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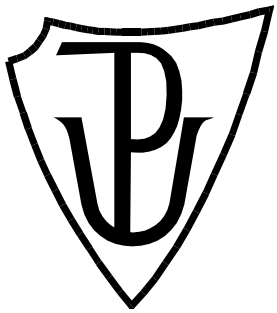
Faculty of Natural Sciences

Department of Biochemistry



Role of auxin-binding proteins ABP1 and ABP4 in regulation of PIN proteins in maize *Zea mays* L.

BACHELOR THESIS

Autor:	**Irena Malichová**
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In Olomouc .....

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Abstrakt

Dřívější studie naznačily, že auxin-binding proteiny (ABP) mohou být zapojeny ve světelné signalizaci během vývoje a růstu rostlin kukuřice. U *Arabidopsis* bylo zjištěno, že PIN proteiny, které hrají klíčovou úlohu v polárním auxinovém transportu (PAT), cyklují mezi endosomy a plazmatickou membránou a jejich rozložení v buňce je regulováno auxinem prostřednictvím ABP1. Je ovšem otázkou, zdali ABP ovlivňují expresi *PIN* genů, a tím i na transkripční úrovni regulují polární auxinový transport. Pro získání více informací o interakci auxinové a světelné signalizace zapojené v růstu a vývoji rostlin kukuřice, jsem studovala, zda proteiny ABP1 a ABP4 ovlivňují expresi genu kódující kukuřičný protein PIN1a. K tomu jsem využila genetického přístupu, který spočíval v analýze 'loss-of-function' mutantů *abp1* a *abp4*. Nejprve jsem zjišťovala, jak mutace v *ABP1* a/nebo *ABP4* genu ovlivňuje fenotyp kukuřičných semenáčků, a to v závislosti na světelných podmínkách. Experimenty ukázaly, že ABP1 a/nebo ABP4 jsou zapojeny v růstu a vývoji rostlin kukuřice, a že účinky těchto genů jsou ovlivňovány kvalitou světla, a to v závislosti na orgánu rostliny. Výsledky dále ukázaly, že ABP1 i ABP4 ovlivňují expresi genu *PIN1a*, a to rozdílně ve tmě a na světle. Pokud ABP proteiny regulují množství transkriptu genu *PIN1a*, lze vyvodit závěr, že ABP pravděpodobně ovlivňují i polární auxinový transport. Data v této bakalářské práci proto podporují předchozí závěry, že ABP1 a ABP4 fungují jako prostředníci mezi signálními drahami auxinu a světla během růstu a vývoje rostlin kukuřice.

Klíčová slova	ABP1, ABP4, auxin, exprese genu, světlo, PIN1, *Zea mays L.*
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Abstract

Previous research in maize suggested a possible involvement of auxin-binding proteins (ABPs) in light-signaling during growth and development. In *Arabidopsis*, it was revealed that PIN proteins, which are the main proteins engaged in polar auxin transport (PAT), cycle between the endosomes and plasma membrane, and their distribution in the cell is regulated by auxin via binding to ABP1. But yet, the question is whether ABPs influence the expression of *PIN* genes, thereby on the transcriptional level influence the polar auxin transport. To obtain more information about the interaction of auxin and light signaling during the growth and development in maize seedlings, I investigated the possible involvement of maize auxin–binding protein ABP1 and ABP4 in expression of genes coding for maize *PIN1a*. For that, I used a genetic approach based on analysis of 'loss-of-function' mutants *abp1* and *abp4*. Firstly, I investigated how the mutation in *ABP1* and/or *ABP4* gene alters phenotype of maize seedlings depending on light conditions. Experiments showed that ABP1 and/or ABP4 play role in the growth and development of maize seedlings, and that their effects and functional relationship are organ- and light-dependent. The results also showed that ABP1 and ABP4 influence the expression of *PIN1a* gene but differentially in the dark and in light. In the case that ABPs regulate the level of *PIN1a* transcript, it can be deduced that ABPs most likely influence the polar auxin transport as well. The results of this bachelor thesis confirm the previous conclusions that ABP1and ABP4 are involved in the interaction between the light and auxin signaling in plant growth and development in maize.

Keywords	ABP1, ABP4, auxin, gene expression, light, PIN1, *Zea mays L.*
Number of pages	54
Number of appendices	0
Language	English

Goals

Theoretical part:

1) Summarize literature information about phytohormones, auxin binding proteins, and light signaling.

Experimental part:

1) Does the mutation in *ABP1* and/ or *ABP4* gene influence the phenotype of maize seedlings?
2) What is the role of light in growth responses of examined genotypes?
3) Does the mutation in *ABP1* and/ or *ABP4* gene influence the gene expression of PIN proteins - thereby influencing polar auxin transport?
4) What is the role of light in gene expression of genes coding for PIN proteins? How the light could influence polar auxin transport?

1. Introduction

Plant hormones auxins are involved in a broad spectrum of growth and developmental processes. For auxin to be able ‘do his job’, it is necessary to bind to an auxin receptor. Two auxin receptors are well explored; a nuclear receptor TIR1/AFB, and the second one Auxin-binding protein1. In maize, at least five ABPs have been identified, but their roles in growth and development are poorly understood.

Theoretical part of this study contains basic information about phytohormones and briefly summarizes so far gathered findings about ABPs. The aim of the experimental part of the thesis was to study possible involvement of maize auxin–binding protein ABP1 and ABP4in expression of genes coding for maize PIN1, main proteins involved in polar auxin transport. The experiments described in this bachelor thesis were performed in the Group of Molecular Physiology in the Laboratory of Growth Regulators at Palacky University in Olomouc. This work was supported by the Operational Programs Education for Competitiveness - European Social Fund (project no.CZ.1.07/2.3.00/30.0004) and by Laboratory of Growth Regulators.

2. Present state of studied issues

2.1 Phytohormones

Plant hormones, also called as phytohormones are signal molecules, whose function is to control and maintain the physiological and developmental processes. They are essential for the right growth, development and reproduction of plants. Disruption in their synthesis or an overproduction of certain phytohormone may cause a change in an inner balance of the plant and result in different phenotype.

Plant hormones differ in many aspects from the animal hormones. Plants don't have specific glands with inner secretion as animals do. The majority of phytohormonesis synthesized in several areas of the plant. The number of plant hormones is significantly lower than animal ones, therefore plant hormones are less specific and their effects are more complex. According to the inner and external conditions, plant hormones may cause a lot of different effects or only a few or just one at the time. Phytohormones cooperate together; the final physiological impact of phytohormones depends on the ratio of two or more phytohormones and also on the sensitivity of a certain part of the plant to these substances. The plant sensitivity to hormones differs, the concentrations of phytohormones may be very low such micro or nanomoles per liter. We can think that there are huge differences between animal and plant hormones signaling, but the study about how the hormones act in plants showed that the mechanism is very close to animal hormones. Phytohormones according their effects are categorized into two groups called stimulators and inhibitors. Stimulators (in appropriate concentration) speed up the growth on the other hand inhibitors slow down the growth and developmental processes (Taiz and Zeiger, 2006).

The primary step of hormone signaling is binding to a receptor. Receptors are protein molecules with a high affinity to bind a specific molecule like hormone and ability to initiate a specific signal pathway. Water soluble hormones bind to receptors placed on plasma membrane. The signal is transduced into the cell due to second messengers (Ca^{2+}, diacylglycerol, etc.) This type of receptor may mediate changes in plasma membrane permeability or activates specific signal pathway, such as gene expression. Lipophilic hormones can penetrate through the plasma membrane into the cytoplasm or in nucleus, where they bind to a receptor and make a complex.

In nucleus, hormones can activate or inhibit an expression of certain genes; which can result in a decrease or an increase in level of certain proteins. Based on an analogy with animal-hormone-working mechanism, five inner growth regulators are considered to be plant hormones. Names of hormone categories are auxins, cytokinins, gibberellins, abscisic acid, and ethylene. There are also substances such as brassinosteroids, polyfenols, polyamines, jasmonate acid, salicylic acid, which influence significantly some of the plant developmental and growth processes.

2.1.1 Auxins

Auxins are compounds with an aromatic ring and a carboxylic acid group. They were the first of the major plant hormones to be discovered. The existence of a growth promoting substance (identified later and called auxins) was observed by Charles Darwin in 1882, but proved was about 40 years later by doctor Frits Went. Wents' experiment identified how a growth promoting chemical causes the coleoptile growth towards the light. Went cut the tips of the coleoptiles and then placed them in the dark, putting a few tips on agar blocks. These blocks absorbed the growth-promoting chemical from the tips. On the top of control cut coleoptiles, he placed a block that lacked the growth-promoting chemical. On others, he placed blocks containing the chemical, either centered on top of the coleoptile to distribute the chemical evenly or offset to increase the concentration on one side. When the growth promoting chemical was distributed evenly the coleoptile grew straight. If the chemical was distributed unevenly, the coleoptile curved away from the side with the cube, as if growing towards light, even though it was grown in the dark. Went later proposed that the messenger substance is a growth-promoting hormone, which he named auxin, derived from the Greek word *auxein* - "to grow/increase", that becomes asymmetrically distributed in the bending region. Went concluded that auxin is at a higher concentration on the shaded side, promoting the cell elongation, which results in a coleoptiles bending towards the light.

Today we know that auxins play a central role in plant growth and development. They induce many physiological processes, including embryogenesis, stem elongation, apical dominance, photo- and gravitropism and lateral root formation. At the cellular level, auxin regulates these processes through changes in cell division, cell expansion, and differentiation. The underlying mechanisms of cell elongation still

remain the subject of debate, although distinct interpretations of auxin-induced growth have been proposed.

2.1.1.1 Biological essence and biosynthesis

Substances marked as auxins have a common or similar biological activity as indole-3-acetic acid (IAA). Plants produce several endogenous auxins for example 4-chloroindole-3-acetic acid (4-Cl-IAA), 2-phenylacetic acid (PAA), indole-3-butyric acid (IBA) and the most important one IAA. The basic chemical structure is made of an aromatic ring with a lateral chain which always contains a carboxylic group with a strong negative charge. Due to a quite simple chemical structure(Fig. 1), artificial auxins were synthesized (Fig. 2). Synthetic auxins are commonly to be found in experimental work, because there are more stable than natural ones. Most used synthetic auxin is 1-naphthaleneacetic acid (NAA), others less common are indole-3-butyric acid(IBA), 2-Methoxy-3,6-dichlorobenzoic acid (dicamba), 4-Amino-3,5,6-richloropicolinic acid (tordon or picloram), 2,4-Dichlorophenoxyacetic acid (2,4-D).

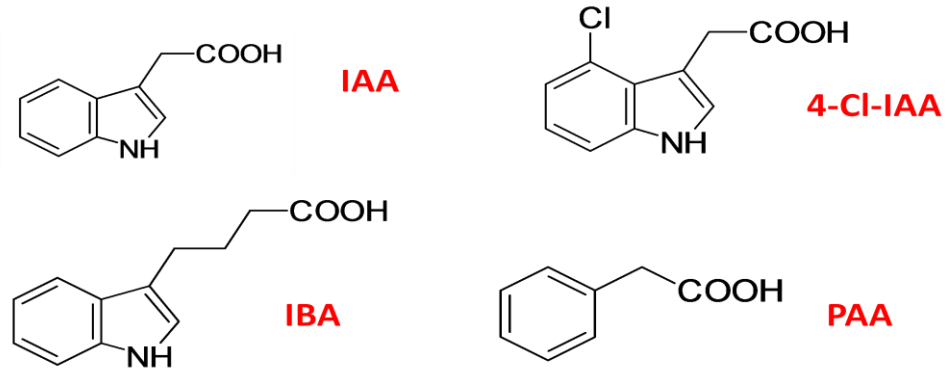

Figure 1
This picture shows the chemical structure of natural auxins.

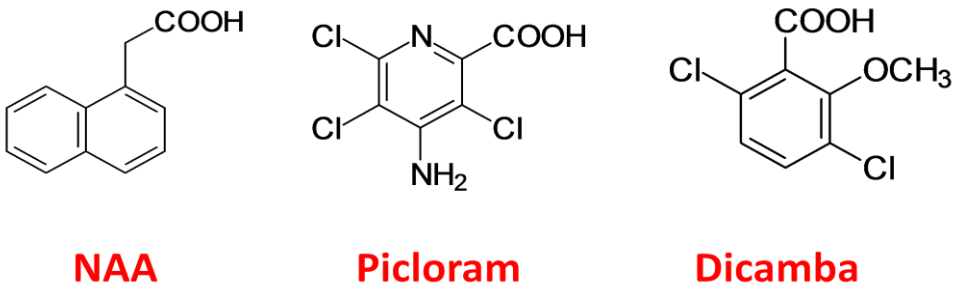

Figure 2
This picture shows the chemical structure of synthetic auxins.

Biosynthesis takes place mainly in growing tissues. IAA is synthesized from tryptophan via multiple pathways (Fig. 3). Most probably, the indole-3-pyruvate (IPA) pathway is the main IAA biosynthetic pathway. In the IPA pathway, tryptophan is converted to IAA by two sequential enzymatic steps using TAA1, a tryptophan amino transferase (Stepanova *et al*., 2008, Tao *et al*., 2008), and YUCCA, a flavin mono-oxygenase (Cheng *et al*., 2006), via IPA as an intermediate. The IAA level is adjusted by *de novo* IAA synthesis from tryptophan, the reversible conversion of IAA-conjugated forms (IAA-conjugated sugars or amino acids) and irreversible catabolism.

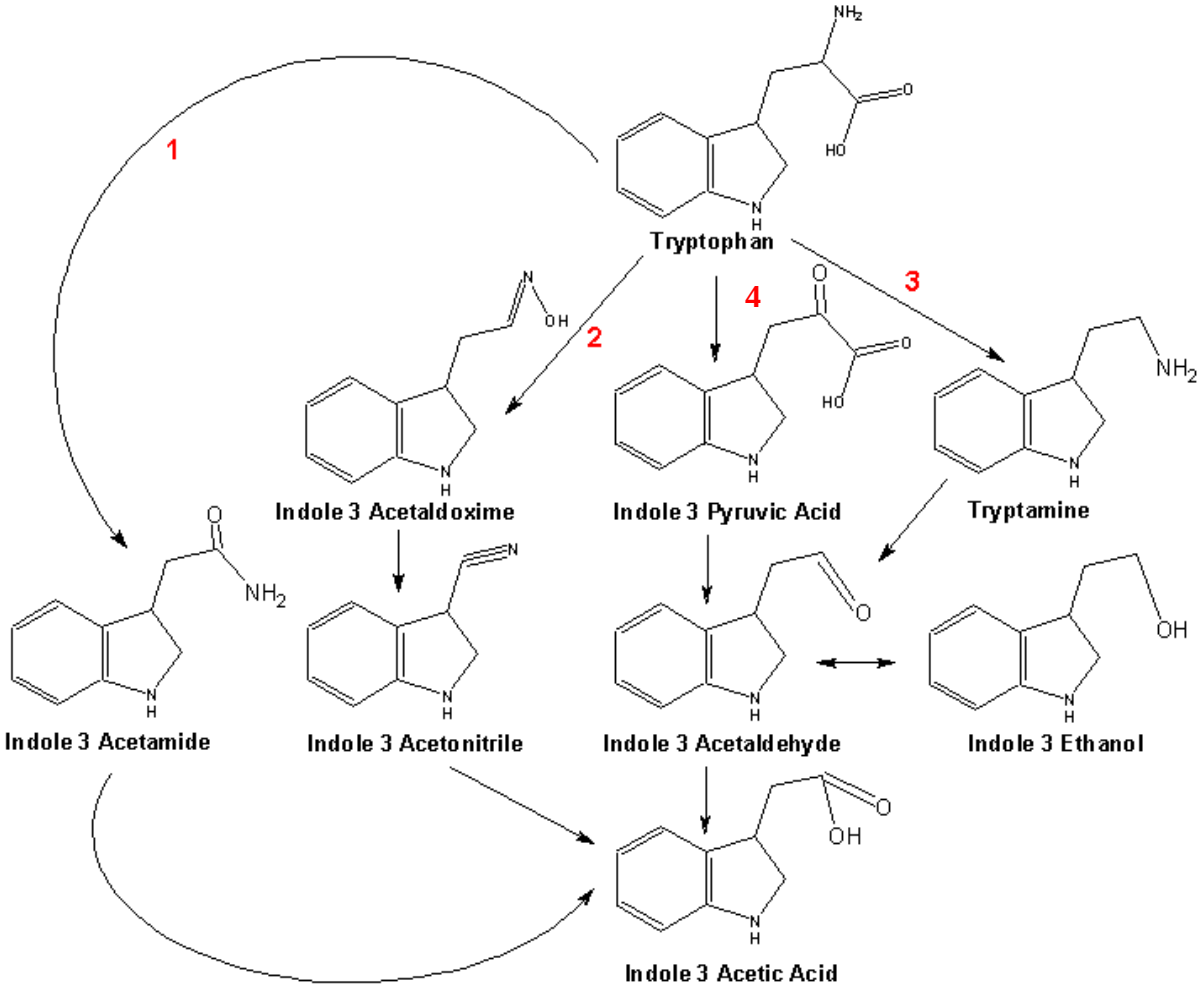

Figure 3
This picture shows the auxin biosynthesis pathways.
1– IAM pathway, 2 – IAN pathway, 3 – TAM pathway, 4 – IPA pathway

2.1.1.2 Auxin transport

Auxin is mainly transported through the cells. The flow direction goes from the apex to the basal parts of the plant. The apex is a major source of IAA. Transport of auxin is sustained by special auxin membrane carriers and requires energy. The influx of auxin is mediated by AUX/LAX transporters. AUX1/LAX1-3 transporters work as a symport H^+ carriers. The efflux of auxin from the cell is provided by PIN-FORMED PROTEIN (PIN) family and several members of the ABCB/PGP transporters (Petrasek and Friml, 2009). PIN proteins dynamically cycle between vesicules and PM, while auxin and auxin-binding proteins were proposed to play important role in this process (Paciorek *et al.*, 2005; Robert *et al.*, 2010) (see also later). The localization and distribution of PIN proteins determine the direction (polarity) of auxin flux.

Four influx AUX/LAX and eight efflux PIN proteins were described in *Arabidopsis thalinana*. These transport proteins establish the auxin gradient. This gradient is regulated by the expression and subcellular localization of auxin transport proteins in response to inner and outer factors.This so called polar auxin transport (PAT) is essential for correct development and growth of the plants. PAT is specific for the active form of auxin; inactive forms of auxin are not transported this way (Taiz and Zeiger, 2006).

Bast (floem) can also play a part in IAA transport; this form of transport doesn't require energy. It is much faster than PAT. The predominant use of passive auxin transport is to control the cell division of cambium, accumulation of calose and roots branching (Taiz and Zeiger, 2006). IAA may cross from floem transport to PAT (Cambridge and Morris, 1996). PAT is regulated by auxin itself, via membrane auxin receptors and subsequent transcriptional and/or non- transcriptional physiological auxin responses of specific cells.

2.1.1.3 Auxin receptors

2.1.1.3.1 TIR1

Around a decade ago a nuclear auxin receptor was introduced to the public: the TRANSPORT INHIBITOR RESPONSE 1/AUXIN BINDING F-BOX PROTEIN (TIR1/AFB). The mechanism of its effect is well known. In the nucleus auxin binds with the TIR1/AFB, TIR1/AFB proteins interact with Aux/IAA transcriptional

repressors. These repressors are targeted for ubiquitination and subsequent degradation in proteasome. Degradation of AUX/IAA repressors results in releasing auxin-regulated promoters and follow-up gene expression; it is the auxin transcriptional pathway (Dharmasiri *et al*., 2005; Kepinski and Leyser, 2005). However, as published, the auxin regulation based on nuclear signaling requires at least 10 – 15 minutes for execution (Badescu and Napier, 2006). Therefore, some rapid cellular auxin effects seem unlikely to be transcriptionally regulated. This suggests a presence of additional auxin signaling pathways, likely via another/other auxin receptor(s). These findings brought the ABP1 putative receptor in consideration and more intense investigation of its effects.

2.1.1.3.2 ABP1

Nearly 40 years ago auxin binding protein (ABP1) was firstly detected in maize coleoptiles due to its ability to bind radiolabeled auxin (Hertel *et al*., 1972) and we know now that ABP1 is present in all green plants (Diekmann *et al.,* 1995, Tromas *et al.,* 2010). It took a long time for ABP1 mysteries to be revealed, but still ABP1 is not fully examined. In crystal structure, ABP1 is a 22kDa dimmer-forming glycoprotein (Fig. 4). Predominant localization of ABP1 is suggested to be in the lumen of endoplasmic reticulum, as suggested from the KDEL sequence on the C-terminus of ABP1. All of ABP proteins contain N-terminus signal peptide with variable sequence. A, B and C conservative 15-20 amino acids- long domains are typical to be found in ABPs. The overall shape of ABP1 is a b-barrel structure that forms a dimmer upon crystallization. Auxin binds to a small binding pocket formed deep within the b-barrel of ABP1. In the auxin-binding site, a metal ion (zinc or copper cation) can be chelated by three histidine residues and one glutamate residue. Auxin interacts with the metal ion through its carboxylic acid, and the aromatic ring of auxin is trapped with the hydrophobic residues inside the barrel. The structure and mechanism of the auxin recognition site of ABP1 is quite different from that of TIR1/AFB receptors, suggesting that some artificial auxins might have selective activity for TIR1/AFB or ABP1. Purified ABP1 has a K_D of approximately 10^{-7}mol/l for NAA at pH 5.5 (Hesse *et al*., 1989).

A small amount of ABP1 is secreted to apoplast, and it is localized close to the plasma membrane (PM). Here at the PM ABP1 is able to bind auxin and cause an auxin

signal perception and transduction. Several sources of evidence support the idea that ABP1 acts as a functional receptor at the PM (Barbier- Brygoo *et al.*, 1989, Leblanck *et al.*, 1999).

ABP1 was shown to be essential for a wide variety of auxin- regulated processes such as cell division, cell expansion, auxin regulated gene expression and early auxin response at the PM. The underlining mechanism of auxin mediated effect on cell elongation remains unclear. If the only *ABP1* gene in *Arabidopsis*is is knockout (homozygous mutant) development of plant embryo stops in globular stadium. Result of this experiment was the finding that ABP1 is essential in cell division and elongation in embryo development (Chen *et al.*, 2001).

The gene expression of ABP1 in plant organs is low. Studies on the function of ABP1 using immunological modulation with an anti-ABP1 antibodies demonstrated that ABP1 is involved in the rapid regulation of membrane potential and ion fluxes at the plasma membrane (Barbier- Brygoo *et al.*, 1989) and in auxin-induced cell swelling of protoplasts derived from *Arabidopsis* hypocotyls (Steffens *et al.*, 2001, Yamagami *et al.*, 2004).

Under-inductive-promoter-ABP1-transformed plants, with overproduction of ABP1,produce auxin in whole leaf, whereas wild type plants produce auxin only in the apex of leaf. The leaf tissue sensitivity to auxin increased in mutants, this process of cell growth is induced by auxin-dependent stimulation (Jones, 1998).

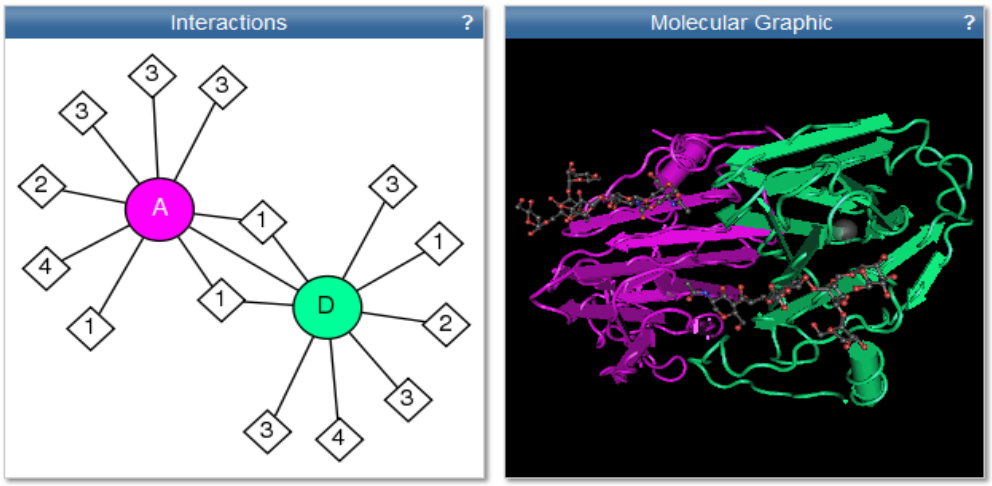

Figure 4
This picture shows the crystal dimmeric structure of the AUXIN BINDING PROTEIN 1.A + D – ABP1; 1 – N acetyl- D glucose amid; 2- 2βD mannose; 3 – 6 D mannose; 4 –$Zn^{2+.}$ The picture was taken from NCBI.

To see how KDEL C-terminus sequence is involved in sensitivity of ABP1 to auxin, transgenic tobacco plants were used. Mutated forms of KDEL to KEQL, KDELGL, HDEL didn't have a significant impact to sensitivity of ABP1 to auxin. This may suggest that ABP1 can be active also inside the cell. Exogenous auxin seems to influence the activity of potassium channels in clutching stomatal cells. In the case of overexpression of *ABP1* gene, the sensitivity of potassium channels to auxin was elevated. This indicates that ABP1 plays a role in stomatal opening (Bauly *et al*.,2000).

In experiment where tobacco cell cultures were producing antibodies against ABP1, which blocked the function of ABP1 receptors, an inhibition of cell cycle was observed (Fellner *et al*., 1996). Further research showed that the majority of cells was discontinued in G1 phase or in changeover between G1 to S phase. This inhibition of cell cycle caused by non-working ABP1 protein was shown to be reversible. These findings strongly indicate that ABP1 is involved in auxin induced cell cycle regulation (David, 2007).

Repression of ABP1 function leads to growth inhibition of apex, slowing down the growth of leaves, decrease in cell expansion and changes in auxin-responding genes. Local repression of ABP1 in apex revealed the role of ABP1 as a control element in arrangement and shape of cells (Braun *et al*., 2008).

A differential auxin distribution within tissues is an important regulatory factor (Vanneste and Friml, 2009). Auxin gradient results from local auxin biosynthesis and directional intracellular auxin transport are triggered by a network of carrier proteins (Petrásek *et al*., 2006; Vieten *et al*., 2007; Yang and Murphy, 2009). The auxin-flow direction depends on the distribution of auxin efflux carriers PIN (Wisniewska *et al*., 2006). Auxin itself via ABP1 inhibits endocytosis of PIN proteins, increasing their levels and activity at the plasma membrane (Paciorek *et al*., 2005; Robert *et al*., 2010) which leads to higher auxin secretion thereby influencing the polar auxin transport (PAT).

In addition to ABP1, at least four other closely related ABPs have been identified in maize so far (Hesse *et al.,* 1989; Schwob *et al.,* 1993), but their roles in growth and development have yet to be elucidated.

Analysis of *abp* mutants suggest that in corn ABPs may be involved in growth of maize seedlings and development of leaf angle (Jones *et al*., 1991; Fellner *et al.,* 2006, Im *et al.*, 2000).

Fellner *et al.* (2006) also reported that the *abp1* and *abp4* mutants are significantly less sensitive than WT seedlings to the inhibitory effect of exogenous auxin (Jurišic- Knezev *et al.,* 2012), and that develop more erect and more horizontal leaves, respectively, than WT plants (Fellner *et al*., 2006). These results therefore suggest that ABP1 and ABP4 play an important role in the development of maize leaf declination. Further research showed that ABP1 is positively involved in the leaf angle development and blade elongation, and it negatively contributes to lateral blade growth. ABP4 then seems to play a negative role in leaf declination, whereas it contributes positively to blade elongation and likely in lateral blade growth. Concurrently, it seems that in all the growth responses tested, ABP4 (or ABP4) suppress the activity of ABP1, whereas for blade elongation, ABP1 (or ABP1) suppress the activity of ABP4 (Jurisic- Knezev *et al.*, 2012).

Jurisic- Knezev *et al.* (2014) provided evidence for a cross-talk between *ABP4*, exogenous auxin, $Ca^{2+,}$ and ZCAX3 (the Ca^{2+} /H^{+} antiporter responsible for regrouping the vacuolar Ca^{2+} in *Zea mays L*.), during growth of etiolated maize mesocotyl. Their model proposes that, in the absence of exogenous auxin, functional ABP4 regulates expression of *CAX3* either directly or via calcium accumulation, and increased accumulation of Ca^{2+} promotes mesocotyl elongation. It is further proposed that in opposite, in the presence of NAA, expression of *ZCAX3* gene is suppressed (Jurisic- Knezev *et al.*, 2014).

Recently, Gao *et al.*(2015) published an article where they put ABP1 on absolutely different position in importance of plant signaling. Due to cellular immunization approaches they prepared *Arabidopsis abp1* mutant that contains a 5-bp deletion in the first exon of *ABP1*. They show that the two newly prepared *abp1* mutants are null alleles. These plants surprisingly do not display any obvious developmental defects. Based on their results, they conclude that ABP1 is not a key component in auxin signaling or *Arabidopsis* development. These findings contradict to so far discovered and gathered information about *ABP1*.

2.2 The Light

Light is an important external factor playing role in many plant physiological processes. Light serves as a stimulus in photomorphogenesis, as well as the main source of energy during plants life. Energy obtained from light is used for synthesis of sugars from CO_2 and water. According to the light conditions, plants can sense what is happening around them. The perception of electromagnetic radiation is mediated by specific receptors called photoreceptors (Fig. 5). Wavelength of sensed light defines the type of photoreceptor (Taiz and Zeiger, 2006).

2.2.1 Cryptochromes (CRYs)

Cryptochromes are receptors of blue light (BL), they perceive BL and UV-A represented by wavelength 320-500 nm. This category consists of two evolutionary old proteins CRY1 a CRY2. CRYs belong to flavoproteins with typical N-terminal photolyase homology domain. Photolyases are bacterial enzymes involved in the repair of UV-induced DNA damage. Cofactor FAD noncovalentlybounds to the photolyase-homology domain. In plants, cryptochromes mediate mainly blue light-induced inhibition of hypocotyl elongation and anthocyanin accumulation (Lin *et al.*, 1996), and they are involved in stomata opening as well (Mao and Zhang, 2005). CRYs are also important in circadian rhythm (Lin and Todo, 2005). They serve as the 'place of light entrance' to the signal pathways controlling the circadian rhythms, but they are not directly involved in the mechanism unlike mammalian cryptochromes (Devlin and Kay, 2000).

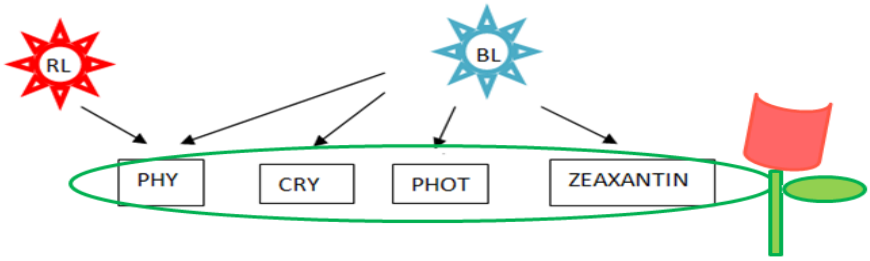

Figure 5
This picture illustrates the receptors of light and the type of light they absorb. RL stands for red light, BL stands for blue light.

CRYs-missing plants showed to be defective in phototropic reaction. On the other hand CRYs-overexpressing plants react more intense in phototropic reaction. This data indicates CRYs influence the phototropism (Ahmad *et al*., 1998).

The concentration levels of CRY1 remained stable in plants germinated in the dark or white light conditions, in young seedlings and in all plant organs of mature plants. This means CRY1 is expressed constantly. An overexpression of *CRY1* gene in *Arabidopsis* resulted as BL-induced inhibition of hypocotyl elongation and anthocyanin accumulation. Generally, higher concentration of CRY1 in the cell has a light-induced inhibition effect on plant growth (Lin *et al*., 1996).

CRY2 as well as CRY1 are water soluble proteins. CRY2 is expressed in all plant tissues, but it is light-unstable. BL, UV-A and also green light negatively regulates CRY2. Gene expression of *CRY1* and *CRY2* is not interdependent. CRY2 together with CRY1 are receptors for BL-induced deetiolization of plants (Lin *et al*., 1998). CRYs mainly work as synergic for example hypocotyl growth inhibition, flowering induction and circadian rhythm regulation. In regulation of the primary root growth CRY1 and CRY2 have antagonist impact. CRY1 supports growth of primary root, while CRY2 doesn't.

2.2.2 Phototropins (PHOTs)

Phototropism is an ability of plants to grow towards the light source or the opposite direction. Phototropism was observed in plants, ferns and also fungi (Taiz and Zeiger, 2006) and key photoreceptors revealed to be involved in phototropic response are called phototropins (Short and Briggs 1994). Two phototropins PHOT1 and PHOT2 were found in *Arabidopsis* and in other plant species.

Phototropins are light-activated serine/threonine protein kinase enzymes. Light sensing by the phototropins is mediated by a repeated motif at the N-terminal region of the protein, known as the LOV domain. LOV domains are structurally similar mammalian oxygen sensors. These domains bind flavin group of flavinmononucleotide (FMN). BL photoexcitation of the LOV domain results in receptor autophosphorylation and an initiation of phototropin signaling (Schwartz and Zeiger, 1984).

Mutant plants with dysfunctional PHOT1 lack phototropic reaction to BL of low intensity, but the phototropic reaction to BL of high intensity remains. Interestingly, in mutant *phot2* phototropic responses to low or high light intensity is similar to the responses of nonmutated plants. Interestingly, double mutant with both nonfunctional phototropins lack phototropic responses to blue light of both intensities (Briggs and Christie, 2002). This demonstrates that PHOT2 is involved in phototropic response to BL of high intensity, but it also suggests a complex functional relation between PHOT1 and PHOT2, which remains to be elucidated.

In stomata, BL radiation exposure blocks transport of Cl^- from the guard cells but activates the proton pump, which carries out H^+ to apoplast. Subsequent opening of K^+ channels allows ions coming into the cell. This process increases osmotic pressure inside the guard cell. In effort to recalibrate the osmotic pressure, water goes inside the cell, which finally results in turgor rise in guard cells and thus in opening of stomata. Mutant with defect in *PHOT1*or*PHOT2*gene shows small changes in BL-induced stomatal opening, but the double mutant *phot1/phot2* lacks this response to BL completely. These data indicate important role of phototropins in BL-induced stomatal movement (Kinoshita *et al.*, 2001).

According to intensity of BL, chloroplasts move to either maintain the photosynthesis maximum by accumulation close to palisade mesophyll perpendicularly to light direction if BL intensity is low. In the case of BL of high intensity, chloroplasts move away from the light be parrally to the light direction in order to protect themselves from the BL. It was proved that PHOTs are involved in BL-induced chloroplast movement (Sakai *et al.,* 2001).

In plants exposed to BL fast inhibition of hypocotyls elongation was observed. Unlike inhibition caused by red light, which is manifested in 8-90 minutes after exposure to RL, the inhibition caused by BL can be observed within 30 seconds. Primary phase of BL-induced inhibition of hypocotyls elongation is mediated by phototropins, where late BL-induced inhibition (after 30 minutes) is mediated by CRYs (Folta and Spalding, 2001).

2.2.3 Phytochromes (PHYs)

Phytochromes serve as red light (RL) receptors. Phytochrome is a blue colour-protein pigment firstly identified in 1959. In 2003 PHY signaling was deeply characterized by

Sullivan and Deng (Sullivan and Deng, 2003). PHYs' perception of red light and far-red includes range from 600 – 750 nm. The phytochrome is a water- soluble protein built up of two subunits, together it has approximately 250kDA.Its structure is made of 125kD apoprotein, which consists of two histidine kinase related domains (HKRD1, HKRD2) located on the C- terminus and PAS domains inside of HKRD1. HKRD1 is necessary for interaction of phytochrome with other proteins. At the N-terminus, pigment chromophore is bound to the apoprotein with covalent thioether linkage. Phytochromes' activity includes light induced serin/threonin protein kinase enzyme activity, which is responsible for self- phosphorylation.

In vivo two photo- reversible forms of phytochrome can be found. Pr form, absorbing red light, exists in plants during night. After exposure to red light, phytochrome converts to form Pfr, the far-red absorbing form that is physiologically active. Pfr can be transferred to Pr form by far-red radiation. The difference between these two forms of phytochrome constitution is the spatial distribution of phytochromes' sections and in the different light absorbing maximum. Absorb maximum for Pr is 666 nm and for Pfr 730 nm.

If the red or white light causes a degradation of phytochrome, this phytochrome is called as type I. The second type of phytochrome, type II is stable after light radiation exposure. In *Arabidopsis* five different genes, encoding phytochrome apoproteins PHYA to E were identified. Type I is represented by PHYA, whereas PHYB-E belongs to type II group.

Phytochromes are responsible for light-induced stimulation of germination and inhibition of elongation; de-etiolization and they also stimulate formation leaf primordia and promote growth of new leaves. The effect of red light (650 – 680 nm) can be eliminated by far red light (710 – 740 nm), but only for a certain period a time, after this time the reaction is not reversible anymore. The R and FR forms of phytochrome are reversible; the phytochrome itself changes its conformation depending on the light conditions. FR phytochrome form is an active form of phytochrome, the absence of FR causes lack of plant response to light.

Responses mediated by phytochromes are categorized into three groups according to the range of fluence rate which provokes the specific reactions. According the photon rate, the responses to RL are divided into three categories:

very low fluencereactions (VLFRs), low-fluence reactions (LFRs) and high-irradiance reactions (HIRs).

VLFRs are involved in induction of seed germination mediated by phytochrome A, stimulation of coleoptiles growth, inhibition of mesocotyl growth (Shimomura *et al.*, 1996). LFRs include germination of lettuce and *Arabidopsis* seeds, regulation of leaves movement. HIRs include for example ethylene production by etiolated sorghum plants. PhyB mediated responses to red and white light in de-etiolating process and PhyA mediated responses to far-red during de-etiolating process in plants. Phytochromes D and E are involved in elongation of a petiole and an internode segments, they also control the flowering period of time. PhyC plays role in de- etiolating process, cotyledons growth and suppression of flowering (Schäfer and Nagy, 2006).

2.3 ABPs in light signalization

The possibility of the involvement of ABPs in cross-talk between light and auxin signaling was firstly brought out by Jones *et al.* (1991). The authors reported that RL reduces the level of free IAA in mesocotyl and abundance of the ABP1, which controls expansion of the cells in maize. Im *et al.* (2000) found that in etiolated corn seedlings expression of *ABP1*was higher than expression of *ABP4*, and that *ABP4* was up-regulated in light-grown seedlings.

Seedlings of modern maize hybrid 3394 with defect in *ABP4* gene expression show changes in its growth responses to auxin and light (Fellner *et al.*, 2006). Based on previous research, Fellner suggests that in corn seedlings *ABP4* may play a role in auxin- and light-induced growth responses. Since *ABP4* can be regulated by auxin as well as by light, its role could be to integrate auxin and light signaling pathways involved in maize growth and development. The association of auxin- and light-induced over-expression of *ABP4* with auxin- and light-induced inhibition of elongation led them further to hypothesize that *ABP4* may function as a negative regulator of elongation growth of corn seedlings.

In 2012 Bořucka and Fellner investigated the gene expression of phytochrome B and phytochrome A in loss-of-function mutants in *ABP1* and *ABP4* genes in maize. They found that knockout of *ABP1* or *ABP4* gene results in essentially reduced expression of *PHYB* gene in dark-grown mesocotyl. White light reduced *PHYB*

expression in WT but not in the ABPs knockout seedlings. The data indicate that ABP1 and ABP4 are positively involved in *PHYB* expression in etiolated mesocotyl

The effect of the dark, blue light, red light, and exogenous auxin on the development of mutant seedlings was also studied. Relative to WT, etiolated *abp* maize mutant seedlings were shorter and showed a reduced responsiveness to exogenous auxin. In BL or RL, the responsiveness of maize seedlings to auxin was distinctly less than in D. The reducing effect of light on seedling responsiveness to auxin is mediated at least by phytochromes. These results posted by Bořucka and Fellner in 2012 support the existence of cross-talk between auxin and light signaling and indicate for the first time the involvement of ABP1 and ABP4 in phytochrome signaling pathways. It was reported that ABP1 in maize binds not only native auxin IAA but also synthetic auxin NAA (Ray and Dohrmann, 1977; Dahlke *et al.,* 2009). Bořucka and Fellner (2012) found that intact etiolated maize seedlings with knockout ABP1 and/or ABP4 are much less sensitive to the inhibitory effect of NAA than plants with the functional ABPs. They further revealed that the level of *PHYB* transcript was significantly reduced by NAA in etiolated WT mesocotyls but not in mesocotyls of the knockout plants. The data suggest that in etiolated mesocotyls, functional ABP1and ABP4 are required for NAA-induced inhibition of the organ elongation and NAA-induced inhibition of *PHYB* expression. However, the molecular mechanism has to be elucidated.

Effendi *et al*. (2013) presented a proof that ABP1 and RL induced- phytochrome regulation of elongation share a common mechanism for organ elongation. NPA, naphthylpthalamic acid- an inhibitor of polar transport, applied under red light revealed that *abp1* mutants in *Arabidopsis* act like phytochrome- deficient mutants in their loss of gravitropic orientation; clearly, auxin transport was disturbed in this loss of gravitropic orientation and NPA acted as an enhancer.

In 2014 Effendi *et al.* isolated two T-DNA insertion mutants for the *pPLAI* gene, encoding small enzyme Phospholipase A. Both null mutants, *pplaI-1*and *pplaI-3,* despite the absence of an obvious phenotype, showed a clear phenotype in delayed auxin-induced expression of key auxin inducible genes. They also included *phyA* and *phyB* null mutants in the analysis. The *phyA* mutant showed a reduced phototropic reaction, similar to *ppla-I-1,* whereas *phyB* reacted indistinguishable from

WT. Effendi *et al.* investigated the inhibition of hypocotyls gravitropism by FR light. Both types of mutants (*pplaI-1*, *pplaI-3* and *phyA*, *phyB)* appeared to be hypersensitive in their responses to low R:FR light and to shade conditions. Lack of *phyA* or *phyB* is known to decrease hypocotyl gravitropism (Robson and Smith, 1996) and root gravitropism (Kunihiro *et al.*, 2011). The *ppla-I* and *abp1* mutants identify a function at the intersection of auxin and light signaling. These mutants have several more common similarities in their phenotypes, which consist of the similar regulatory defects in auxin-dependent responses like gene regulation, phototropism and gravitropism (Effendi and Scherer, 2011; Effendi *et al*., 2011, 2013). Additionally, both *ppla-I-1* and *abp1/ABP1* flower early. This suggests that phospholipase A might be involved in light-induced ABP1 auxin signaling pathway.

In 2015 Effendi *et al.* engineered strong point mutation alleles by complementation of the knockout to aid investigation of ABP1 functions. The mutated binding sites showed that the protein surface contacting 1-NAA is distorted in all mutants; therefore all of them were knock-out mutants. It is known that the most of auxin actions are interwoven with changes in polar auxin transport (Petrasek and Friml, 2009). In these mutants and in *ABP1*/*abp1* (Effendi *et al.*, 2011, 2015) the authors showed that basipetal auxin transport in root tips of *abp1* mutants was delayed. Functions such as lateral root formation, tropisms (Petrasek and Friml, 2009), and emergence and growth of epidermal cell lobes (Xu *et al.*, 2010) were all affected in *abp1* mutants and are all dependent on polar auxin transport. This supports the suggestion that ABP1 plays a role in auxin transport.

abp1 mutants are compromised in a number of phyB functions (elongation in R, apical dominance, early flowering, inhibition of gravitropism, misregulation of shade marker genes in LR similar to a *phyB* mutant), and some phyA functions (broad leaves, elongation in FR, inhibition of gravitropism, misregulation of shade marker genes in HR similar to a *phyA* mutant). ABP1 seems to have a dual role, repression of elongation in the light in conjunction with phytochromes, but supporting elongation in the dark. This study indicates that ABP1 can crosstalk with phyB and phyA, even though these are located in the cytosol and nucleus. By testing the gene expression, a

frame work for the mechanics of functional interaction of ABP1 and phyB to trigger interwoven signaling pathways, was provided (Effendi *et al.*, 2015).

3. Material and methods

3.1 Plant material

Zea mays L. was used as a plant material in all experiments. The loss-of-function mutants in *ABP1*, *ABP4* and *ABP1/4* genes containing the Robertson's Mutator transposable elements in these genes were used (Im *et al.*, 2000). Near isogenic line inbred line A619 was used as a control genotype (here called WT). Seeds of the mutant and WT were a gift from Alan M. Jones (The University of North Carolina, USA).

3.2 Growth experiments

The seeds of control plant (WT) and mutants were surface sterilized and grown *in vitro*. Seeds were put in plastic 50 ml tubes with 70% ethanol and after 3 minutes they were rinsed several times with distilled water. Seeds were placed in 100 ml Erlenmeyer flasks containing SAVO (5% sodium hypochlorite; BOCHEMIE, Czech Republic) and few drops of detergent TWEEN 20. Seeds were shaken on a stirrer for 40 minutes. Further work took place in sterile flow box. After stirring, seeds were rinsed with sterile distilled water for 5 times. Then with sterile tweezers, seeds were planted in Magenta boxes (77 x 77 x 196 mm; Sigma-Aldrich, Prague CZ), filled with 60 ml of basal Murashige and Skoog medium (MS; Murashige and Skoog, 1962). Six seeds were sown in one box. Magenta boxes with the seeds were placed in growth chambers (Snijders, The Netherlands) with different light conditions (dark – boxes covered with aluminum foil, Blue Light- Philips TLD-36W/18-Blue, Red Light- Philips TLD-36W/15-Red) and temperature about 23 °C. Seeds were incubated for 7 days and then parts of the seedlings, such as mesocotyl, roots (main and seminal) and coleoptile were measured with a ruler with accuracy 1mm.

For preparation of 4 l MS medium following ingredients were used: 40 g of sucrose, 17.208 g MS medium (Caisson), 0.7808 g of MES buffer and approximately 4 l of distilled water. After proper dissolution of each chemical, pH was adjusted to 6.1 using 1M KOH. Medium was poured into 400ml glass bottles and to each of the bottles 2.8 g of phytoagar was added. The lid of these bottles was loose and covered with aluminum foil and the medium was than sterilized in autoclave.

3.3 RNA extraction and cDNA synthesis

Seven days after germination, mesocotyls were harvested, weighted out (around 200mg FW), frozen immediately in liquid nitrogen and grounded in liquid nitrogen using a mortar and pestle. Extraction of total RNA from mesocotyls was performed using Isolate II RNA Plant Kit (Bioline) according to manufacturer instructions. An additional DNaseI treatment took place just after extraction of RNA. The DNaseI treatment of purified RNA in solution was performed using Isolate II RNA Plant Kit (Bioline) according to manufacturer instruction; only the time of incubation with DNase enzyme was prolonged from 10 to 20 minutes and precipitated with 96% Ethanol overnight in – 80°C. The purity of extracted total RNA was checked by PCR reaction (Tab. 1 and Tab. 2) consisting in amplification of Actin gene (*MAZ81*, accession number U60511) using specific primers (ZmAct81; Tab. 3), which covered an intron sequence of the gene. The sample was colored with 6x Loading Dye (1µl), load on the agarose gel (1,5g of agarose dissolved in 100ml of 0,5x TBE buffer). PCR product was size fractionated by electrophoresis in a 1.5 % (w/v) agarose gel stained with GelRed (5µl).

In case of contamination of RNA with genomic DNA, bands with size of 419 bp, should be visible and DNase treatment had to be repeated. In the case that no fragments were visible RNA was used for cDNA synthesis. cDNA was synthesized from 1µg of total RNA. To take the proper amount of RNA used for cDNA synthesis, concentration of RNA was measured with spectrophotometer. The reverse transcription reaction was performed by Prime Script 1st strand cDNA synthesis Kit (Takara) according to manufacturer instruction (Tab. 4). The cDNA integrity was checked in PCR reaction (Tab. 5) with the same primers as used before in checking RNA purity (ZmAct81) and in addition with primers specific for housekeeping gene GAPDH (Tab. 6, Tab. 2) (accession no. Zm3765). cDNA was diluted 1/5 for this reaction. In the case of bands with the same intensity, cDNA was used for analysis of expression of *PIN1* genes.

3.4 Gene expression

Gene expression was performed with primers specific for gene *ZmPIN1a* adopted from article (Forestan *et al*., 2012). These primers formed a lot of strong primer dimmers, which did not allow correct evaluation of gene expression. Several modifications of PCR procedure were tested, such as decreasing the primer or cDNA concentration, or fewer cycles or touchdown PCR, but none of these helped (see results- problem solver). Therefore

new primers were designed using NCBI pick up primers program. After optimization, the new primers (IR-ZmPIN1a) were shown to be functioning. The PCR reaction was performed using 5x My Tag buffer 4µl, forward primer 10 µM 0,5 µl, reverse primer 10 µM 0,5 µl, My Taq polymerase 0,1 µl, nuclease free- water 13 µl and 2 µl of non-diluted cDNA per 1 reaction. PCR was performed under conditions as described in Tab. 7. PCR was performed in 20 µl volumes. PCR products were loaded into the 1.5% agarose gel and electrophoresis was run (300mV, 80A, for approx. 75 minutes). After wards, the picture of gel was taken (Gel Doc ^{TM} EZ Imager, BIO- RAD) and the intensity of signal was determined using the ImageJ program (Abramoff *et al.*, 2004). Normalization was assessed by expression values of housekeeping gene *GAPDH* (accession No. Zm3765). In PCR reaction for *GAPDH* gene cDNA diluted 1/10 was used. The *PIN1a* gene expression was expressed as relative to gene expression observed in WT.

Tab. 1: PCR mix per 1 reaction

Item	V/ µl
5x My Taq buffer	4
F primer 10 µM	0,5
R primer 10 µM	0,5
My Taq polymerase	0,1
Nuclease free water	13
RNA or cDNA	2

Tab. 2: PCR program RNA purity

Temperature / °C	Time / s	
94	300	
94	30	45 cycles
45	30	
72	45	
72	300	
15	∞	

Tab. 4: cDNA synthesis per 1 reaction

Item	V/ µl
50µM oligodT Primer	1
10mM dNTP	1
5x PrimeScript buffer	4
Rnase inhibitor (40U/ µl)	0,5
PrimeScriptRtase	1
Rnase-free water	4,5
RNA + H_20	8

Tab. 5: PCR program cDNA integrity

Temperature / °C	Time / s	
94	300	
94	30	35 cycles
45	30	
72	45	
72	300	
15	∞	

Tab. 6: PCR program GAPDH

Temperature / °C	Time / s	
94	180	
94	30	35 cycles
60	30	
72	45	
72	300	
15	∞	

Tab. 7: PCR program PIN1a

Temperature / °C	Time / s	
94	180	
94	30	30 cycles
57	45	
72	30	
72	300	
15	∞	

Tab. 3: List of specific primers used in the bachelor thesis

Primer	Sequence	Product size / bp
ZmAct81 F	5'-ACACAGTGCCAATCT-3'	419
ZmAct81 R	5'-ACTGAGCACAATGTTAC-3'	
ZmPIN1a F	5'-GTCAAGGAGGTCCGCATGGCCGTCGCC -3'	701
ZmPIN1a R	5'-CGCTGTTGGCCTGCGGGAACGAGCAGC-3'	
IR-ZmPIN1a F	5'-GCTTCAACCACACCGACTTC -3'	713
IR-ZmPIN1a R	5'-AGGTGTTGGGGTTACGGATG -3'	
GAPDH F	5'-TGATCCGCCACATGTTCAAGACC -3'	79
GAPDH R	5'-CGGCATACACAAGCAGCAACC -3'	

4. Results

4.1 Problem solver

4.1.1 Sterilization

The loss of function mutants in *ABP1* and *ABP4* genes in maize were frequently contaminated with a mould (Fig. 6). If one seedling in the Magenta box didn't resist to inner pathogens, the rest of the seedlings in the box were infected. To correctly evaluate the growth reactions and gene expression, only 'clean' non-infected plants can be used. Therefore a right technique of sterilization, which wouldn't be too aggressive and wouldn't alter the results, was sought. Firstly, different timing and concentration of ethanol and SAVO were tested (see Tab. 8).

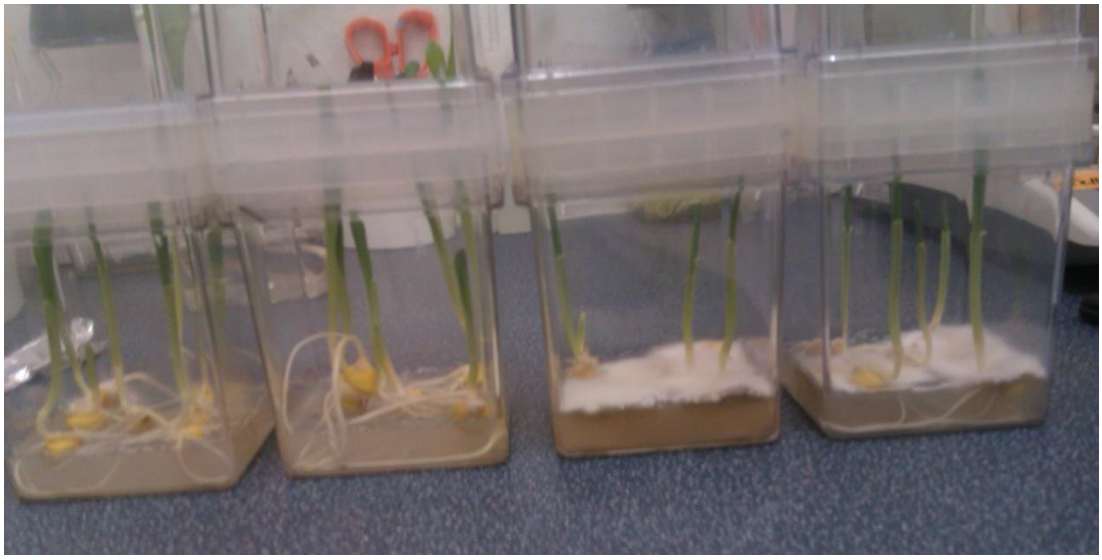

Figure 6
This picture shows the problem of infection in 7-day-old maize seedlings. On the left there are two Magenta boxes with WT while on the right there are two Magenta boxes with *abp1* mutant. Seed of both genotypes germinated on the red light and were sterilized for 3 minutes in 70% EtOH, rinsed with sterile distilled water, kept in 3 % SAVO for 30 minutes, and finally rinsed 5 times with sterile distilled water.

Tab. 8: Different variations of sterilization of maize seeds with Ethanol and SAVO

Exp.	Ethanol		SAVO		Infected seeds in the box
	Concentration %	Time / min	Concentration %	Time / min	
1	70	3	3	40	2/4
2		5		40	2/4
3		5		55	3/4
4		**3**	**5**	**40**	**1/4**
5	96	3	3	40	3/4
6		5		40	**1/4**
7		5		55	3/4
8		10		30	2/4
9	3 min vortex			55	2/4
10				60	3/4

Then a mordant used in agriculture for oilseed rape was tried out. Maize seeds were surface covered in mordant (0,25 ml of mordant per 8g of seeds) and then dried on air in a flowbox. All examined genotypes of maize seeds germinated poorly and the contamination was high (Fig. 7), therefore this way of sterilization was rejected.

Figure 7
This picture shows the problem of infection in 7-day-old maize seedlings. Seeds were treated with a mordant for oilseed rape. From left: WT, *abp1*, *abp4*, *abp1/4*.

Another idea was to let the seeds overnight in water, then to remove the surface of the seeds (testa) and then to perform the sterilization with ethanol and SAVO. In half of the seeds the testa was removed with surgical scalpel and in the second half the testa was etched for 18 minutes with 30% sodium hydroxide. Then both groups of seeds were kept for 3 minutes in 70% ethanol, rinsed with sterile water, then kept 40 minutes in 3% SAVO and finally rinsed 6 times with sterile distilled water. Seeds sterilized with hydroxide started to germinate on the 7th day after sowing or didn't germinate at all. Seeds with removed testa were all infected and moldy.

Although the infection of loss-of-function mutants was persistent, some ways of sterilization showed to be better than others. Therefore the sterilization with 70% Ethanol for 3 minutes and 5% SAVO for 40 minutes was selected. Also the amount of planted seeds was tripled for higher yield of non-infected seedlings.

4.1.2 PCR optimization

Firstly, the gene expression was performed with specific primers (ZmPIN1a) adopted from article (Forestan *et al.*, 2012). The melting temperature was calculated to 76°C. cDNA was diluted 1/10 and 35 cycles were used for PCR. These primers formed a lot of strong primer dimmers (Fig. 8), which make impossible the correct evaluation of the gene expression.

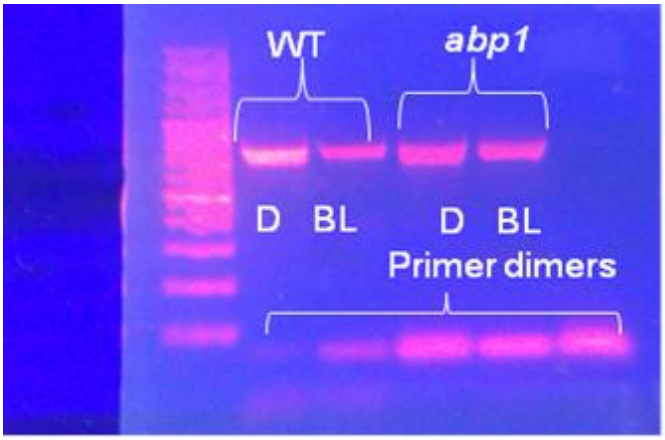

Figure 8
This picture shows the problem of primer dimmers formed during polymerase chain reaction to express the *PIN1a*gene in 7-day-old maize seedlings. The PCR reaction was performed using primer 10 µM (0.5 µl), 2 µl cDNA diluted 1/10. In the PCR, the DNA was denatured at 94°C for 3minutes, followed by 35 cycles of amplification (30s at 94°C, 30s at 76°C, 45s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes.

Figure 9
This picture shows the PCR temperature gradient from 83 to 76 °C with sample 3 (*abp1*dark). PCR was performed to express the *PIN1a*gene in 7-day-old maize seedlings. The PCR reaction was performed using primer 10 µM (0.5 µl), 2 µl cDNA diluted 1/10. In the PCR, the DNA was denatured at 94°C for 3minutes, followed by 30 cycles of amplification (30s at 94°C, 30s at X°C, 45s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes.

Then the concentration of primers was lowered from 10µM to 1µM. Times and the temperatures were the same as before. This concentration of primers was insufficient. Therefore, gradient of annealing temperature from 76 to 83°C was tested in the next PCR optimization with the original concentration o primers (10µM).

Weak band corresponding with *PIN1a* gene and no dimer was visible at 78.8 °C (Fig. 9). Therefore, PCR with annealing temperature 79°C with higher concentration of cDNA (1/5) was consequently tested. However, no visible bands were observed in that case. Another temperature gradient was therefore performed (79-69°C). Temperature 69.7°C (Fig. 10) rounded to 70°C seemed reasonable to use. So it was finally used as the annealing temperature in next PCR with 1-4 samples (WT-D, BL, *abp1*- D, BL).

Because in two of the samples the primer dimmers were still formed during PCR, I decided to try a touchdown type of PCR. TCH PCR should prevent formation of unspecific bands and primer dimmers. Samples 1-4 were used for this experiment at the concentration of cDNA= 1/10, concentration of primers 10µM, TCH from 82°C, decreasing 0,4°C every cycle (29cyles), then 19 cycles with stable annealing temperature at 70°C. Unfortunately, TCH PCR didn't help as the primer dimmers were still present. I therefore decided to design a new primers using NCBI pick up primers program.

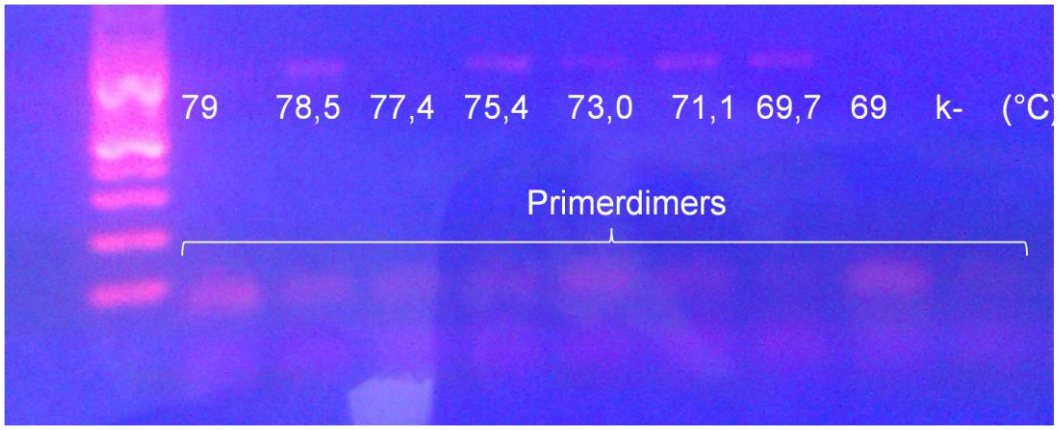

Figure 10
This picture shows the PCR temperature gradient from 79 to 69 °C with sample 3 (*abp1* D). PCR was performed to express the *PIN1a*gene in 7-day-old maize seedlings. The PCR reaction was performed using primer 10 µM (0,5 µl), 2 µl cDNA diluted 1/10. In the PCR, the DNA was denatured at 94°C for 3minutes, followed by 30 cycles of amplification (30s at 94°C, 30s at X°C, 45s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes.

Newly designed primers (IRZmPIN1a) with calculated Tm= 59°C were tried out on samples 1-4. In samples 1 and 2 (WT- D, BL) primer dimmers were visible (not shown). Therefore, sample 1 and 2 were selected for further optimization of PCR and temperature gradient (68- 57°C) was performed. In temperature 57°C primer dimmers were not so strong, but still visible. However, the temperature 59.2°C where a half primer concentration was used (1/20), was interesting. At this temperature the primer dimmers were not so obvious (Fig. 11, in right).

This temperature gradient was performed once again, and the only difference was that the annealing time 30s was prolonged to 45s (Fig. 12).

Figure 11
This picture shows the PCR temperature gradient from 68 to 57 °C with sample 1 (WT D). The PCR reaction was performed using primer 10 µM (0,5 µl), 2 µl cDNA diluted 1/10. In the PCR, the DNA was denatured at 94°C for 3minutes, followed by 30 cycles of amplification (30s at 94°C, 30s at X°C, 45s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes. At the right side (59.2°C) is PCR product where the primer concentration was lowered to 5 µM (0,5 µl), otherwise no differences in PCR were made.

I was wondering how the annealing and extension time would influence the PCR reaction, so I firstly reduced the extension time from 45s to 30s. No changes were observed. So I decided to put the extension time back to 45s, and I prolonged the time of annealing from 30s to 45s, and boom primer dimmers were gone. Further PCRs with IRZmPIN1a primers were with annealing time 45s.

I was still curious how the samples would look like in lower temperature range. It is why; I tested the PCR response at the temperature gradient (59- 50°C).Based on this gradient PCR, temperature 57.5°C (Fig. 13) was finally selected as an annealing temperature for further expression analysis using IRZmPIN1a primers.

Figure 12
This picture shows the PCR temperature gradient from 68 to 57 °C with sample 3 (WT D). PCR was performed to express the *PIN1a*gene in 7-day-old maize seedlings. The PCR reaction was performed using primer 10 µM (0,5 µl), 2 µl cDNA diluted 1/10. In the PCR, the DNA was denatured at 94°C for 3minutes, followed by 30 cycles of amplification (30s at 94°C, 45s at X°C, 30s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes.

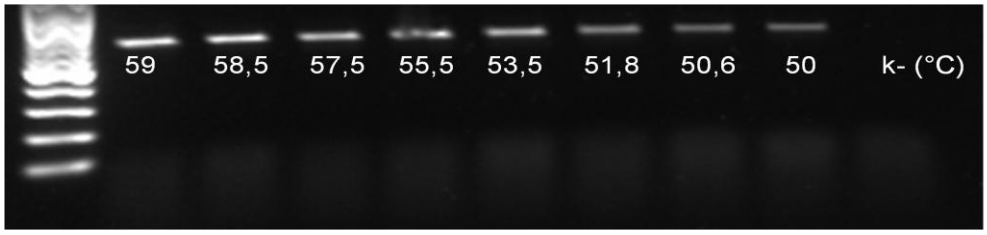

Figure 13
This picture shows the PCR temperature gradient from 59 to 50 °C with sample 1 (WT D). PCR was performed to express the *PIN11a*gene in 7-day-old maize seedlings. The PCR reaction was performed using primer 10 µM 0.5 µl, 2 µl cDNA diluted 1/10. In the PCR, the DNA was denatured at 94°C for 3minutes, followed by 30 cycles of amplification (30s at 94°C, 45s at X°C, 30s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes.

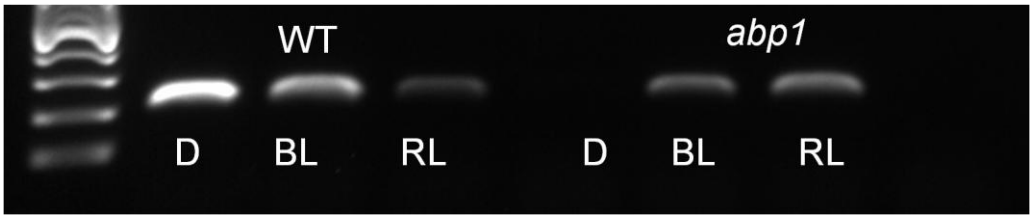

Figure 14
This picture shows the PCR of Ubi, which should be stable, if it was used as a housekeeping gene. PCR was performed to express the Ubiqutin gene in 7-day-old maize seedlings. The PCR reaction was performed using primer 5 µM (0.5 µl), 2 µl cDNA diluted 1/10. In the PCR, the DNA was denatured at 94°C for 3minutes, followed by 35 cycles of amplification (30s at 94°C, 45s at 70°C, 30s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes.

Important for relative gene expression is the housekeeping gene. In the article according which the original primers were synthesized, Ubiquitin was used. Unfortunately Ubi didn't have the same gene expression in all examined genotypes (Fig. 14). Some modifications of PCR were tried out, but the gene expression of Ubi was different among genotypes. Therefore, a new housekeeping gene was selected

After performing some tests (Fig. 15), the *GAPDH* gene coding for glyceraldehyde-phosphate dehydrogenase was selected as the referential housekeeping gene.

Figure 15
This picture shows the gene expression of *GAPDH* gene. PCR was performed to express the *GAPDH* in 7-day-old maize seedlings. The PCR reaction was performed using primer 10 µM (0.5 µl), 2 µl cDNA diluted 1/10. In the PCR, the DNA was denatured at 94°C for 3minutes, followed by 35 cycles of amplification (30s at 94°C, 30s at 60°C, 45s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes.

4.2 Growth responses

The goal of the growth experiments was to find out whether the mutation in *ABP1* and/or *ABP4*gene causes changes of phenotype in maize seedlings. The effect of the mutation on light-dependent growth responses was also examined. In the experiments, the loss-of-function mutants in *ABP1*, *ABP4* and *ABP1/4* genes and corresponding isogenic line were used. Maize seeds germinated on the basal MS medium without any additives. Seedlings were cultivated for 7 days in the growth chambers with different light conditions (BL, RL, D).

4.2.1 Coleoptile length

The differences among genotypes:

In the dark (D), the WT had the longest coleoptiles among all examined genotypes, i.e. 40 mm in average. Etiolated mutant plants *abp1*, *abp4*and *abp1/4* had the length of coleoptiles approximately 25% shorter than etiolated WT seedlings. Among the mutants, no significant difference in coleoptiles length was observed, as the length of coleoptiles of all examined mutant plants varied from 24- 30 mm (Fig. 16).

In red light conditions (RL), WT had the longest coleoptiles among all four examined genotypes (38 mm in average). The mutant plants *abp1*, *abp4*and *abp1/4* had the coleoptiles about 30 mm long. Under blue light (BL), WT plants reached approximately same length of coleoptiles as the double mutant *abp1/4*, the values were around 25 mm. Single mutants *abp1*and *abp4* have slightly shorter coleoptiles than WT - the length fluctuates around 20 mm (Fig. 16).

The effect of light:

The length of coleoptiles in WT and *abp1* remained pretty much the same as observed in plants in dark. The red light very slightly stimulates the elongation of coleoptiles in single mutant *abp4* (26%) and less in double mutant *abp1/4(18%*) - (Fig. 16).

Plants exposed to blue light show distinct reduction of coleoptiles length. In WT the reduction of coleoptile growth was the most obvious, as it was nearly about 40% shorter than WT coleoptiles developed in D. In *abp1* and *abp4* mutant, BL inhibited coleoptile growth by approx. 30% and 20%, respectively, whereas it has no inhibitory effect in the double mutant (Fig. 16).

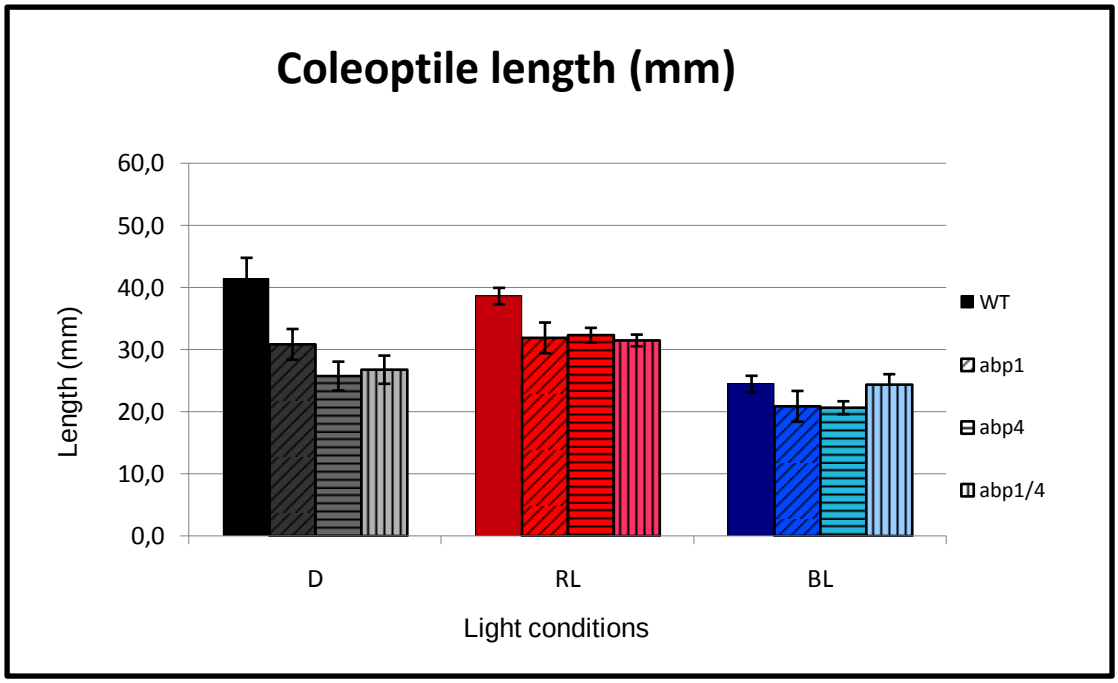

Figure 16
The graph represents the length of coleoptiles in the experimental maize seedlings germinated on different light conditions such as dark, red light and blue light. It shows the differences among the genotypes and also the effect of light. For each genotype, 15 seedlings were measured in each experiment. Values represent mean ± SE of 9 independent experiments.

4.2.2 Mesocotyl length

The differences among genotypes:

In the dark, *abp4* mutant developed the longest mesocotyl among examined genotypes, i.e. about 50% longer than mesocotyl of WT seedlings. Single mutant *abp1*and double mutant *abp1/4* showed mesocotyl about 20% shorter than that developed in WT plants (Fig. 17).

In red light, all four genotypes had approximately the same length of mesocotyl, i.e. around 10-15mm. In blue light, WT, *abp1* and *abp4* had the length of mesocotyl very short, around 6 to 7 mm. The double mutant *abp1/4* developed mesocotyl twice as long as WT plants (Fig. 17).

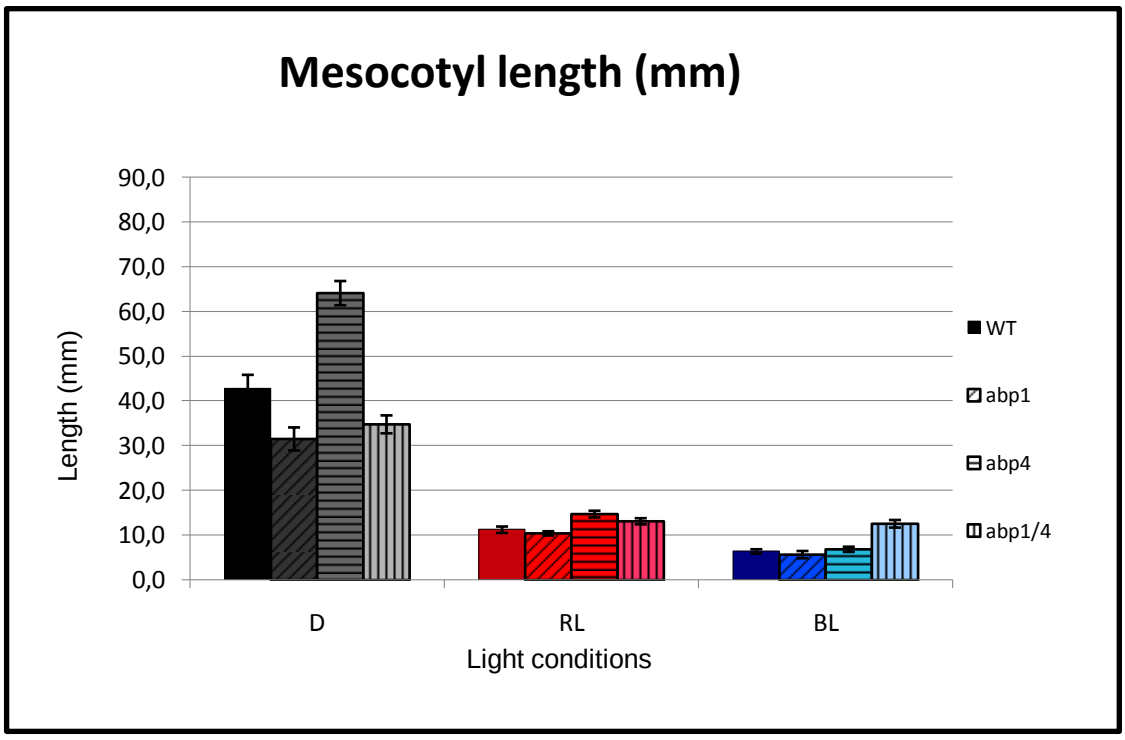

Figure 17
The graph represents the length of mesocotyls of maize seedlings germinated on different light conditions such as dark, red light and blue light. It shows the differences among the genotypes and also the effect of light. For each genotype, 15 seedlings were measured in each experiment. Values represent mean ± SE of 9 independent experiments.

The effect of light:

Mesocotyls of maize seedlings were strongly inhibited in their growth by red as well as blue light. Plants of WT grown under the red light had about 3- 4 times shorter mesocotyl than that in etiolated plants, i.e. about 72% shorter (Fig. 17). Single mutant *abp1* and double mutant *abp1/4* were less sensitive than WT to the inhibitory effect of red light as they developed mesocotyl about 65% shorter than in the dark. Differently, mesocotyl of red light-grown double mutant was about 77% shorter than mesocotyl of etiolated plants (Fig. 17).

The reduction of mesocotyl length was even more distinct in seedlings grown under the blue light. In WT and single mutants, the mesocotyl was about 85% shorter than those developed in the dark. Mesocotyl of double mutant *abp1/4* showed reduced sensitivity to blue light, as its length was inhibited by blue light by approximately 60% (Fig. 17).

4.2.3 Number of seminal roots

The differences among genotypes:

In the dark, WT and *abp1* seemed to have about 3 seminal roots, while in*abp4* and *abp1/4* the number of seminal roots was a little bit lower. In red light, WT has 4 seminal roots, while mutants have usually 3 seminal roots. In blue light, *abp1* and *abp4* had similar number of seminal roots as WT, whereas double mutant has even lower number of seminal roots (Fig. 18).

The effect of light:

In WT, formation of seminal roots was increased by red light. Red light or blue light had no effect on number of seminal roots in mutant *abp1* and in double mutant. Differently, in *abp4* seedlings both lights slightly stimulated seminal root formation (Fig. 18).

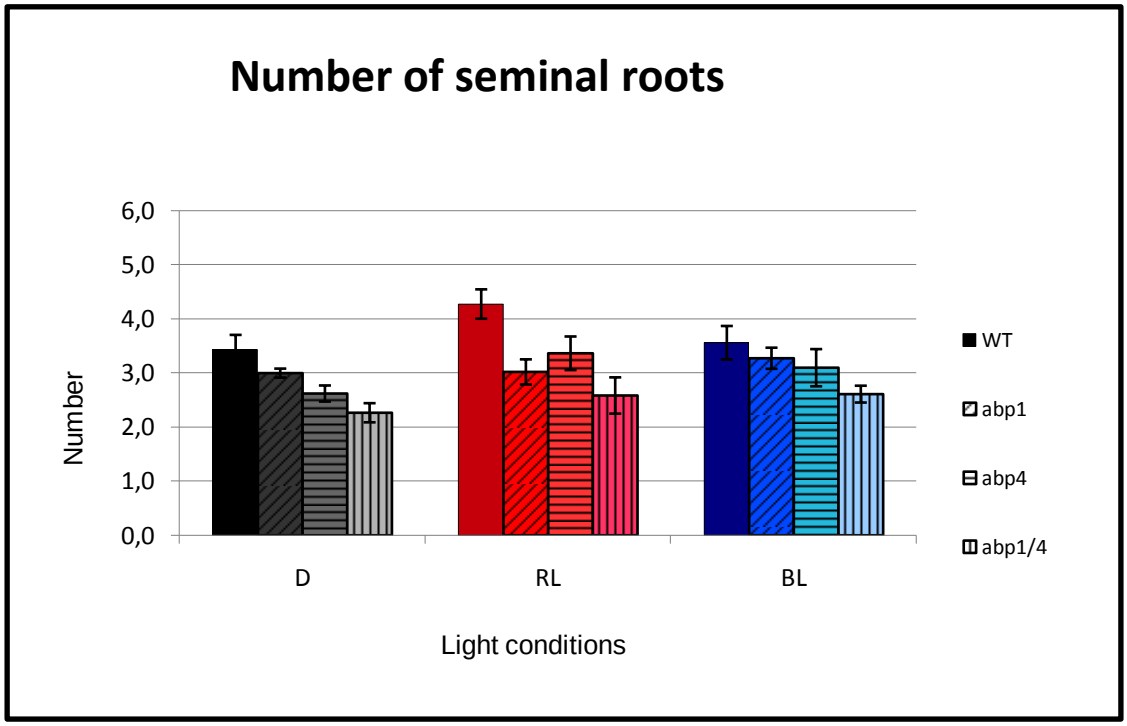

Figure 18
The graph represents the number of seminal roots of maize seedlings germinated on different light conditions such as dark, red light and blue light. It shows the differences among the genotypes and also the effect of light. For each genotype, 15 seedlings were measured in each experiment. Values represent mean ± SE of 9 independent experiments.

4.2.4 Length of seminal roots

The differences among genotypes:

In the dark, *abp1* developed distinctly longer seminal roots than WT and other mutants, while *abp4* and double mutant developed seminal roots of the similar length as the WT. Similar situation was observed in red and blue light, but the trend was that both in lights the double mutant developed the shortest roots (Fig. 19).

The effect of light:

Red or blue light had no marked effect on elongation of seminal roots in WT and *abp1* mutant. In mutant *abp4*, blue but not red light inhibited root elongation (approx. by 37%), while both lights slightly(approx. by 20%) reduced root length in double mutant (Fig. 19).

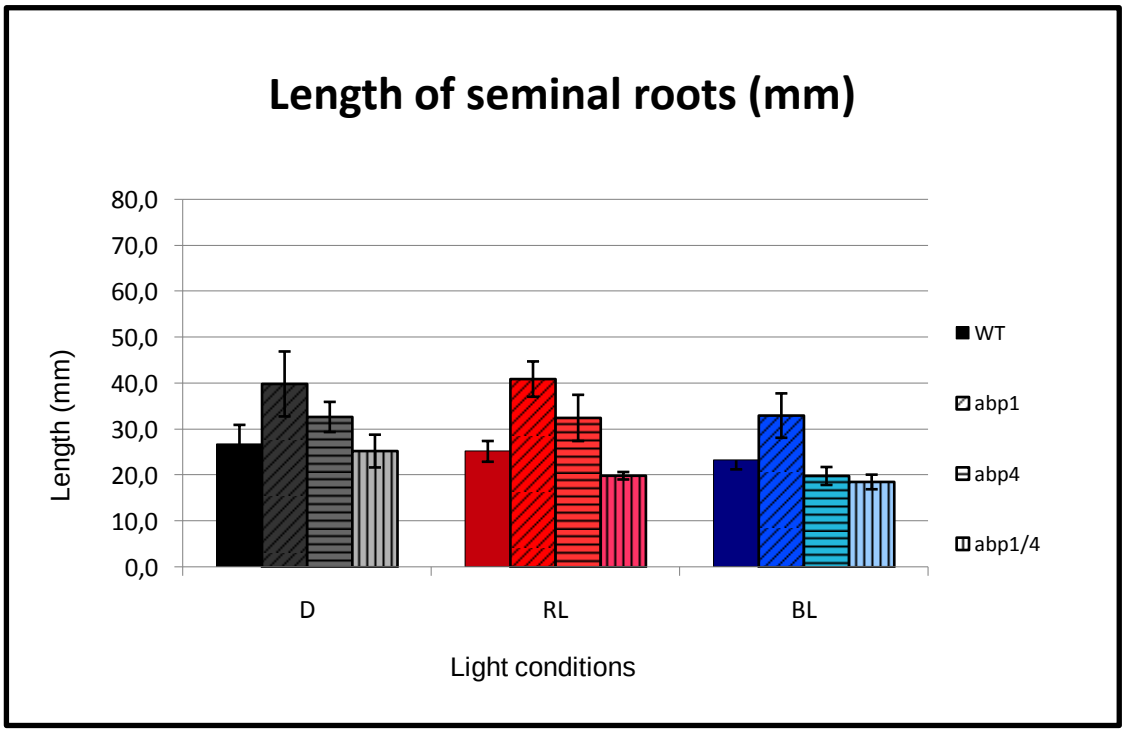

Figure 19

The graph represents the length of seminal roots of maize seedlings germinated on different light conditions such as dark, red light and blue light. It shows the differences among the genotypes and also the effect of light. For each genotype, 15 seedlings were measured in each experiment. Values represent mean ± SE of 9 independent experiments.

4.2.5 Length of primary root

The differences among genotypes:

In the dark, WT had the length of primary root around 68 mm, so similar to the root length observed in mutant *abp1* and *abp4*. The defect in both genes let to shortening of the primary root in etiolated plants to 44 mm (Fig. 20).

Similar situation was observed in red light conditions, and again double mutant showed markedly shorter primary root than WT and the single mutants.

In blue light, values of primary root length in WT and *abp1* are alike, both around 50mm, whereas *abp4* and double mutant *abp1/4* have weakly shorter primary root than WT and *abp1* (Fig. 20).

The effect of light:

Plants exposed to red light don't show any significant difference in length of the primary roots from those grown in the dark. Differently, primary root in WT, *abp1* and *abp4* seedlings was inhibited by blue light approx. by 24, 20 and 30%, respectively, whereas double mutant was insensitive to the blue light-induced inhibition of root elongation (Fig. 20).

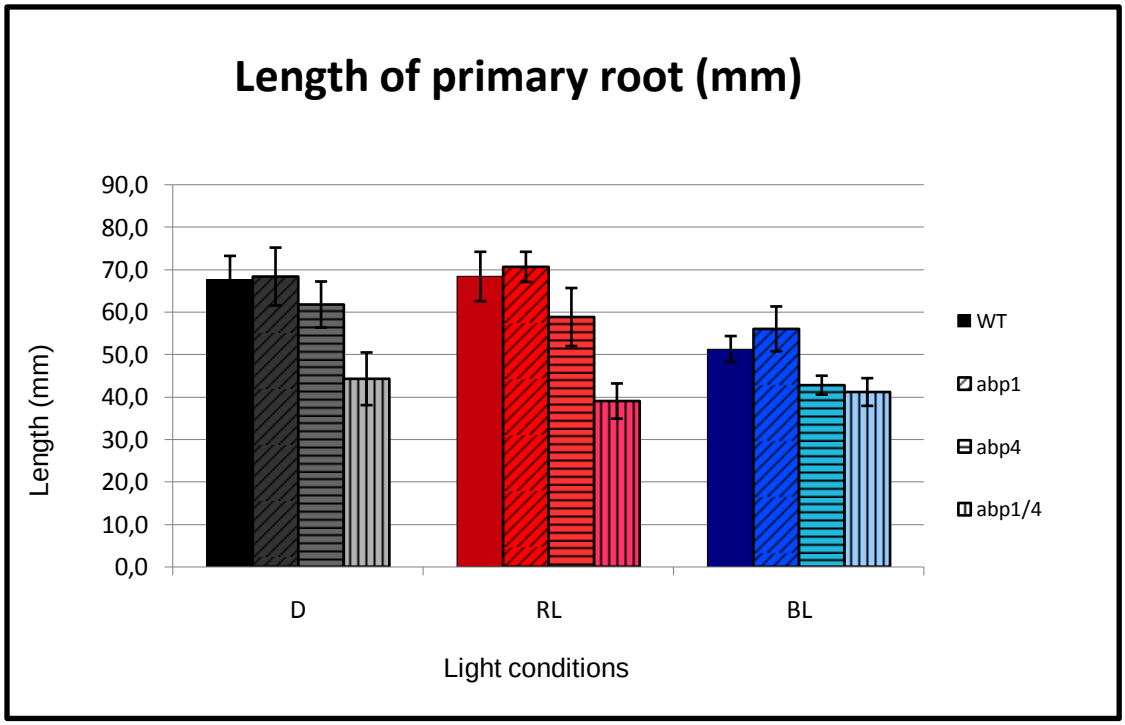

Figure 20

The graph represents the length of primary root of maize seedlings germinated on different light conditions such as dark, red light and blue light. It shows the differences among the genotypes and also the effect of light. For each genotype, 15 seedlings were measured in each experiment. Values represent mean ± SE of 9 independent experiments.

4.3 Gene expression

PIN proteins are important auxin efflux carriers. In *Arabidopsis*, ABP1 was found to promote PIN protein endocytosis, whereas auxin via binging to ABP1 seems to inhibit PIN protein internalization (Robert *et al.*, 2010). Preventing internalization of PIN proteins causes higher amount of PINs in plasma membrane thereby higher auxin efflux transport. The aim of this study was to find out if the mutation of *ABP* genes in maize influences the gene expression of PIN proteins. With the reference to the work of Bořucká and Fellner (2012), my interest was also to test if expression of PIN genes is regulated by light. The gene expression of *PIN1a* was studied with method semi-quantitative RT-PCR in WT and three mutant plants (*abp1*, *abp4*, *abp1/4*). Seeds germinated on the basal MS medium, and after 7 days the mesocotyls were used for isolation of total RNA as described in chapter material and methods.

The graphs of relative expression of *PIN1a* gene show that in the dark the expression of *PIN1a* gene was not affected in mutant *abp1*(Fig. 21),but it was strongly reduced in single *abp4* and in double mutant *abp1/4* (Fig. 22).

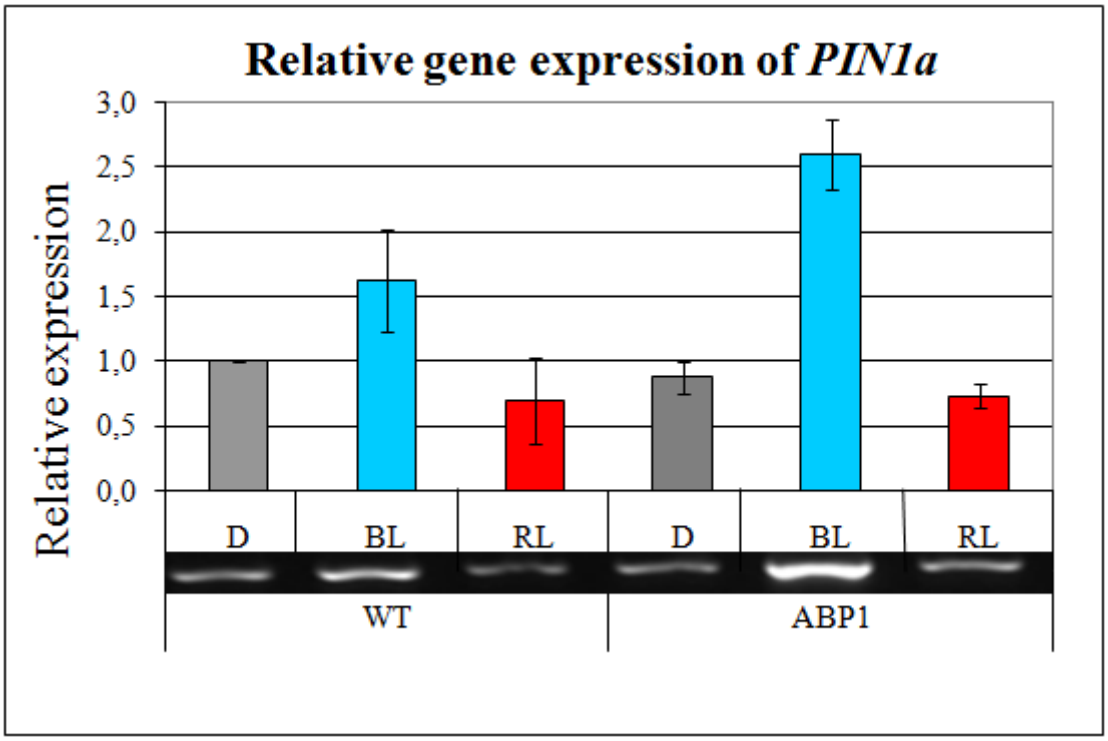

Figure 21
The graph represents the relative gene expression of *PIN1a* gene in 7 day-old- maize seedlings germinated on MS medium in different light conditions i.e. dark, red light and blue light. The graph shows the differences among the genotypes and also the effect of light. Values represent mean ± SE of 5 technical replicates.

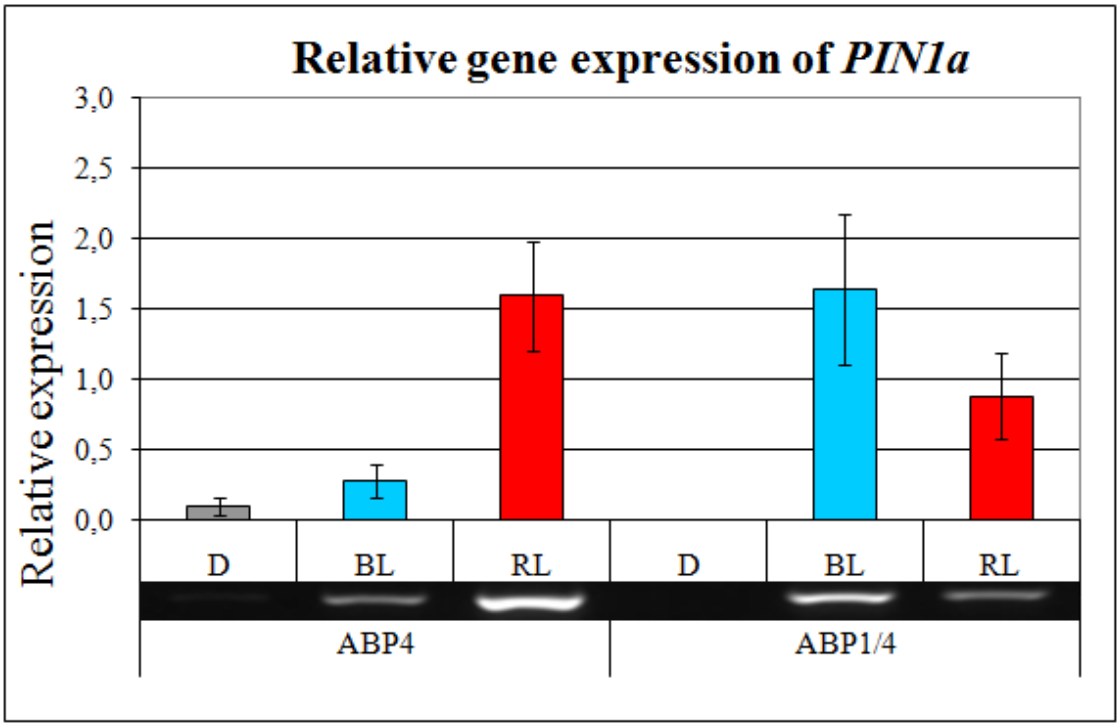

Figure 22
The graph represents the relative gene expression of *PIN1a* gene in 7 day-old- maize seedlings germinated on MS medium in different light conditions i.e. dark, red light and blue light. The graph shows the differences among the genotypes and also the effect of light. Values represent mean ± SE of 2 technical replicates.

Blue light very slightly stimulated *PIN1a* expression in WT and *abp4* mutant, whereas knockout of *ABP1* gene or both genes *ABP1* and *ABP4* led to marked increase of *PIN1a* transcript level (Fig. 21 and 22). Red light had no effect on *PIN1a* expression in WT and *abp1* mutant (Fig. 21), whereas it stimulated expression of *PIN1a* in *abp4* and in double mutant (Fig. 22). The different light conditions affected *PIN1a* gene expression (Fig. 23), whereas the level of *GAPDH* transcript remained the stable in all tested genotypes (Fig. 24).

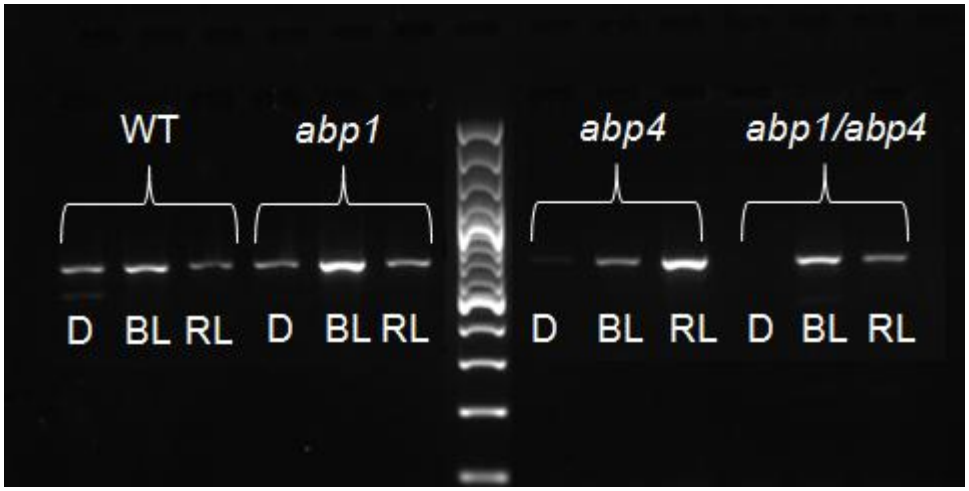

Figure 23

This picture shows the gene expression of *PIN1a* gene in 7 day-old- maize seedlings germinated on MS medium in different light conditions i.e. dark, red light and blue light. The PCR reaction was performed using primer 10 µM (0,5 µl), 2 µl cDNA diluted 1/10 (WT,abp1) and non- diluted (*abp4, abp1/4*). In the PCR, the DNA was denatured at 94°C for 3 minutes, followed by 35 cycles of amplification (30s at 94°C, 45s at 57°C, 30s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes.

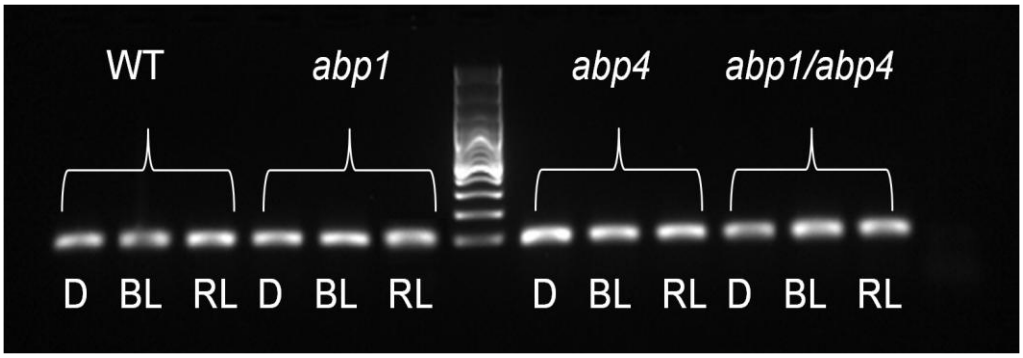

Figure 24

This picture shows the stable gene expression of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in 7 day-old- maize seedlings germinated on MS medium in different light conditions i.e. dark, red light and blue light.The PCR reaction was performed using primer 10 µM (0,5 µl), 2 µl cDNA diluted 1/10. In the PCR the DNA was denatured at 94°C for 3minutes, followed by 35 cycles of amplification (30s at 94°C, 30s at 60°C, 45s at 72°C). The final incubation lasted 5minutes and the reaction was cooled and kept at 15°C. PCR was performed in 20 µl volumes.

5. Discussion

The aim of this study was to find out if the mutation in *ABP*1 and/or *ABP4* genes in maize affects the phenotype of seedlings and whether the mutations influences expression of gene coding PIN1a protein. The effect of the mutation on light-dependent growth responses was also examined. In the experiments, the loss-of-function mutants in *ABP1*, *ABP4* and *ABP1/4* genes and corresponding isogenic line were used. It was also reported that available loss-of-function mutants in ABP1 and ABP4 genes do not show a distinct phenotype (Im *et al.,* 2000), suggesting functional redundancy of APBs in maize. Previous studies suggested that ABP1 and ABP4 are involved in maize leaf growth (Fellner *et al*., 2006; Jurisic-Knezev *et al*., 2012). It was also reported that ABP1 and/or ABP4 are involved in the growth of maize seedlings, and that their effects and functional relationship are organ- and light-dependent (Jurisic-Knezev *et al.,* 2012).

In my thesis, I confirmed that the mutation in *ABP1* and/or *ABP4* gene influences the phenotype of maize seedlings grown in the dark and also in blue and red light. In the case of nonfunctional *ABP1* and/or *ABP4*, a shortening of coleoptiles in etiolated and RL-grown seedlings was observed, which indicates that functional ABP1 and ABP4 are positively involved in coleoptiles elongation in the dark and RL.

It is evident that the mutation in *ABP1* or *ABP4* gene affects coleoptile growth generally, i.e. reduction of the growth is observed in all light conditions. However, coleoptiles in all genotypes still respond to the inhibitory effect of BL, i.e. they are shorter than those developed in the dark and in RL. As obvious, BL inhibited coleoptile elongation in WT more than in *abp1* and *abp4* mutant. Interestingly, double mutant developed in BL coleoptile of the same length like observed in the dark, i.e. the coleoptile in double mutant was almost insensitive to the inhibitory effect of BL. It is therefore possible that functionality of both genes *ABP1* and *ABP4* is required for BL-induced inhibition of coleoptile elongation.

In the dark, plants of single mutant *abp4* developed almost two times longer mesocotyl than WT. It seems that a functional ABP4 negatively regulates the mesocotyl growth of etiolated plants. This is also supported by the fact that in *abp1* mutant, where *ABP4* gene is functional, an inhibition of mesocotyl length was observed. An inhibition of mesocotyl length in double mutant *abp1/4* doesn't correspond with this assumption, but it is possible that knockout of *ABP1* and *ABP4* triggers alternative signal pathway(s). According to Jurisic-Knezev (2012), ABP1 has a positive effect on

mesocotyl elongation of etiolated plants. There is also another possibility how to interpret the data. Considering that the *abp4* mutant contains high level of the ABP1 protein (Im *et al.,* 2000), it seems that ABP1 is a positive factor in mesocotyl elongation of etiolated plants. It is also in agreement with an inhibition of mesocotyl growth observed in double mutant *abp1/4* in D. The higher mesocotyl elongation in the double mutant *abp1/4* grown in BL leads to the conclusion that functional ABP1 and ABP4 are partially involved in BL-induced inhibition of the mesocotyl growth.

Our data further suggest that whatever the light conditions, ABP1 and ABP4 are positively involved in formation of seminal roots, whereas functional ABP1 negatively regulate their elongation (Fig. 18 and 19).

The development of significantly shorter primary root in double mutant *abp1/4* indicates the requirement of both, ABP1 and ABP4, in primary root elongation in all tested light conditions. Differently from dicotyledons, BL essentially reduced root elongation. The percentage inhibition of root growth by BL was similar in WT, *abp1* and *abp4* mutant (25, 20 and 31%, respectively), whereas the knockout of both genes led to insensitivity of primary root to BL. It indicates that ABP1 and ABP4 are required for maintaining the mesocotyl sensitivity to BL.

Most auxin actions are interwoven with changes in polar auxin transport (Petrasek and Friml, 2009). Several studied showed that in *abp* mutants, many auxin-dependent processes were affected. This supports the idea that ABP1 is required for auxin transport- dependent functions (Effendi *et al.,* 2015; Petrasek and Friml, 2009; Xu *et al.,* 2011). To gain a new insight into the effect of mutation in *ABPs* on major auxin efflux carriers, the gene expression of PIN was investigated in this bachelor thesis.

Compared to WT, etiolated mesocotyls of single mutant *abp4* and double mutant *abp1/4* have significantly reduced level PIN1a transcript. Differently, *abp1* mutant did not show any essential reduction of *PIN1a* expression. The data suggests that the in the etiolated mesocotyl, the functional ABP4 positively influences the expression of *PIN1a* gene, thereby likely sustaining polar auxin transport active.

In WT mesocotyls, BL slightly stimulated and RL slightly inhibited expression of *PIN1a*. The fact that BL-induced expression of *PIN1a* was exaggerated in *abp1* and double mutant, but not observed in *abp4*, indicates that in BL-conditions, functional ABP1 is a negative regulator of *PIN1a* expression. It is also in agreement with the observation that *abp4* has much increased level of ABP1 (Im *et al*. 2000).

Thus, without measurement of polar auxin transport, I can speculate that BL-induced reduction of polar auxin transport (Liu et al. 2011) can be mediated by ABP1-reduced expression of *PIN1a*. Gene expression of *PIN1a* was significantly altered by RL only in single mutant *abp4*. This suggests that in RL conditions, functional ABP4 negatively regulates *PIN1a* expression. Considering that the *abp4* mutant contains high levels of the ABP1 protein (Im *et al.*, 2000), another possible explanation is that in RL conditions, functional ABP1 stimulates the *PIN1a* expression. Of course, both signaling pathways are conceivable. However, it is also possible that in the case of *ABP1* and *ABP4* knockout, an alternative signal pathway is activated to sustain *PIN1a* expression. In tomato hypocotyls, polar auxin transport was significantly increased in the *cry1* mutant after BL exposure, suggesting a negative role of *cry1* in polar auxin transport (Liu *et al*., 2011). In root system, Laxmi *et al*., (2008) proposed that light via blue light receptor pathways positively influences the activity of the auxin efflux carrier by controlling the intracellular distribution of PIN2, maintaining its plasma membrane location. Also for roots, Zeng *et al*., (2010) deduced that *Arabidopsis cry1* reduces expression of *PIN1*. However, as far as I know, no more data about the regulation of PIN proteins by light were reported, therefore this work, about ABPs and PINs interaction in plant auxin- light signaling, is beneficial.

6. Conclusion

The mutations in *ABP1* and/or *ABP4* gene differentially influence the phenotype of maize seedlings grown in dark and in light conditions. Data indicates that functional ABP1 and ABP4 are positively involved in coleoptiles elongation in the dark and RL. Also, functionality of both genes *ABP1* and *ABP4* is very likely required for BL-induced inhibition of coleoptile elongation. Further, it seems that a functional ABP4 is a negative regulator, whereas ABP1 is a positive regulator, of the mesocotyl growth of etiolated plants. It is suggested that ABP1 and ABP4 participate in maintaining the mesocotyl sensitivity to BL. Both, ABP1 and ABP4, seem to be required for primary root elongation in all tested light conditions.

The mutations in *ABP1* and/or *ABP4* gene alter expression of gene for PIN1a proteins, while the data suggest that in the etiolated mesocotyl the functional ABP4 positively influences the expression of *PIN1a* gene, thereby likely sustaining polar auxin transport active.

Light alters the expression of *PIN1a* gene, therefore likely influencing polar auxin transport. It is suggested that functional ABP1 is a negative regulator of *PIN1a* expression in BL, whereas functional ABP4 seems to be a negative regulator of *PIN1a* expression in RL. However, since the *abp4* mutant contains high level of the ABP1 protein (Im *et al.*, 2000), it is possible that in RL conditions functional ABP1 stimulates the *PIN1a* expression. All together, the data indicate that both, ABP1 and ABP4, are involved in maize seedling responses to light.

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8. Abbreviation list

ABP1- auxin binding protein 1 loss of function mutant
ABP4- auxin binding protein 4 loss of function mutant
ABP1/4- auxin binding protein 1 and 4 loss of function double mutant
BL- blue light
D- dark
RL- red light
CRYs- cryptochromes
FW- fresh weight
PHYs- phytochromes
PHOTs- phototropines
IAA- indole-3-acetic acid
IBA- indole-3-butyric acid
4-Cl-IAA- 4-chloroindole-3-acetic acid
PAA- 2-phenylacetic acid
IPA- indole-3-pyruvate
TAA1- tryptophan amino transferase
YUCCA- flavin mono-oxygenase
NAA- 1-naphthaleneacetic acid
dicamba- 2-Methoxy-3,6-dichlorobenzoic acid
picloram- 4-Amino-3,5,6-trichloropicolinic acid
2,4-D-2,4-Dichlorophenoxyacetic acid
TIR1- Transport inhibitor response 1 (a protein)
PAT- polar auxin transport
PM- plasma membrane
FMN- flavinmononucleotide
LOV- Light, Oxygen, or Voltage sensing domain
VLFR- very low frequency reactions
LFR- low frequency reactions
HIR- high intensity reactions
PCR- polymerase chain reaction
TCH- touch down
WT- wild type