPCPINGLPIVRNISDLPQDNYGRPGLSHMTVAG 91
mutation2 SASSSPIVVVFSVALLLFYFSETSLGAPCPINGLPIVRNISDLPQDNYGRPGLSHMTVAG 91
mutation3 DNYGRPGLSHMTVAG 15
mutation4 IVVVFSVALLLFYFSETSLGAPCPINGLPIVRNISDLPQDNYGRPGLSHMTVAG 54
mutation5 - 1
mutation6 SASSSPIVVVFSVALLLFYFSETSLGAPCPINGLPIVRNISDLPQDNYGRPGLSHMTVAG 97
mutation7 SASSSPIVVVFSVALLLFYFSETSLGAPCPINGLPIVRNISDLPQDNYGRPGLSHMTVAG 110
mutation8 SASSSPIVVVFSVALLLFYFSETSLGAPCPINGLPIVRNISDLPQDNYGRPG 97
mutation9 SASSSPIVVVFSVALLLFYFSETSLGAPCPINGLPIVRNISDLPQDNYGRPGLSHMTVAG 120
Consensus SASSSPIVVVFSVALLLFYFSETSLGAPCPINGLPIVRNISDLPQDNYGRPGLSHMTVAG
ABP1 SVLHGMKEVEIWLQTFAPGSETPIHRHSCEEVFVVLKGSGTLYLAETHGNFPGKPIEFPI 137
mutation1 SVLHGMKEVEIWLQTFAPGSETPIHRHSCEEVFVVLKGSGTLYLAETHGNFPGKPIEFPI 151
mutation2 SVLHGMKEVEIWLQTFAPGSETPIHRHSCEEVFVVLKGSGTLYLAETHGNFPGKPIEFPI 151
mutation3 SVLHGMKEVEIWLQTFAPGSETPIHRHSCEEVFVVLKGSGTLYLAETHGNFPGKPIEFPI 75
mutation4 SVLHGMKEVEIWLQTFAPGSETPIHRHSCEEVFVVLKGSGTLYLAETHGNFPGKPIEFPI 114
mutation5 HGMKEVEIWLQTFAPGSETPIHRHSCEEVFVVLKGSGTLYLAETHGNFPGKPIEFPI 57
mutation6 SVLHGMKEVEIWLQTFAPGSETPIHRHSCEEVFVVLKGSGTLYLAETHGNFPGKPIEFPI 157
mutation7 SVLHGMKEVEIWLQTFAPGS 170
mutation8 SVLHGMKEVEIWLQTFA 114
mutation9 SVLHGMKEVEIWLQTFAPGSETPIHRHSCEEVFVVLKGSGTLYLAETHGNFPGKPIEFPI 180
Consensus SVLHGMKEVEIWLQTFAPGSETPIHRHSCEEVFVVLKGSGTLYLAETHGNFPGKPIEFPI
ABP1 FANSTIHIPINDAHQVKNTGHEDLQVLVIISRPPIKIFIYEDWFMPHTAARLKFPYYWDE 197
mutation1 FANSTIHIPINDAHQVKNTGHEDLQVLVIISRPPIKIFIYE 211
mutation2 FANSTIHIPINDAHQVKNTGHEDLQVLVIISRPPIKIFIY 211
mutation3 FANSTIHIPINDA QVKNTGHEDLQVLVIISRPPIKIFIYEDWFMPHTAARLKFPYYWDE 135
mutation4 FANSTIHIPINDAHQVKNTGHEDLQVLVIISRPPIKIFIYEDWFMPHTAARLKFPYY 174
mutation5 FANSTIHIPINDAHQVKNTGHEDLQVLVIISRPPIKIFIYEDWFMPHTAARLKFPYY 117
mutation6 FANSTIHIPINDAHQVKNTGHEDLQVLVIISRPPIKIFIYEDWFMPHTAARL 217
mutation7 FANSTIHIPINDAHQVKNTGHEDLQVLVIISRPPIKIFIYEDWFMPHTAARLKFPYYWDE 230
mutation8 114
mutation9 FANSTIHIPINDAHQVKNTGHEDLQVLVIISRPPIKIFIYEDWFMPHTAARLKFPY 240
Consensus FANSTIHIPINDAHQVKNTGHEDLQVLVIISRPPIKIFIYEDWFMPHTAARLKFPYYWDE
ABP1 QCIQESQAAADYKDDDKSAWSHPQFEKSAKDEL*
mutation1 QCIQESQAAADYKDDDKSAWSHPQFEKSAKDEL*
mutation2 QCIQESQAAADYKDDDKSAWSHPQFEKSAKDEL*
mutation3 QCIQESQAAADYKDDDKSAWSHPQFEKSAKDEL*
mutation4 QCIQESQAAADYKDDDKSAWSHPQFEKSAKDEL*
mutation5 QCIQESQAAADYKDDDKSAWSHPQFEKSAKDEL*
mutation6 QCIQESQAAADYKDDDKSAWSHPQFEKSAKDEL*
mutation7 QCIQESQAAADYKDDDKSAWSHPQFEKSAKDEL*
mutation8 -
mutation9 QCIQESQAAADYKDDDKSAWSHPQFEKSAKDEL*
Consensus QCIQESQAAADYKDDDKSAWSHPQFEKSAKDELX

Supplement 2 – Morphological comparison of abp1 mutants