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Diploma Thesis

Olomouc 2010 **Jana Bořucká**

UNIVERZITA PALACKÉHO V OLOMOUCI

Diplomová práce

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PALACKY UNIVERSITY IN OLOMOUC
Faculty of Science
Department of Cell Biology and Genetics

Functional analysis of interaction between auxin-binding proteins and phytochromes in corn

Diploma Thesis

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Study program: **Biology**
Field of study: **Molecular and Cell Biology**

Supervisor: Doc. RNDr. Martin Fellner, Ph.D.

Olomouc 2010

UNIVERZITA PALACKÉHO V OLOMOUCI
Přírodovědecká fakulta
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Funkční interakce auxin-binding proteinů a fytochromů ve vývoji kukuřice

Diplomová práce

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Studijní program: **Biologie**
Studijní obor: **Molekulární a buněčná biologie**

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Olomouc 2010

DECLARATION

I declare that I elaborated this diploma thesis independently under supervision of Associate Prof. Martin Fellner, Ph.D. and using only information sources mentioned in the References chapter.

In Olomouc ……

Signature

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SUMMARY

My diploma thesis was focused on study of role of auxin-binding proteins (ABPs) in light signaling during development of corn seedlings. Using genetic approach consisting of analysis of hybrids and loss-of-function mutants in *ABP1* and *ABP4* genes in maize (*Zea mays L.*), I investigated expression of red light receptors phytochrome A and phytochrome B as a function of light quality. In addition, I studied whether exogenous auxin can influence expression of the phytochromes and whether auxin effect could be mediated by ABP signaling pathways. Obtained results support previous hypothesis that modern corn hybrid responds to light less than the older hybrids. Namely, I found that in opposite to the older hybrid 307, white light was not able to reduce expression of *PHYA* gene in the modern hybrid 3394. Experiments on *abp* mutants showed that auxin can reduce expression of *PHYA* and/or *PHYB* genes, and that ABP1 and/or ABP4 mediate the effects of auxin. Interestingly, I also disclosed that blue light could decrease or block the expression of both phytochromes, and the results suggest that at least in the case of *PHYA*, ABP1 and ABP4 may be positively involved in the phytochrome gene expression. The results of my experiments support the existence of cross-talk between light and auxin signaling pathway, and provide some evidence that ABPs may function as elements of light signaling pathways involved in development of maize seedlings.

SOUHRN

Diplomová práce byla zaměřena na studium role auxin-binding proteinů (ABPs) v signální dráze světla během vývoje semen kukuřice. Využitím genetického přístupu spočívajícího v analýze hybridů a loss-off-function mutantů v genech *ABP1* a *ABP4* u kukuřice (*Zea mays* L.) jsem zkoumala expresi receptorů červeného světla fytochromu A a fytochromu B. Dále jsem studovala, zda exogenní auxin může ovlivňovat expresi fytochromů a zda může být účinek auxinu zprostředkován signálními drahami ABP proteinů. Získané výsledky podporují předešlou hypotézu o moderním hybridu kukuřice, který se jeví jako méně citlivý ke světlu než starší hybrid. Konkrétně jsem zjistila, ze narozdíl od staršího hybrida kukuřice 307, bílé světlo neredukovalo expresi *PHYA* u moderního hybridu 3394. Experimenty na *abp* mutantech ukázaly, že auxin může redukovat expresi *PHYA* a/nebo *PHYB*, a že ABP1 a/nebo ABP4 mohou zprostředkovávat účinky auxinu. Rovněž jsem došla k závěru, že modré světlo může snížit nebo blokovat expresi obou fytochromů a výsledky naznačují, že alespoň v případě *PHYA* mohou být ABP1 a ABP4 pozitivně zahrnuty v expresi fytochromů. Zjištěná fakta z mých experimentů podporují existenci vzájemné interakce mezi signálními drahami světla a auxinu a zároveň poskytují důkaz, že ABPs mohou fungovat jako elementy signální dráhy světla zapojené ve vývoji mladých rostlin kukuřice.

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1 INTRODUCTION

The auxin signaling pathway is not completely well-known and discovered process. It includes primary binding of auxin to receptor, an auxin-binding protein (ABP). The role of *ABP1* as auxin receptor is known in *Arabidopsis*, where is essential for many developmental and growth responses. However, little is known about ABP functions in corn. The aim of my diploma thesis was to study the interaction between auxin-binding proteins and phytochromes. Using genetic approach I studied loss-of-function mutant in *ABP1* and *ABP4* in maize *Zea mays* L. and their potential influence on expression of *PHYA* and *PHYB* genes. The study was based on previous research of Martin Fellner (Fellner *et al.,* 2003, 2006), and it was part of the Czech-USA research project: "Interaction between auxin and light signaling in seedling growth and development of leaf angle in the corn: supported by Ministry of Education, Youth and Sports of the Czech Republic (project no. 1P05ME792; 2005-2008). The project involved collaboration of Laboratory of Molecular Physiology at Palacky University in Olomouc and Department of Biology, University of Washington, Seattle (Prof. Elizabeth Van Volkenburgh). The experiments in of diploma thesis were also supported by an EU FP6 Marie Curie Research Training Network VaTEP (grant No. MRTN-CT-2006-035833; 2006-2010).

In my thesis, I followed hypothesis that modern corn hybrid 3394 is resistant or less sensitive to auxin- and light-induced responses of etiolated seedlings in comparison with the older hybrids, such as 307. My study was further focused on determination of possible role of ABPs as a common element in auxin and light signaling pathways.

Goals of my study were:

1) To determine effect of light and exogenous auxin on expression of genes coding for phytochromes phyA and phyB in maize mesocotyls.

2) To determine whether loss of genes *ABP1* and/or *ABP4* affects light- or auxin-regulated expression of *PHYA* and *PHYB* in maize mesocotyls.

2 BACKGROUND

2.1 AUXIN

Auxins as plant hormones belong to important signaling molecules that regulate physiological as well as morphological response in plants. They act in low concentrations and they are required for plant growth and development (Davies, 2004; Vanneste and Friml, 2009).

Auxin regulates three essential cellular processes in morphogenesis: elongation, division and differentiation. At the plant level auxin controls embryo and fruit development, organogenesis, vascular tissue differentiation, root patterning, elongation and tropism growth, apical hook formation and apical dominance (Kepinski and Leyser, 2005; Paciorek and Friml, 2006; Chapman and Estelle, 2009). Very rapid cellular responses, such as plasma membrane depolarization, K^+ channel expression and apoplastic acidification have been also connected to auxin (Chen *et al.*, 2001).

Natural auxins, e.g. IAA (indole-3-acetic acid) and IBA (indole-3-butyric acid) are the most abundant in plant tissues (Taiz and Zeiger, 2006). However, scientists and industrial laboratories are able to synthesize a wide range of auxins, such as NAA (1-naphtylacetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid) that are usually used in experiments.

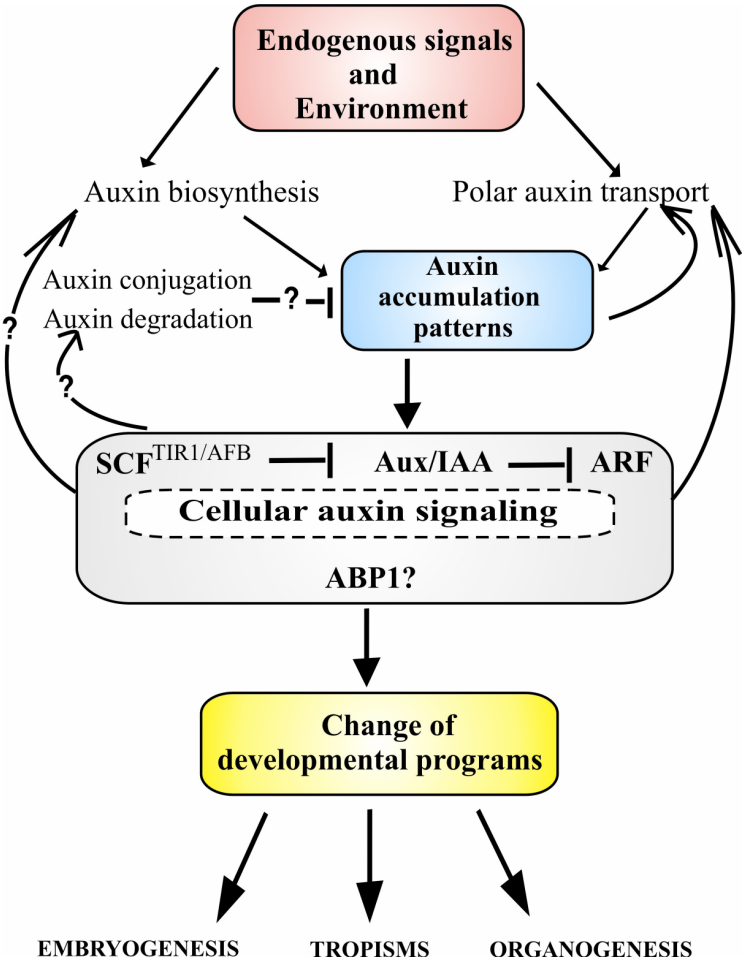


Fig. 2.1: Auxin action (according to Vanneste and Friml, 2009). Endogenous and exogenous signals modulate auxin biosynthesis, polar auxin transport, accumulation and degradation. As result of this cascade of processes, some developmental programs and changes can be triggered.

2.1.1 Auxin biosynthesis and degradation

Various environmental (e.g. gravity and light) and endogenous signals (e.g. ethylene) can cause changes in auxin distribution through their effects on the local auxin biosynthesis and intracellular auxin transport (Štěpánová *et al.*, 2008; Tao *et al.*, 2008; Eckardt, 2010). IAA biosynthesis is associated with rapidly dividing and growing tissues, especially in meristems, young leaves, developing fruits and seeds. In the cell accumulating auxin, a change on the developmental program is triggered while the change seems to be already preprogrammed, for example by different cell type-specific combination of auxin-dependent transcriptional regulators in a given cell type.

In this way, auxin accumulation selects a cell or cells at a particular position and at particular time for a change in developmental program.

Auxin IAA is structurally related to the amino acid tryptophan (Trp). Plant can convert Trp to IAA by several pathways, namely by one Trp-independent and four Trp-dependent pathways: IAM (indole-3-pyruvic acid), IAN (indole-3-acetonitrile) pathway, IAOx (indole-3-acetaldoxime), TAM (tryptamine pathway), and IPA (indole-3-pyruvic acid) (Zhao *et al.*, 2001, Cohen *et al.*, 2003).

Auxin is distributed differentially within plant tissues, and in the cells it appears to be regulated largely by pH (Rubery and Sheldrake, 1974; Raven, 1975; Taiz and Zeiger, 2006). Auxin accumulates at the location of organ initiation (Dubrovsky *et al.*, 2008; Vanneste and Friml, 2009) and then auxin gradient is established along the growth axis of the developing primordium with the maximum at the tip (Benková *et al.*, 2003; Vanneste and Friml, 2009). This way of auxin transportation is called polar auxin transport. Differential auxin distribution within plant tissues accompanies important developmental decisions. In other words, the timing and locations of auxin accumulation determine spatio-temporal aspects of developmental reprogramming.

2.1.2 Auxin transport

Auxin is able to move between cells in a directional manner. This process is crucial for the regulation of plant physiology and development. The scientists have described two distinct major pathways to translocate auxin. The first one, called rapid or long-distance source-to-sink transport is from young shoot tissues toward sink tissues (Marchant, 2002). The second one, slower type of transport, polar auxin transport, is important for auxin transport over shorter distances. It depends mainly on specific influx and efflux carrier proteins. Direction of this transport is strictly controlled within a given tissue.

Auxins are weak acids and they are easily protonated in the relatively acid environment of the apoplast and later become lipophilic and able to diffuse through cell membrane. In the neutral cytoplasm, auxin become deprotonated and trapped inside the cell because it is not able to permeate through the plasma membrane. This mechanism called diffusion-based is connected with active auxin-uptake by specific influx carriers represented by protein AUX1 (Stoma *et al.*, 2008). On the other hand, specific efflux carriers exist in the cell, represented by PIN1 protein located at the

basal part of the cell (Smith, 2008). Efflux carrier transports auxin through the plasma membrane outside the cell (Weijers and Friml, 2009).

Auxin transport machinery includes P-glycoproteins of the ABCB transporter family as well. Together with PIN proteins as auxin-efflux carriers they act in auxin efflux. ABCB protein efflux can control the amount of auxin available in auxin channels and this transport is predominantly non-directional (Mravec *et al.*, 2008). In contrast, switch in PIN polarity means that auxin fluxes can rapidly redirect (in response to environmental or endogenous signals). PIN-dependent auxin transport is regulated by auxin at multiple levels so that auxin can influence transcription, turnover and PIN-proteins localization on the plasma membrane as well (Petrášek *et al.*, 2006; Zažímalová *et al.*, 2007).

2.1.3 Auxin signaling pathway

Individual cells interpret auxin largely by a nuclear signaling pathway that involves the F-box protein TIR1 acting as an auxin receptor (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005). The nuclear signaling pathway involves a plant-specific receptor mechanism in which the ligand (auxin) does not trigger an allosteric change in the receptor (Tan *et al.*, 2007). Binding of auxin to the receptor TIR1 (Transport Inhibitor Response 1) stabilizes its interaction transcriptional repressors called Aux/IAAs (Kepinski and Leyser, 2005) targeting them afterwards for proteolysis via 26S proteasome. Thus, auxin triggers developmental changes in plant cells (Vanneste and Friml, 2009). This mechanism seems to play an important role in conveying positional information during developmental processes such as embryogenesis, organogenesis, tissue patterning and tropisms (Vanneste and Friml, 2009).

Auxin signaling is interpreted by two classes of transcriptional regulators. The first one, Aux/IAA negatively regulate auxin signaling due to their protein structure, involves four highly conserved domains. These domains are important for their function as transcriptional repressors and they interact with the other important class of transcriptional regulators, the ARFs (auxin response factors) (Kim *et al.*, 1997; Hardtke *et al.*, 2004; Vanneste and Friml, 2009). The ARFs are a class of plant specific transcription factors that mediate auxin-dependent transcriptional regulation. The ARF multigene family includes members that can activate or repress transcription, depending on the amino acid sequence in a non-conserved central domain (Guilfoyle

and Hagen, 2007). Active ARFs are negatively regulated by interaction with Aux/IAA (Szemenenyei *et al.*, 2008). Different ARFs play a role in diverse growth responses including embryogenesis, root and floral development and senescence (Napier, 2004).

The mechanism of auxin perception and the identity of auxin receptor can answer the question in auxin signaling. The protein ubiquitination machinery including Skp1-cullin-F-box protein (SCF) E3 ubiquitin ligase and its component TIR1 protein plays very important role (Quint and Gray, 2006). The crystallographic analysis of this interaction shows that TIR1 contains a hydrophobic pocket in which auxin and various auxin analog can bind. Auxin does not change the conformation of TIR1 but instead acts as molecular glue by filling a hydrophobic cavity at the interaction interface, thereby enhancing TIR1-Aux/IAA interaction (Tan *et al.*, 2007). This demonstrates that TIR1 is the auxin receptor.

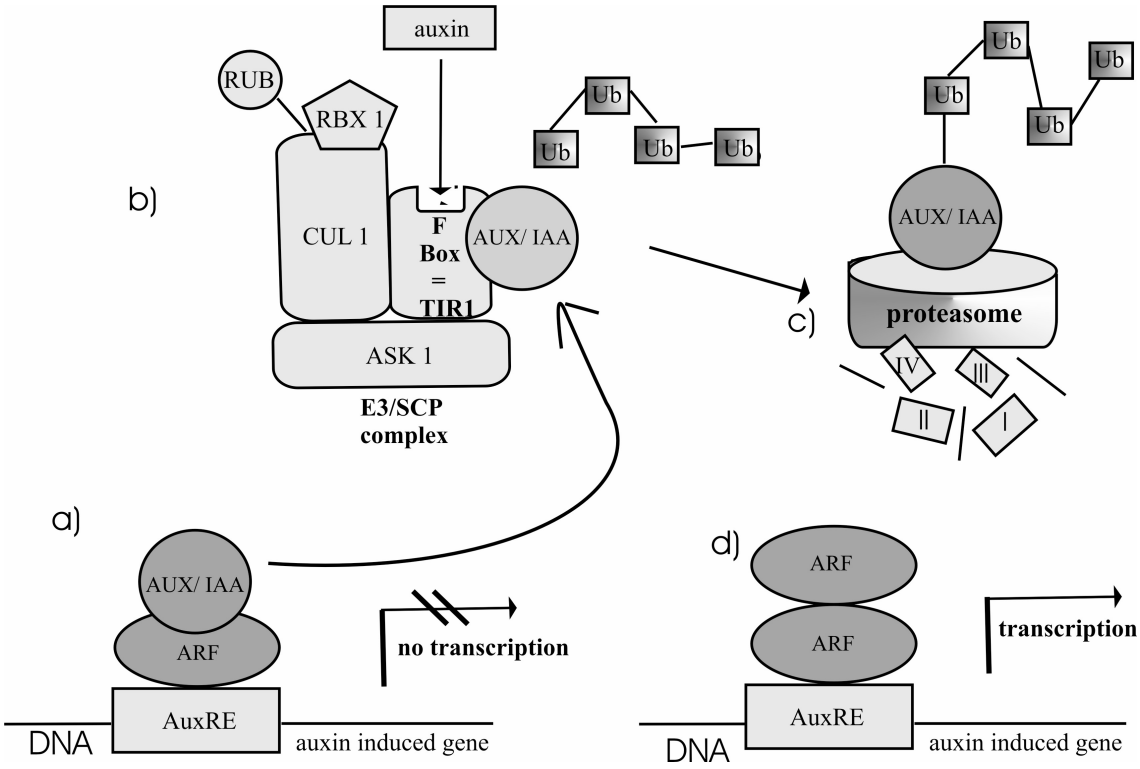


Fig. 2.2: Function of TIR1 as a receptor controlling auxin-induced gene expression (according to Christian *et al.*, 2006). a) Auxin-response elements binding together with ARFs control auxin-induced genes what may black transcription. b) Auxin binds to the TIR1 (F-box protein) which is part of E3/SCF complex involved in ubiquitination of Aux/IAA proteins that are rapidly degraded by proteasome. c) This process removes the transcriptional block.

2.1.4 Auxin receptor ABP1

ABP1 is a low-abundance, soluble 22kDa glycoprotein, ubiquitous amongst green plants, found in the greatest amounts in the elongation zones of aerial tissues (Jones, 1994; Diekmann *et al.*., 1995; Napier and Venis, 1995; Napier, 2004). ABP1 is located predominantly in the endoplasmic reticulum, where the pH is too high for efficient auxin binding, but together with its ligand may interact with a plasma membrane (Henderson *et al.*, 1997; Chen *et al.*, 2001; Paciorek and Friml, 2006).

ABP1 as the auxin receptor triggers early modification of ion fluxes across the plasma membrane in response to auxin, mediates auxin-dependent cell expansion and embryonic development. ABP1 also plays a critical role in the regulation of the cell cycle by acting at both the G1/S and G2/M checkpoints as well as requiring for entry in the cell cycle (David *et al.*, 2007). ABP1 is likely to be involved in the two key developmental processes resulting from cell division-associated growth and cell expansion, f.e. ABP1 is a key regulator of root growth and it is required for the auxin-mediated response (Chen *et al.*, 2001; Jones *et al.*, 1998; Tromas *et al.*, 2009). ABP1 is a major regulator for auxin action on the cell cycle and regulates auxin-mediate gene expression and cell elongation in addition to the already known TIR1-mediated ubiquition pathway.

Structure of ABP1 and the mechanism of interaction with auxin

ABP1 belongs to the ancient and functionally diverse germin/seed storage 7S protein superfamily. Among typical structural features belong metal ion inside of the binding pocket and further protein fold and β-jellyroll barrel formed by two antiparallel β-sheets (Woo *et al.*, 2002). The binding pocket of ABP1 is predominantly hydrophobic with metal ion, zinc, deep in the pocket coordinated by a cluster of histidine residues and one glutamic acid residue, leaving one coordinated axis free. In the auxin-unbound form this is filled by a water molecule. Auxin binding displaces the water and auxinic carboxylic acid group makes a contact with the zinc ion, orientating the auxin deep inside an otherwise hydrophobic pocket (Napier, 2004).

Auxin enters the binding pocket of ABP1 through the N- and C-terminal extension (Woo *et al.*, 2002). The C-terminus contains a KDEL (lysine-aspartic acid-glutamic acid-leucine) -motif that acts as retention signal for the ER lumen, where is found bulk of ABP1 (Dahlke *et al.*, 2009). The selectivity of ABP1 for auxins is

explained by the characteristic of the binding pocket (Woo *et al*., 2002; Campanoni and Nick, 2005). The three main routes by which auxin molecule can enter or leave the ABP1 binding site are possible. One of the routes leads to the membrane and the other two to ABP1 aqueous surroundings (Bertoša *et al*., 2008).

ABP1 signaling pathway

ABP1 signaling pathway is still very poorly understood. It is supposed that auxin-induced conformational changes in ABP1 might alter interaction with other membrane proteins such as heterotrimeric G-proteins. Another characteristic feature of ABP1 that could contribute to explanation of signaling pathway is that there is no transmembrane domains in ABP1, and majority of ABP1 is localized in the ER. Small amount of ABP1 escapes KDEL retention signal and it is excreted to the apoplastic space probably by vesicular transport. After binding extracellular auxin, ABP1 can interact with putative transmembrane docking protein, which transduces the signal into the cell. The docking protein theory was proposed by Klämbt (1990). Secretion of ABP1 into the cell wall is probably via secretory system.

It was demonstrated that ABP1 mediates very early the auxin responses including the modifications of ion fluxes across the plasma membrane, resulting in the activation or deactivation of ion channels (K^+ anions) or transporters (H^+) (Barbier-Brygoo *et al.,* 1989; Leblanc *et al.,* 1999; Napier *et al.* 2002). Constitutive over-expression of *ABP1* in maize or tobacco cells resulted in larger cells and this effect was auxin dependent, consistent with ABP1 having an auxin receptor function (Jones *et al.,* 1998; Im *et al.,* 2000; Chen *et al*., 2001). A role for ABP1 in cell division has also been evoked (Fellner *et al*., 1996) and characterization of the *Arabidopsis abp1* loss-of-function mutant supports a dual role of the protein in cell expansion and cell division during early embryogenesis. Recently, it was shown that ABP1 acts on the control of cell cycle (David *et al.,* 2007). It was demonstrated that cell cycle arrest provoked by *ABP1* inactivation could not be bypassed by exogenous auxin suggesting that ABP1 mediates the auxin control of the cell cycle (David *et al.,* 2007). Until 2009, no evidences were provided if the ABP1 is involved in auxin-regulated gene expression. However, recently Tromas *et al.* (2009) demonstrated that ABP1 is required for auxin-induced expression of a subset of *Aux/IAA* genes in roots. The authors present a model in which ABP1 is the major regulator of auxin action on the cell cycle and regulates

auxin-mediated gene expression and cell elongation in addition to the TIR1-mediated ubiquitination pathway.

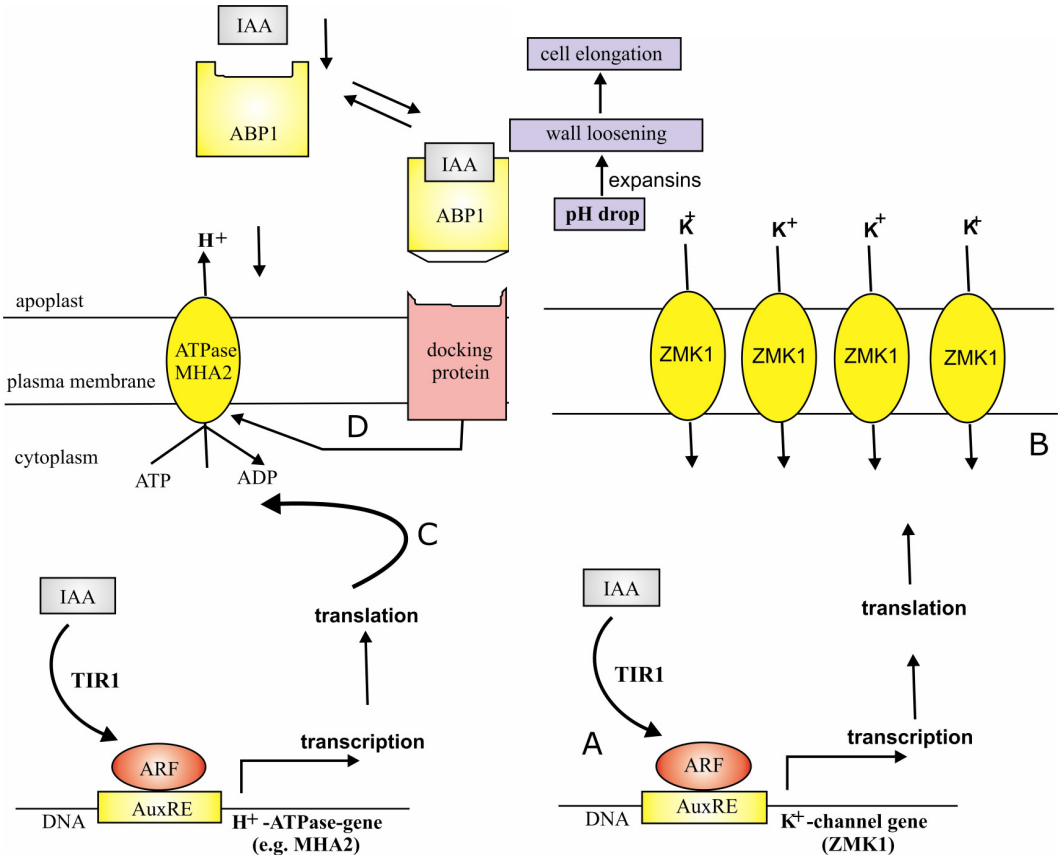


Fig. 2.3: Possible model of cross-link between TIR1 and ABP1 signaling pathway (according Christian *et al.*, 2006).

A) This model involves ZMK1 gene expression as response to auxin signal through TIR1/AFB receptors. This increases the density of channels on the membrane surface. B) This is important for charge compensation for the protons pumped by the H^+-ATPase. C) ABP1 as auxin receptor can be involved in ATPase activity stimulation by auxin. D) Auxin induced *de-novo* synthesis of ATPase. This process could be connected with apoplastic acidification and auxin-induced gene expression via the TIR1/AFB pathway.

The interaction of two auxin signaling pathways including TIR1 and ABP1 receptors remain uncovered. TIR1 binds auxin at physiologically relevant concentrations and auxin-binding to TIR1 results in the regulation of expression of auxin-regulated genes. Rapid auxin responses such as rapid activation of ion fluxes at the plasma

membrane involve another putative auxin receptor ABP1. ABP1 appears predominantly in the endoplasmic reticulum, whereas TIR1 and Aux/IAA substrates are mainly located in the nucleus, thus physical interactions between TIR1 and ABP1 proteins are highly unlikely (Vanneste and Friml, 2009; Tromas *et al*., 2009). But Christian *et al.* (2006) proposed possible model of connection between TIR1 and ABP1 signaling pathways (see **Fig. 2.3**).

2.2 PHYTOCHROMES

Plants utilize light as a source of energy and as a source of information about their environment. There are significant differences between dark-grown (etiolated) and light grown seedlings. Upon light exposure, seedlings undergo de-etiolation. The cotyledons open, expand and begin to photosynthesize and the hypocotyl elongation is inhibited in vegetative meristems (Schroeder *et al*., 2002). This phenomenon is known as the photomorphogenesis.

In plants, light-dependent responses are controlled by a series of photoreceptors, classified into phytochromes, cryptochromes and phototropins (Quail, 2002) that integrate numerous light signals to monitor the time of day and season and the gauge the proximity of neighboring vegetation (Sheehan *et al*., 2007).

2.2.1 Phytochrome characterization and structure

Phytochromes are a family of chromoproteins that exist in two photoconvertible conformations, Pr (a red-light-absorbing form) and Pfr (a far-red-light absorbing form). Plant phytochromes are conjugates of PHY apoprotein covalently attached to their linear tetrapyrrole chromophor 3E-phytochromobilin P-B that is synthesized from heme in chloroplast and assembled with nuclear-encoded PHY apoprotein in the cytosol. There, it forms the inactive red-absorbing Pr form of phytochromes (Kircher *et al*., 1999; Kim *et al*., 2005; Sawers *et al*., 2005). Phytochromes are predominately in the Pfr form because sunlight is enriched in red light and this converts each to the Pr-form during periods of darkness (dark reversion). Photoconversion back to Pr-form can be mediated by pulses of FR as well (Schäfer and Bowler, 2002).

The localization of phytochrome is very specific. In dark, phytochrome appears in the Pr-form and it is localized within the cytoplasm. During conversion to Pfr it is translocated to the nucleus where it activates signaling pathways (Kim *et al.*, 2005; Sawers *et al.*, 2005).

The phytochrome molecule consists of two structural domains, the globular N-terminal chromophore-binding domain and the conformationally open or extended C-terminal domain. The two domains are connected via a flexible hinge region. The N-terminal domain is necessary and sufficient for photoperception and possesses the bilin lyase domain, which allows the attachment of the chromophore to apo-phytochrome. Several conserved subdomains and sequence motifs have been identified in the phytochrome molecules. Some experiments were performed demonstrating that chromophorebearing N-terminal domains of phyA and phyB determine their photosensory specificity and differential light lability (Kim *et al.*, 2005).

In angiosperms, phytochromes represent small gene families classified into subfamilies as PHYA, PHYB/D, PHYC/F and PHYE. Different family members have distinct, overlapping and even antagonistic roles during plant development (Franklin and Whitelam, 2004; Sawers, 2005). Although the monocot lineage has probably lost the PHYE sub-family, there is a divergence of monocots and dicots. Phytochromes are usually present in single copies, but in maize each phytochrome is duplicated probably due to allotetraploid event so that the gene family consists of 6 phytochromes: PhyA1, PhyA2, PhyB1, PhyB2, PhyC1 and PhyC2 (Mathews and Sharrock, 1996; Sheehan *et al.*, 2004; Sawers *et al.*, 2005).

2.2.2 Phytochrome A, phytochrome B

PhyA is the most abundant phytochrome in dark-grown seedlings although is rapidly degraded upon exposure to light (Nagy and Schäffer, 2002). The other phytohromes are expressed in much lower levels and their expression is not strongly influenced by light. PhyB is the most abundant phytochrome in sunlight due to phyA degradation. Phytochromes (especially phyA) are very sticky and can interact non-specifically with a range of proteins and RNA *in vitro* (Quail, 1994). PhyB plays a central role in mediating responses to light in the control of plant height, transition to flowering and axilary branch meristem development as well as of elongation of sheath and stem tissue of

mature plants, of seedling development, mesocotyl elongation, stand density and chloroplast gene expression (Sheehan *et al.*, 2007). PhyA is predominantly responsible for de-etiolation in continuous far-red light, whereas phyB for de-etiolation in response to continuous red light (Canton and Quail, 1999). Predicted molecular weight of phyA (125 kDa) is less than phyB (128 kDa), but phyB proteins migrate more quickly through the gel perhaps due to different post-transcriptional modification. In addition, the expression of phyA is regulated by phyB (Canton and Quail, 1999; Sheehan *et al.*, 2007).

2.2.3 Phytochrome phosphorylation: key part of plant signaling

The signal-transducing photoreceptors in plants are all phosphoproteins and/or protein kinases, suggesting that light-dependent protein phosphorylation and dephosphorylation play important roles in the function of the photoreceptors (Kim *et al.*, 2005). The phosphorylation and dephosphorylation serve as a signal modulation mechanism in the regulation of cellular activities. The attachment to or removal of phosphate group from a protein often has profound effects on the structure, thereby affecting the functional properties of the protein such as enzymatic activity, intracellular localization, protein-protein interactions and stability (Watson, 2000; Luan, 2003). It was demonstrated that protein phosphorylation is regulated by light. Namely, red, far-red and blue light control the phosporylation level of some proteins, both in the nucleus and in the cytoplasm. It suggests that protein kinases and protein phosphatases are involved in the plant light signaling.

Phytochromes are phosphoproteins or light-regulated protein kinases that catalyze the phosphorylation of photoreceptors. The protein can be phosphorylated either by autophosphorylation or by other protein kinases, and can be dephosphorylated by protein phosphatases. Since phytochromes are phosphoproteins and kinases, they can be phosphorylated by autophosphorylation and by other protein kinases (Colón-Carmona *et al.*, 2000; Shalitin *et al.*, 2003).

Kim *et al.* (2005) suggested that reversible phosphorylation of phytochrome is a key biochemical mechanism in early light signaling in plants. However, there are also other important mechanistic events to complete phytochrome signaling and cytoplasmic events such as changes in ionic conductance across the plasma membrane.

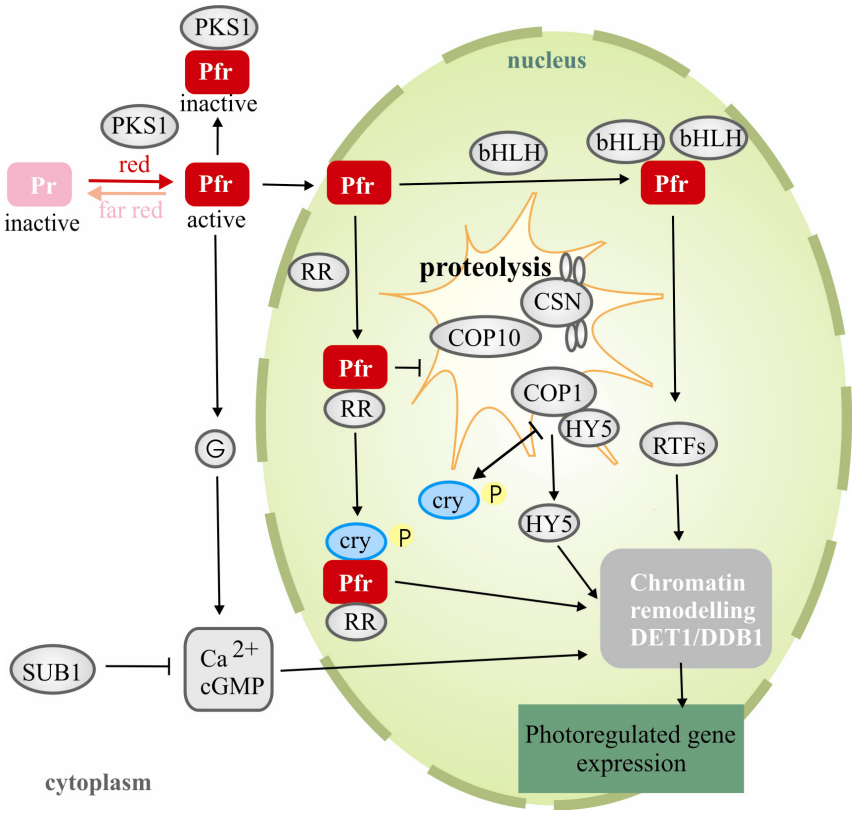


Fig. 2.4: A model of phytochrome signal transduction (according to Kim *et al.*, 2005). Activated phytochrome (Pfr) may regulate transcription through several parallel pathways. A rapid response involves Pfr translocation to the nucleus where it binds transcription factors (bHLH family) that are subsequently activated. Second possible pathway involves binding response regulators (RR). They stabilize them in the activated form and can induce light-regulated gene expression by inhibiting COP1-dependent proteolysis of the HY5 transcriptional factor and by binding activated cryptochromes. In the cytoplasm, phytochrome may activate gene expression through G-proteins, calcium and c-GMP-dependent pathway (regulated by SUB1).

2.2.4 Interaction between light and auxins

A number of interactions have been observed between light and auxin action during various physiological processes but detailed description of many cross-talks are not well understood.

As I have shown in previous chapter, phytochrome phosphorylation is involved in plant signaling. Recent studies suggest two possible roles of phytochrome phosphorylation in the control of protein-protein interaction between phytochromes and downstream signal transducers. For example, phytochrome can stabilize Aux/IAA proteins that can serve phytochrome kinase substrate. Several Aux/IAA proteins interact with phytochromes and are phosporylated by phytochrome on the N-terminal domain of the proteins. These phosporylations might be connected with the regulation of Aux/IAA protein via protein degradation pathways. Such pathways can provide an example of molecular mechanism for the cross-talk between auxin and light signaling in the plant development (Kim *et al.*, 2005). Phytochrome kinase activity plays a critical role in the cross-talk between light and other signaling. The phytochrome-mediated light signaling in plants is initiated by conformational changes in phytochrome molecules triggering by the incoming light. The light signal can elicit cellular responses through the action of downstream signal elements that constitute intricate signaling networks (Kim *et al.*, 2004). Example of this interaction could represent red light-induced reduction of ABP1 abundance as well as light-induced decrease of auxin transport in correlation with the decrease in growth (Jones *et al.*, 1991; Jones *et al.*, 1998; Fellner *et al.*, 2003).

Many of growth and developmental plasticity are achieved by light modulation of auxin systems. Light imposes a strong influence in multiple facts of the auxin system, controlling auxin levels, transport and responsivess as well as the distribution of auxin through the seedling and the response to auxin within individual cells (Salisbury *et al.*, 2007). Recent studies have shown that phytochromes impose a strong influence on auxin levels in plants by regulation both SUR2 suppressor, and TAA1 enhancer of IAA biosynthesis. Active phyB (Pfr) reduces IAA levels by coordinated activation of SUR2 and repression of TAA1 transcript levels. Reduced levels of phyB induced by low R:FR-ratio light trigger the reciprocal control with a consequential increase in IAA levels (Tao *et al.*, 2008). Dual control of both positive and negative regulators of IAA biosynthesis enables phyB to exert strong control on IAA synthesis. Aux/IAA, SAUR and GH3 genes are probably regulated by light. Some analyses have shown that they are rapidly

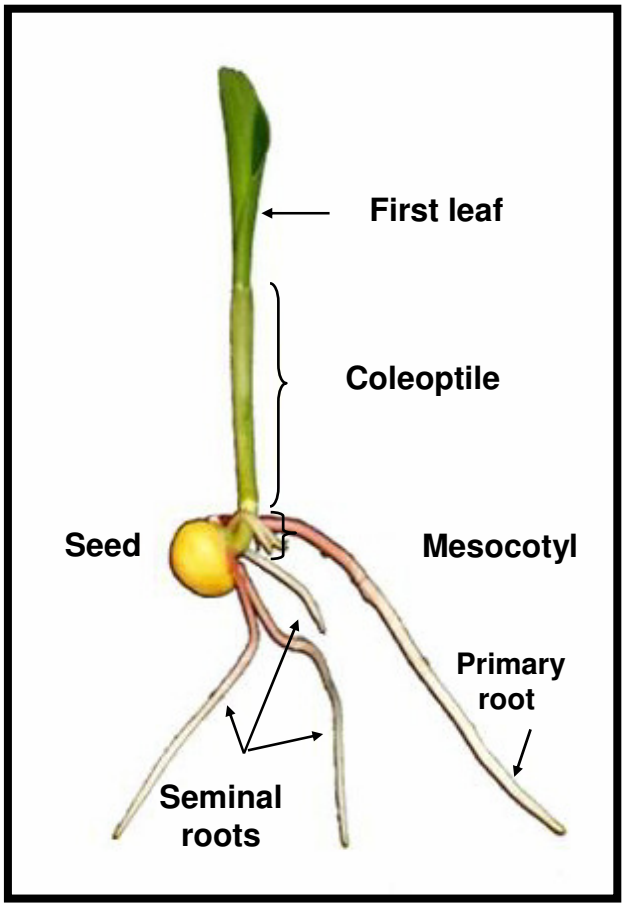
regulated by phytochrome during seedling de-etiolation (Tepperman *et al.*, 2006). It was also suggested that turnover of Aux/IAAs may be an important factor modulating the degree between skotomorphogenic versus photomorphogenic development (Sorin *et al.*, 2005). The strongest connections between light and auxin signaling have been observed in plants exposed to low R:FR ratio light, which triggers the shade avoidance syndrome. In this case, transcript abundance of numerous auxin responsive genes increases rapidly in response to low R:FR-ratio conditions (Halliday *et al.*, 2009).

2.3 MAIZE AS MODEL TO STUDY

Maize (*Zea mays* L.) as a major world crop was first domesticated from teosinte, a wild grass that looked quite different from modern corn. Teosinte grows in Mexico and Central America as a bushy plant with many spikes (ancestor to corn cob) (Matsuoka *et al.*, 2002). Because of people migration throughout America, new varieties were selected to grow in local environments and these varieties show genetic diversity that form basement for research as well as traits for crop breeding.

Due to ideal domesticated traits, maize was chosen as a genetic model plant in the early 20th century. Maize also serves as model grass to develop methods for optimizing plant-based biofuel production and increasing yield despite different environmental conditions. Using markers (as controlling seeds and plant colours) in cytogenetics, we could study patterns of inheritance, functions of genes, the origin of nucleus, the properties of telomers, epigenetic silencing, imprinting, transposition and we can also observe the distinctive meiotic chromosomes of maize. Tools including cytogenetics, transposons and gene expression profiles provide the necessary foundation to sequence the maize genome.

Maize has very dynamic genome (the size is 2.3 gigabases) including 10 chromosomes. Important sight for evolution of maize is a big variability among varieties. Analysis of the sequenced genome demonstrates that this ancestral duplicated genome underwent significant rearrangement. The genome analysis demonstrates that landraces and worldwide varieties are highly diverse, making preservation of their unique molecular heritage important for cultural, scientific and agricultural reasons (Gore *et al.*, 2009; Schnable *et al.*, 2009; Sylvester *et al.*, 2009; Swanson-Wagner RA *et al.*, 2009).



Scheme of maize seedling

(Dejana Jurišić-Knežev, 2010, unpublished results)

3 MATERIALS AND METHODS

3.1 PLANT MATERIAL

In my experiments, I used seeds of *Zea mays L*. hybrids 307 and 3394 and loss-of-function mutants in *ABP1* and *ABP4* genes: single mutants *abp1, abp4,* double mutant *abp1abp4*, and corresponding WT (i.e. isogenic line).

ABP1 and *ABP4* are the two most highly expressed genes among ABP multigene family in maize. In order to reveal the role of *ABP1* and the other members of the *ABP* gene family, Im *et al.* (2000) used a reverse genetic approach by screening an F1 population of maize plants mutagenized by the *Mutator* elements (Robertson's mutator transposable elements). A reverse genetic strategy was applied to identify loss-of-function alleles of *ABP1* gene (Bensen *et al*., 1995). All the loss-of-function mutants are homozygous in this gene (Im *et al*., 2000) and were kindly provided by Alan M. Jones from University of North Carolina.

3.2 CULTURE CONDITION: *IN VITRO*

Seed sterilization

For experiments *in vitro*, maize seeds (stored in the fridge) were rinsed in 70% ethanol for 3 minutes using magnetic stirrer followed by washing three times with distilled sterile water. After that seeds were surface sterilized by soaking for 30 minutes (hybrids) or 60 minutes (mutants) in commercial SAVO solution (BOCHEMIE, s.r.o., Czech Republic) enriched by drop of detergent Tween20 (Calbiochem, USA). Afterwards, seeds were washed five times with sterile distilled water. For better sterilization results air drying in sterile Petri dish in laminar hood was applied.

Plant growth

Seeds of the experimental plants were sown on solid basal Murashige-Skoog medium (Murashige and Skoog, 1962). The medium contained macroelements, microelements (i.e. 4.3 g/l Murashige and Skoog basal mixture MS, Sigma M5524), 1% (w/v) sucrose, 1mM MES (2-(*N*-morpholino)-ethanesulfonic acid), and 0.7% (w/v) agar. The pH of the medium was adjusted to 6.1 by 1M KOH before autoclaving. Five seeds of each genotype were sown into the sterile Magenta boxes (Sigma-Aldrich, USA) containing 60

ml of basal medium. In the experiments with auxin, the basal medium was supplemented with appropriate concentration (5.10^{-5} and 10^{-6} mol.l^{-1}) of synthetic auxin NAA (1-Naphthaleneacetic acid). Seeds in the boxes were placed into the growth chamber (Snijders Scientific B.V., Netherlands) and incubated in the dark, white light (Percival PGC-10, IA, USA, The Netherlands; total photon fluence rate 150 mmol.m^{-2}.s^{-1}) and blue light (Microclima MC1000E Snijders Scientific; Philips tubes TLD-36W/15-Blue; max. irradiance at 420 nm; total photon fluence rate 10 mmol.m^{-2}.s^{-1}) for 7 days at the temperature of 23°C.

3.3 GENE EXPRESSION

Expression of genes coding for phytochromes was studied on maize mesocotyls of 7-day-old seedlings grown *in vitro* on the basal medium in the absence or the presence of auxin in the dark, white light and under blue light.

Total RNA was extracted from the mesocotyl of experimental plants using RNeasy Plant Mini RNA kit (Qiagen Inc., USA, Valencia, CA) according to the manufacturer's instructions. After RNA extraction was performed traces of genomic DNA from a batch of RNA were removed by a DNaseI treatment using RQ1 RNA-free DNase (Promega, USA) for 40 min at 37℃ in water-bath. RNA was then mixed with phenol:chloroform:isoamylalcohol (25:24:1) vortexed and spun to allow the two phases to form. Afterwards, supernatant was transferred into new tube and the volume completed with 20 µl of 1M acetic acid and 550 µl of 96% EtOH. The mixture was incubated overnight and then washed with EtOH (70% and 96%, centrifuge each time for 5 min; rest of EtOH was removed with pipette and the pellet was air-dried for 5 min). RNA quantity and quality was then measured by spectrophotometer Smart Spec Plus 2000 (BioRad, Czech Republic) in RNase-free water as blank.

A reverse transcription reaction was performed with 1µg of total RNA by adding 4µl of 5x FS (First Strain) buffer, 1 µl of 10 mM dNTP, 1 µl of 0.1M DTT, 1 µl of 50 µM oligo(dT)20 primer, 0.5 µl of RNaseOUT (InVitrogen, USA) and 1 µl of SuperScript III Reverse Transcriptase (InVitrogen Co., USA, Carlsbad, CA). The volume was filled up to 20 µl with RNase-free water. The reaction mixture was incubated in a thermocycler (MJ Mini Gradient Thermal Cycler, BioRad, Czech Republic) programmed for 50℃ for 60 min followed by step at 85℃ for 5 minutes and ended by cooling for 15 minutes.

The cDNA product was directly used in PCR (Polymerase Chain Reaction). PCR amplification was performed in a mixture containing: 4 µl of 5X GoTaq polymerase buffer, 2 µl of 1 mM dNTP, 1 µl of 10 nM of each gene-specific primer, 1 ul of GoTaq polymerase (Promega, USA) and 1 µl of diluted cDNA.

Table 1: List of gene-specific primers used for PCR

Primer	Sequence
PHYA – F	5'-GAG AGA TCC ATG AAG CAA AAG GTT TAC-3'
PHYA – R	5'-GAA GGT TGA CAT GCC CAG CTT CCC TGA G-3'
PHYB – F	5'-GTT TTG GCT GAC TTC GCT AAG CAT G-3'
PHYB – R	5'-GGA CGA TGA GGA AGA AAC TCC GCT CTG-3‘
ZmACT81 - F	5‘-ACA CAG TGC CAA TCT-3‘
ZmACT81 – R	5‘-ACT GAG CAC AAT GTT AC-3‘
18S rRNA – F	5‘-ACG AAC AAC TGC GAA AGC-3‘
18S rRNA – R	5‘-CGG CAT CGT TTA TGG TTG-3‘

Table 2: PCR program for *PHY* genes (according to Sheehan et al., 2004; modified)

Initial step for denaturation	95℃	15 min	
PCR amplification	94℃	30 s	24 cycles
	55℃	30 s	
	72℃	1 min	
Final elongation step	72℃ 4℃	10 min forever	

Table 3: PCR program for *ACT81* gene

Initial step for denaturation	94℃	5 min	
PCR amplification	94℃	30 s	35 cycles
	45℃	30 s	
	72℃	45 s	
Final elongation step	72℃¨ 15℃	5 min forever	

Table 4: PCR program for 18S gene (according to Shou *et al.*, 2004, modified)

Initial step for denaturation	94°C	3 min	
PCR amplification	94°C	30 s	22 cycles
	55°C	45 s	
	72°C	30 s	
Final elongation step	72°C	5 min	
	15°C	forever	

PCR products were size fractionated by the electrophoresis in a 1% (w/v) agarose gel stained with ethidium bromide. Received bands were evaluated by the software ImageJ to get relative gene expression.

4 RESULTS

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4.1 EXPERIMENTS WITH MAIZE HYBRIDS

4.2 EXPERIMENTS WITH MAIZE MUTANTS

5 DISCUSSION

6 CONCLUSION

7 ABBREVIATIONS

1-NAA	1-naphtyl acetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
ABP	auxin-binding protein
abp	auxin-binding protein loss-off-function mutant
ADP	Adenosinediphosphate
ARE	Auxin Responsive Elements
ARF	Auxin Response Factor
ATP	Adenosinetriphosphate
Aux/IAA	Auxin Resistant
BL	blue light
cDNA	complementary DNA
cFR	continuous far red light
COP1	Constitutive Photomorphogenic 1 signalosome
CUL1	Cullin homologue 1
cry	cryptochrome mutant
D	dark
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide
DTT	dithiothreitol
E3	ubiquitin ligase
ER	endoplasmic reticulum
EtBr	ethidium bromide
G1/S	transition between growth 1 (G1) and synthetic (S) phases of cell cycle
G2/M	transition between growth 2 (G2) and meta-phase (M) of cell cycle
IAA	indole-3-acetic acid
IAM	indole-3-acetamid
IAN	indole-3-acetonitrile
IAOx	indole-3-acetaldoxime

IBA	indole-3-butyric acid
IPA	indole-3-pyruvic acid
KDEL	endoplasmic reticulum retention signal
mRNA	messenger ribonucleic acid
MAPK	mitogen-activated protein kinase
MS medium	Murashige and Skoog medium
PCR	polymerase chain reaction
Pfr	far red light absorbing form
PHY A-E	phytochrome A-E
PIN1	pin-formed protein 1
Pr	red light absorbing form
RNA	ribonucleic acid
RNase	ribonuclease
RL	red light
RUB1	ubiquitin-related protein
SCF complex	Seventeen kilodalton protein Cullin F-box
SE	standard error
TAM	tryptamine pathway
TIR	Transport Inhibitor Response
Ub	ubiquitin
WT	wild-type
ZMK1	*Zea mays* K+ channel

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