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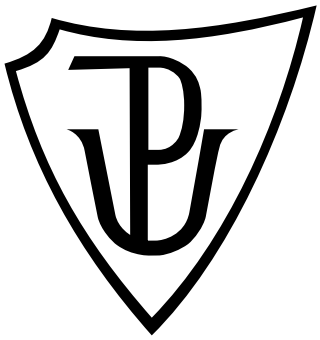

The role of light and auxin-binding proteins during the development of leaf angle in maize

PhD. Thesis

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I hereby declare that this thesis has been fully worked out by myself with the use of cited references.

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Abstract: Maize (*Zea mays* subsp. *mays*) is the second most cultivated crop in the world and, naturally, it represents a very important object of breeding programs. Increasing yield potential is one of the main targets of breeding techniques as well as cultural practices. Maize hybrids developed and released by Pioneer Hi-Bred International from 1930 to 2001 were selected primarily for their higher yield in high-density planting conditions; however the selection resulted not only in a higher yield but also in changes in morphological traits, such as smaller and more erect leaves and enhanced tolerance to abiotic stress present in such a condition. The role of auxin and light in this process was proposed few years ago. In this thesis, the interaction between light and auxin signaling pathways was investigated in four old Pioneer hybrids (307, 317, 3306 and 3366) and one modern hybrid (3394). Juvenile seedlings of the modern hybrid developed more erect leaves with a smaller leaf area than seedlings of all the old hybrids and showed altered cross-talk between blue light and auxin signaling pathways. A putative auxin receptor – auxin-binding protein 1 was hypothesized to be involved in this process, but its role as a linkage element between the light and auxin signaling pathways that was altered during the breeding of modern hybrid was not confirmed. However, the analysis of maize *abp1*, *abp4* single mutants and *abp1abp4* double mutant revealed, that auxin-binding protein 1 and 4 are involved in the free IAA homeostasis and their action is BL- and tissue-dependent, what provides a novel insight into their role in developing maize seedlings.

Keywords: light, auxin, auxin-binding protein 1, maize

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Abstrakt: Kukuřice (*Zea mays* subsp. *mays*) je druhou nejvíce pěstovanou plodinou na světě, a představuje velmi důležitý předmět šlechtitelských programů. Zvyšování produkce je jedním z hlavních cílů šlechtitelských a pěstitelských technik. Hybridy kukuřice vyšlechtěné americkou firmou Pioneer Hi-Bred International v letech 1930 – 2001 byly selektovány především pro jejich vyšší výnos v podmínkách s vysokou hustotou výsadby, ale tato selekce měla za následek nejen vyšší výnos, ale i změny v morfologických vlastnostech hybridů, jako například menší a více vztyčené listy a větší toleranci k abiotickým stresům přítomným v těchto podmínkách. Role auxinu a světla v tomto procesu byla navržena před několika lety. V této práci byla zkoumána interakce mezi signálními dráhami světla a auxinu u čtyř dřívějších hybridů (307, 317, 3306 a 3366) a jednoho moderního hybridu (3394) firmy Pioneer. Juvenilní rostliny moderního hybridu se vyznačovaly více vzpřímenými listy s menší listovou plochou než rostliny všech dřívějších hybridů, a pozměněnou interakcí mezi signálními dráhami modrého světla a auxinu. Předpokládalo se, že receptor auxinu – auxin-vázající protein 1 je zapojen do tohoto procesu. Jeho role jako spojovacího článku mezi signální dráhou světla a auxinu, která byla pozměněna v průběhu selekce moderního hybridu, se však nepotvrdila. Analýza *abp1*, *abp4* „single“ mutantů a *abp1abp4* „double“ mutanta nicméně ukázala, že auxin-vázající proteiny 1 a 4 jsou zapojeny do homeostáze volného auxinu a jejich působení je závislé na typu rostlinného pletiva, což nabízí nový pohled na jejich roli ve vývoji klíčenců kukuřice.

Klíčová slova: světlo, auxin, auxin-vázající protein 1, kukuřice

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1 INTRODUCTION

1.1 AUXIN

The plant hormone auxin is considered to be the first growth hormone discovered in plants, since its action in the phototropic bending was published in 1881 by Charles and Francis Darwins in the impressive book *The Power if Movement in Plants*. Auxin regulates various developmental processes during the all life of the plant, such as stem elongation, lateral roots initiation, vascular differentiation, fruit development, apical dominance, cell division and the cell cycle regulation. The main auxin present in higher plants is indole-3-acetic acid (IAA). All parts of young developing seedlings are able to synthesize IAA - young leaves, newly fertilized embryos and primary roots (Ljung et al. 2001).

1.1.1 Metabolism of auxin

Biosynthesis of IAA emanates from the amino acid tryptophan (Trp) or alternatively from indole or indole-3-glycerol phosphate (Fig. 1) (reviewed in Woodward and Bartel 2005, Mano and Nemoto 2012). Up to now, five Trp-dependent pathways were proposed: IPA, TAM, IAN, IAM and two-step pathway (Fig.1, except the IAM pathway). Biosynthesis of auxin through the IPA (indole-3-pyruvic acid), the IPA pathway, is known from bacteria (Koga 1995, Minamisawa et al. 1996). IPA was also detected in Arabidopsis, and three Arabidopsis genes coding for Trp transaminases that catalyze conversion of IPA from Trp were identified – *TAA1* (*TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1*), *TAR1* and *TAR2* (*TRYPTOPHAN AMINOTRANSFERASE RELATED1, 2*) (Tao et al. 2008, Stepanova et al. 2008). On the contrary, IPA decarboxylation and formation of indole-3-acetaldehyd was not proven in plants so far (Ye and Cohen 2009). No TAA1-like genes were identified in monocots, however *vt2* (*the vanishing tassel2*) mutant of maize may be the first candidate (McSteen 2010, Phillips et al. 2011). The TAM (tryptamine) biosynthetic pathway of auxin seems to be used by both dicots and monocots. The rate-limiting step of TAM pathway is catalyzed by flavin monooxygenase-like enzymes YUCCA that were identified in Arabidopsis *yucca* mutants with elevated level of auxin and also in monocots – rice and maize (YUCCA-like) (Zhao et al. 2001, Yamamoto et al. 2007, Gallavotti et al. 2008a). The IAN (indole-3-acetonitrile) pathway in plants is poorly

characterized. In Arabidopsis, enzymes converting Trp to indole-3-acetaldoxime and nitrilases that convert IAN to IAA (Zhao et al. 2002, Pollmann et al. 2006) are known. Nitrilase genes were characterized also in maize (Park et al. 2003), but indole-3-acetaldoxime was not detected in maize seedlings (Sugawara et al. 2009), thus IAN pathway does not seems to be a common pathway in plants. There is only little information about the IAM (indole-3-acetamide) pathway. Genes for enzymes that convert Trp to IAM are not known, but in Arabidopsis the conversion of IAM to IAA seems to be catalyzed by amidase, encoded by *AMI1* gene (Pollmann et al. 2003). In monocots, no genes involved in this pathway have been reported yet, but IAM as a metabolite was detected in lot of species including maize (Sugawara et al. 2009, Mano and Nemoto 2012). Very recently, new and simple two-step pathway of auxin biosynthesis has been established in plants (reviewed in Zhao 2012, Mano and Nemoto 2012).

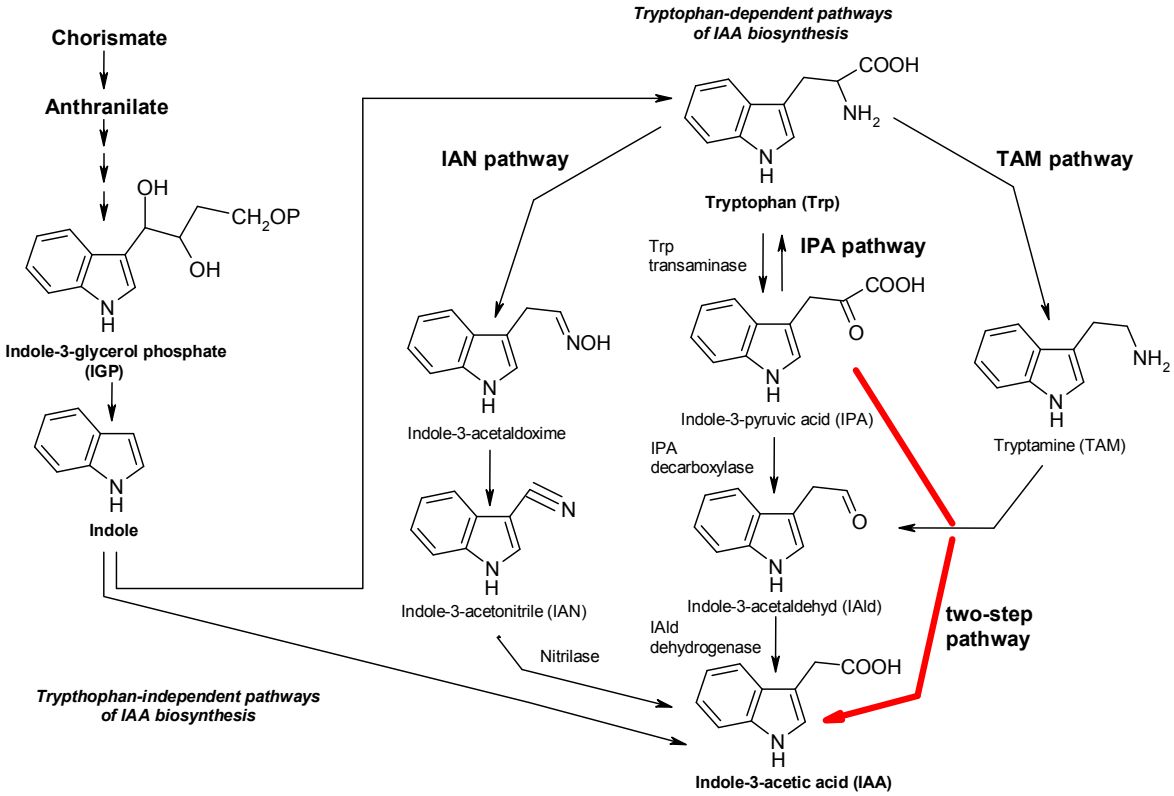


Fig. 1 Scheme of IAA biosynthetic pathways

Simplified diagram of Trp-dependent IAA biosynthetic pathways (right part), which includes indole-3-acetonitrile pathway (IAN), indole-3-pyruvic acid (IPA) pathway, tryptamine pathway (TAM) and the two-step pathway (Zhao et al. 2012). At the left side, the diagram of the tryptophan-independent pathway of IAA biosynthesis is shown (scheme adopted from Woodward and Bartel 2005).

This pathway includes conversion of Trp to IPA catalyzed by TAA amino transferases and subsequent conversion of IPA to IAA catalyzed by enzymes from YUCCA family (Won et al. 2011) (Fig. 1 – red arrow). Trp-independent biosynthetic pathways of IAA were found in variety of plant species (Woodward and Bartel 2005). The first evidence about Trp-independent synthesis of IAA comes from maize *orangepericarp* mutant that is defective in Trp synthesis, but genes involved in this pathway have still not been identified (Wright et al. 1991, Normanly 2009).

Auxin homeostasis is regulated through the conjugation and degradation of the conjugates. Conjugated forms of IAA are inactive and can either be degraded or serve as storage compounds. IAA can be conjugated in variety of ways, e.g. forming esters, amides and glycosides. IAA conjugating enzyme IAGLU that conjugates IAA to glucose was characterized for the first time in maize (Szerszen et al. 1994, Ludwig-Müller et al. 2005).

Auxins are used in agriculture as selective weed killers and herbicides that destroy broad leaved weeds but do not affect mature monocotyledonous plant (Strachan et al. 2010). They are also used for inducing of rooting, flowering, parthenocarpy, prevention of premature fruit drop and in some species to abort dormancy (Serrani et al. 2007, Cohen 1996, Repčák 2002). For agricultural purposes, synthetic auxins naphthalene-1-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Fig. 2) are used most frequently.

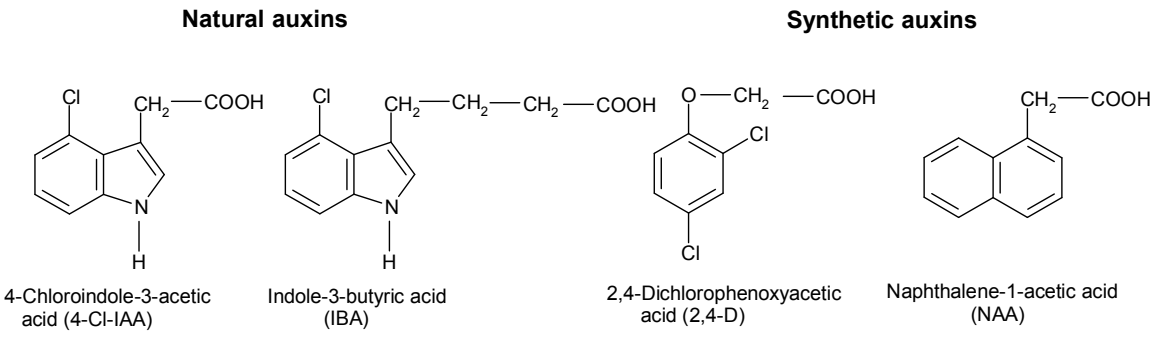


Fig. 2 Structure of two natural and two synthetic auxins

1.1.2 Auxin transport

Transport of auxin from the sites of synthesis is performed through a polar auxin transport (PAT), or for long distances nonpolarly in the phloem. PAT is the unique transport from cell to cell that is not affected by gravity and is characteristic only for auxin. In shoots, PAT is directed basipetally, being localized mainly in the vascular parenchyma tissue. In roots, auxin is transported acropetally toward the root tip and then basipetally through the epidermal and cortical tissues to the elongation zone (Rashotte et al. 2000). Transport from cell to cell requires an active form of auxin that is not conjugated – free IAA. Chemiosmotic hypothesis that explains mechanism of PAT was independently proposed by Rubery and Sheldrake (1974) and Raven (1975). According to the model, PAT proceeds through auxin influx and efflux carriers that are localized at the plasma membrane (PM) of the transporting cells (Fig. 3). Polarity of the transport is determined by the localization of auxin efflux carriers at the basal ends of the conducting cells. Protonated form of IAA (IAAH) can enter the cell passively by diffusion, but an involvement of uptake carriers in this process was also suggested (Rubery and Sheldrake 1974). Mutant studies helped to discover putative auxin efflux and influx carriers. The principal auxin influx carrier in Arabidopsis is AUX1 (AUXIN RESISTANT 1), which belongs to the gene family named *LAX* (*Like Aux1*) coding for a variety of additional possible auxin influx carriers (Bennett et al. 1996, Parry et al. 2001). Most of IAAH molecules dissociate in the neutral pH of the cytoplasm, but IAA^- anions cannot diffuse through the PM, hence an action of auxin efflux carriers is needed (Rubery and Sheldrake 1974). In Arabidopsis, two gene families coding for candidates of auxin efflux carriers are known – PINs (pinformed) and PGPs (P-glycoproteins) (Friml and Palme 2002, Geisler et al. 2005, Blakeslee et al. 2005).

Homologs of auxin influx and efflux carriers have been identified also in the maize genome. Localization of the expression of AUX1 homolog indicates that it may play a role similar to Arabidopsis AUX1, but experimental evidence is lacking (Hochholdinger et al. 2000, Brooks et al. 2009). The maize PIN homologue ZmPIN1 was confirmed to function as an auxin efflux carrier (Gallavotti et al. 2008b) and its gene expression was found to be strictly associated with differentiating vascular tissues (Carraro et al. 2006). Moreover, *ZmPIN1a* gene expression is up-regulated at the site of each axillary meristem or lateral organ primordium, thus during every branching

process in maize (Gallavotti et al. 2008b). PGPs, members of ATP-binding cassette (ABC) transporters are also involved in the auxin transport in maize (Multani et al. 2003).

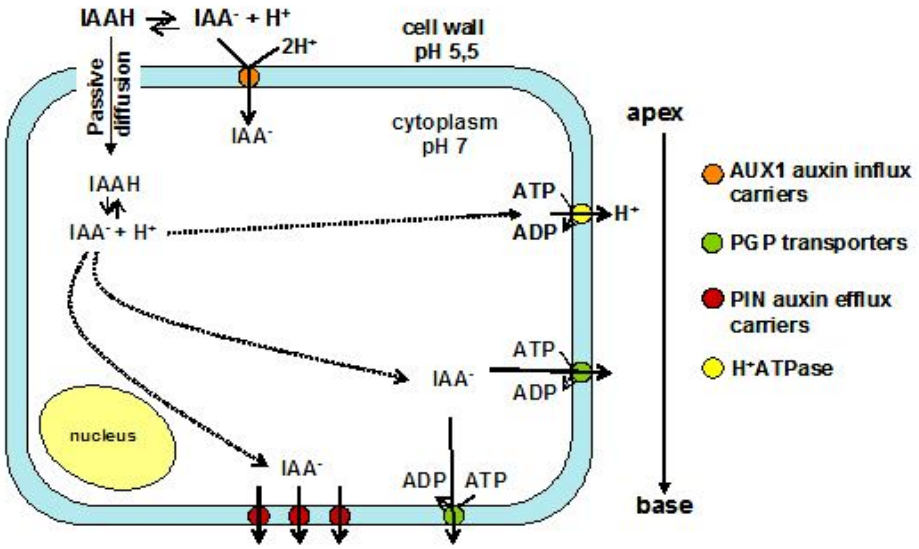


Fig. 3 Scheme of polar auxin transport
Activity of H^+-ATPase results in a decrease of pH at the outer side of PM. Hence the portion of IAA molecules that is present in protonated form (IAAH) may enter the cell passivelly, through the diffusion. However, deprotonated form has to pass over the auxin influx carrier AUX1. In the higher pH of cytoplasm, the molecules of auxin are deprotonated and cannot pass across the PM. Therefore, IAA^- is transported out of the cell through the auxin efflux carriers – PINs or PGP transporters.

Recently, a new member of PIN family in Arabidopsis – PIN5 was identified. It is not involved in polar auxin transport, but mediates intracellular transport of auxin. PIN5 is localized to endoplasmic reticulum (ER) and is believed to interpose auxin flow from the cytosol to the lumen of ER, therefore participating in the regulation of auxin homeostasis and metabolism (Mravec et al. 2009, Friml and Jones 2010, Zažímalová et al. 2010).

1.1.3 Auxin action

Auxin regulates the number of physiological processed in plant tissues. Dramatic effect of auxin on the elongation of cells represents a classical physiological auxin response. To explain the mechanism of auxin-induced elongation, the "Acid growth theory" was formulated by Hager and his colleagues in 1971. They proposed that auxin acting in cooperation with the PM-bound H^+-ATPases causes acidification of the cell wall that leads to the increased activity of the cell wall loosening enzymes and

thereby to the cell elongation. This theory was supported by the observation that auxin can directly stimulate activity of H^+-ATPases and also can induce *de novo* synthesis of the major H^+-ATPase isoform in maize coleoptiles (Rück et al. 1993, Hager et al. 1991, Frías et al. 1996). Additionally, auxin regulates expression of genes coding for the enzymes that participate in the reorganization of cellulose-xyloglucan framework that allows cell expansion in elongating hypocotyls and growing fruits of tomato plants (Catalá et al. 1997, Catalá et al. 2000). Efflux of protons into the cell wall through the IAA-activated and multiplied proton pumps requires compensation for the positive charges inside the cell. Supporting evidence has been shown that potassium uptake serves to balance the charge of secreted protons. Auxin-induced growth of maize coleoptile segments was shown to be dependent on the availability of potassium ions in the presence of Ca^{2+} and their uptake via K^+ inward channels (Claussen et al. 1997). The same dependence was shown also for the cell elongation induced by fusicoccin, a strong stimulator of PM H^+-ATPase (Tode and Lüthen 2001). Following works searched for a role of K^+ channels in the auxin-mediated elongation growth. Philippar et al. (1999) found that auxin induces expression of the maize gene *ZmK1* encoding K^+ channel, but does not influence the channel properties. Moreover, it was also demonstrated that expression of *ZmK1* followed lateral distribution of IAA after gravitropic and phototropic stimulation of maize coleoptiles (Philippar et al. 1999, Fuchs et al. 2003). Interestingly, auxin-stimulated coleoptile growth and protoplasts swelling showed virtually identical dependency on K^+ concentration (Christian et al. 2006a). Auxin-dependent increase of expression that was also detected for two Arabidopsis genes coding for K^+ inward channels *KAT1* and *KAT2* supports the importance of K^+ efflux during auxin-promoted growth (Philippar et al. 2004). However, anion channels seem to be also linked to auxin-dependent growth (Thomine et al. 1997).

1.1.4 Auxin signaling

Induction of auxin action requires its perception leading to activation of a signaling pathway that triggers realization of the specific response. One of the dominant functions of signaling pathways in general is the regulation of gene transcription through activations of transcriptional factors. Activated preexisting transcriptional factors trigger expression of targeted genes called primary response genes. An important function of primary response genes is to regulate expression of

secondary response genes that are required for long-term responses (McMahon and Monroe 1992). In the auxin signaling pathway, five classes of primary responsive genes have been identified: *AUX/lAA* family (*AUXIN/INDOLE-3-ACETIC ACID*), *SAUR* family (*SMALL AUXIN-UP RNA*), *GH3*-like family, *ACS* (encoding 1-aminocyclopropane-1-carboxylic acid synthase, a key enzyme in ethylene biosynthesis) and *GH2/4*-like family (encoding glutathione S-transferases) (Abel et al. 1996). Transcripts of all primary responsive genes accumulate rapidly after auxin exposure (Woodward and Bartel 2005). *AUX/lAA* gene family encodes a short lived proteins involved in the SCF^{TIR1} mediated auxin signaling pathway discussed below (Abel et al. 1994, Gray et al. 2001). *GH3* gene family appears to be involved in the regulation of IAA conjugation, because some members of this family encode IAA-amino acid conjugation enzymes (Staswick et al. 2005). The function of the *SAUR* family is still not revealed.

For initiation of a signaling pathway, the perception of particular growth factor is required. The perception of auxin is proposed to be carried by the three protein families that act as auxin receptors: TIR1/AFB (TRANSPORT INHIBITOR RESPONSE 1/ AUXIN SIGNALING F-BOX) family, ABP (AUXIN-BINDING PROTEIN) family, and SKP2A (S-PHASE KINASE-ASSOCIATED PROTEIN 2A).

1.1.4.1 TIR1 mediated signaling

TIR1 gene was identified during screening for mutant of Arabidopsis, which showed altered response to auxin transport inhibitors (Ruegger et al. 1997). The *tir1* mutant is defective in a variety of auxin-controlled processes during development, thus TIR1 is important for a normal auxin action. The TIR1 protein possesses series of leucine-rich repeats and F-box motive related to ubiquitin-mediated processes (Ruegger et al. 1998). The idea that ubiquitin-mediated protein degradation is involved in the auxin signaling came from different genetic studies identifying genes required for a normal auxin response. One candidate gene *AXR1* (*AUXIN RESISTANT 1*) encodes a protein related to the first enzyme in the ubiquitin conjugation pathway, ubiquitin-activating enzyme (E1) (Leyser et al. 1993). Ubiquitin-dependent selective breakdown of proteins uses a set of the three enzymes: E1, E2 and E3. The third one, E3, is the ubiquitin protein ligase, which recognizes target substrate and labels it with the ubiquitin molecules. When a short chain of ubiquitins is formed, the labeled protein is targeted to proteasome and degraded (Vierstra 2003). Auxin response was shown to be

dependent on the E3 complex called SCF^{TIR1} (Gray et al. 1999). Substrates for the SCF^{TIR1} complex are AUX/IAA proteins, which operate as transcriptional regulators (Gray et al. 2001). *AUX/IAA* genes are known to be rapidly expressed after the auxin treatment (Abel et al. 1995). This proteins consist of four domains (I, II, III, IV), from which domain II is necessary for a sufficient interaction with SCF^{TIR1} and domains III and IV mediate homo- and heterodimerisation between AUX/IAAs and heterodimerisation with other proteins such as ARFs (AUXIN RESPONSE FACTORs) (Gray et al. 2001, Kim et al. 1997, Ulmasov et al. 1997b).

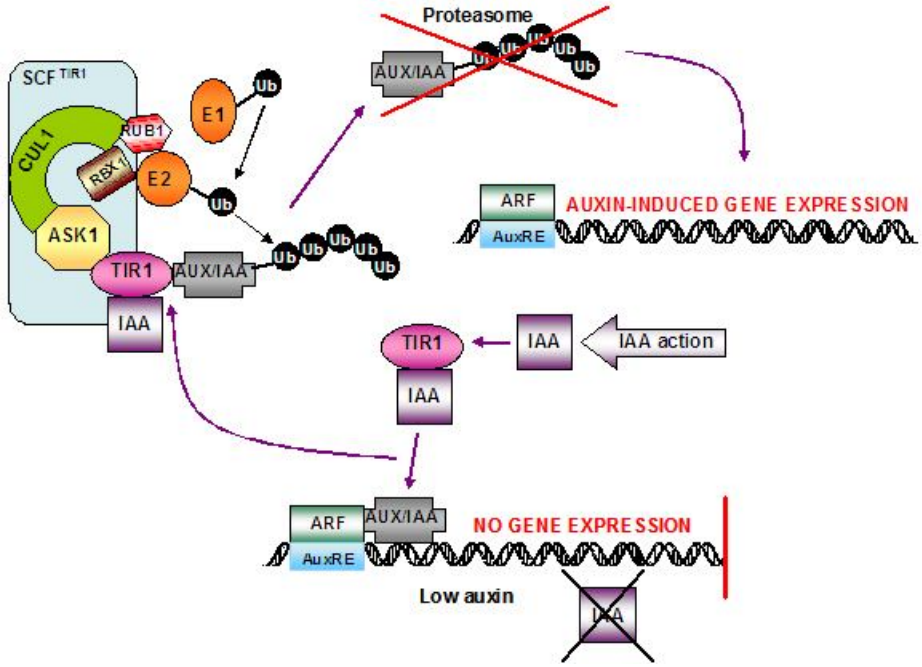


Fig. 4 Scheme of the TIR1-mediated auxin signaling
When auxin level is low, AUX/IAAs interact with ARFs, which are bound to the AuxREs of target genes and block their expression. When auxin level becomes high, IAA binds to the TIR1 and initiates protein degradation machinery. TIR1-IAA complex recognizes target proteins AUX/IAAs, which are immediately labeled by ubiquitin molecules in the SCF^{TIR} complex and consequently degraded in the proteasome. Thus, removed AUX/IAAs no longer block auxin-induced genes expression (scheme adopted from Paciorek and Friml 2006).

ARFs function as transcriptional regulators, which interact with auxin response elements (AuxREs) present in auxin responsive genes (Ulmasov et al. 1997a). AUX/IAA proteins act as active repressors of the auxin-induced gene expression due to heterodimerisation with positive regulators ARFs that are bound to AuxREs domains of affected genes (Ulmasov et al. 1997b, Tiwari et al. 2001). In 2005, TIR1 was identified as an auxin receptor, its role in SCF^{TIR1} complex being proposed to bind auxin and then promote AUX/IAA – SCF^{TIR1} interaction enabling expression of targeted genes (Fig. 4) (Kepinski et al. 2005, Dharmasiri et al. 2005a).

It is obvious that TIR1 is not the only auxin receptor that mediates the regulation of gene expression because mutation in the Arabidopsis *TIR1* gene is not lethal. Therefore, others proteins with the same function have to be involved in the Arabidopsis auxin signaling machinery. Arabidopsis genome contains almost 700 genes encoding F-box proteins, and three of them AFB 1, 2 and 3 were shown to interact with AUX/IAA proteins in auxin-dependent manner and collectively with TIR1 mediate auxin-regulated transcription throughout the development of seedlings (Gagne et al. 2002, Dharmasiri et al. 2005b). In maize, the homologue of *TIR1* was identified and its expression was observed in young leaf primordial, but its functional characterization is still lacking (Zhang et al. 2007).

TIR1-signaling pathway requires regulation of a gene expression and *de novo* protein synthesis. However, some auxin-induced responses are too rapid to be mediated by the TIR1. A very rapid auxin-induced response is for example the membrane hyperpolarization in protoplasts, which could be measured immediately after auxin application (Barbier-Brygoo et al. 1989, Barbier-Brygoo et al. 1991). Moreover, studies on the triple mutant *tir1-1/afb1-3/afb2-3* and quadruple mutant *tir1-1/afb1-3/afb2-3/afb3-4* showed that the rapid phase of cell elongation induced by auxin is not mediated through the TIR1 receptors family (Schenck et al. 2010). Therefore, the existence of another auxin receptor that mediates rapid auxin responses is obvious.

1.1.4.2 Auxin-binding protein – mediated signaling

1.1.4.2.1 ***Auxin-binding proteins***

The story of auxin-binding proteins (ABPs) began when the first auxin binding activity was detected in the crude membrane fraction from etiolated maize coleoptiles by Hertel et al. (1972). In 1985, ABP was purified for the first time by Löbler and Klämbt (1985) and subsequently detailed characterization of its binding activity and structure became the topic of several studies (reviewed in Jones 1994, Timpte 2001, Napier et al. 2002). Generally, this protein was designated as an auxin-binding protein 1 (ABP1), but others synonyms are also found in literature such as Zm-ERabp1 (*Zea mays* endoplasmic reticulum auxin-binding protein 1), 22 kDa ABP, or ABPzm1. The gene coding for ABP1 in maize belongs to a small multigene family consisting of at least five members. Hesse et al. (1989) purified three ABPs from maize coleoptiles membrane fractions, of which ABP2 and ABP3 together represented less than 5% of

the amount of ABP1. The gene encoding ABP1 was cloned and translation of its full cDNA showed that ABP1 is a luminal component of ER bearing targeting C-terminal tetrapeptide sequence -Lys-Asp-Glu-Leu (-KDEL) (Hesse et al. 1989, Lazarus et al. 1991, Schwob et al. 1993). KDEL motif, which represents ER luminal residence signal well known in animal cells (Koch 1987, Pelham 1989), was thus for the first time detected in plants. In 1993, Schwob et al. cloned, sequenced and compared three maize genes encoding ABP1, ABP4 and ABP5. In the predicted amino acid sequences of all three proteins, signal peptide sequence, C-terminal KDEL sequence, glycosylation and auxin binding sites are present. Sequences of ABP4 and ABP5 are very similar since there are only three amino acid substitutions, two of them occurring in the signal peptide. On the gene level, only six nucleotide differences are present in the protein coding region of *ABP4* and *ABP5* genes, three of them being silent. Moreover, introns of the two genes are also highly conserved (Schwob et al. 1993). For ABP2, only a partial sequence of 29 amino acids from N-terminus of a mature protein is known (Hesse et al. 1989) and current knowledge about ABP3 is not very clear as well. In the genome of Arabidopsis, the only gene coding for an auxin-binding protein (*ABP1*) is present and its homozygous mutation results in embryo lethality (Chen et al. 2001). On the contrary, mutation in one or two genes in maize does not hamper normal development of seedlings. Up to now, three maize *abp* mutants were isolated by means of the Robertson´s *Mutator* transposable element system (Im et al. 2000). In contrast to Fellner et al. (2006) (see page 30) the authors did not detect obvious phenotypic aberration in the single mutants *abp1* and *abp4*, and double mutant *abp1abp4*, suggesting the functional redundancy in this gene family (Im et al. 2000). Because ABP1 was found to be ubiquitous in vascular plants, it became the most extensively studied auxin-binding protein (Napier et al. 2002).

1.1.4.2.2 ***Localization of ABP1 in maize organs and tissues***

In maize, ABP1 was detected in coleoptiles, mesocotyls, roots, leaves, tassels, and ears (reviewed in Jones 1994). It was observed that its occurrence correlates with growing regions of etiolated seedlings. This means that the highest levels of ABP1 can be found in the apical region of mesocotyls, basal region of coleoptiles and young leaves (Jones 1994). Abundance of ABP1 is also a tissue specific, e.g. its higher content was detected in epidermal tissues than in other residual tissues of coleoptile (Löbler and Klämbt 1985, Shimomura et al. 1988). According to the “epidermal-

growth-control" theory of the stem elongation (Kutschera and Niklas 2007), elevated accumulation of ABP1 in epidermis is rational and supports its proposed function during the elongation growth. Interestingly, it was reported that red light (RL) decreases level of free auxin and abundance of ABP1 in epidermis of maize shoots. However, an early kinetics of the ABP1 decrease did not correlate with an early kinetics of RL-regulated growth (Jones et al. 1991). Thereby, the function of ABP1 in epidermis during auxin-driven elongation growth was not confirmed.

1.1.4.2.3 ***ABP1 function***

ABP1 is a soluble 22 kDa glycoprotein containing a high-mannose-type oligosaccharide (Hesse et al. 1989). Purified ABP1 bounds 1-NAA with affinity between 50 and 200 nM, whereas its affinity to 1-IAA is 100 times lower (Hertel 1995, Badescu and Napier 2006). ABP1 binds 1-NAA at the optimal pH 5.0 – 5.5, thus it is proposed to act as an auxin receptor at the cell surface, in the appropriate pH conditions (Napier 1995). Data from several works suggest that ABP1 can mediate auxin-induced electrical responses of PM (Barbier-Brygoo et al. 1989, Barbier-Brygoo et al. 1991, Rück et al. 1993). Moreover, auxin may activate PM H^+-ATPases through the ABP1 located at the outer side of PM (Rück et al. 1993). ABP1 was shown to be engaged in auxin-induced modulation of K^+ channels activity in *Vicia faba* L. guard cells (Thiel et al. 1993), anion channel activity (Zimmermann et al. 1994) and stomatal opening coupled with altered cytoplasmic pH in *Phaphiopedilum tonsum* L. (Gehring et al. 1998). Purified ABP1 is able to bind to maize coleoptile plasma membrane vesicles *in vitro* and specifically binds 1-NAA (Schiebl et al. 1997). Therefore, the existence of some docking protein located at the PM is imperative. One candidate was proposed by Shimomura (2006), who used photoaffinity crosslinking assay to identify an extracellular glycosylphosphatidylinositol (GPI)-anchored plasma membrane protein from *Zea mays* named CBP1 (C-terminal peptide-binding protein 1). CPB1 is homologous to the *Arabidopsis* GPI-anchored glycoprotein SKU5 (Shimomura 2006), which is involved in the cell elongation processes (Sedbrook et al. 2002). But how the protein, whose predominant localization and the main pool are within the ER, can be active only at the outer side of PM? Few reports brought the direct evidence about the localization of small fraction of ABP1 at the PM surface (Jones and Herman 1993, Diekmann et al. 1995), but few attempts operating with different techniques of visualization could not support these findings (Napier et al. 1992, Henderson et al.

1997). Searching for a possible escape of ABP1 from ER and its secretion to PM and the cell wall brought again two more or less contradictory results. Jones and Herman (1993), using immunochemical assay, found that ABP1 is secreted in maize cell cultures via the secretory system sensitive to brefeldin A (drug that specifically blocks secretion) and this secretion does not require loss of KDEL signal. On the other hand, Henderson et al. (1997), who used immunofluorescence assays, could not detect secretion of ABP1 in maize suspension cultures, but they could quantify the percentage of secreted ABP1 in baculovirus-infected insect cells. They found that less than 15% of ABP1 ever escapes from ER and less than 2% overcome secretory pathway. Up to now, only indirect evidences for auxin receptor function of ABP1 are available.

ABP1 was shown to mediate auxin responses associated with the elongation. Antibodies directed against Box A (a suggested auxin-binding domain, Fig. 5) and synthetic ABP1-C-terminal oligopeptides (Fig. 5) are able to evoke a protoplast swelling as well as auxin, while antibodies against the C-terminus of ABP1 have the opposite effect (Steffens et al. 2001). Overexpression of *ABP1* in tobacco cells causes altered sensitivity to auxin (Bauly et al. 2000, Chen et al. 2006), leads to the increase of free auxin accumulation and results in the greater expansion of cells containing nuclei that underwent endoreduplication (Jones et al. 1998, Chen et al. 2006).

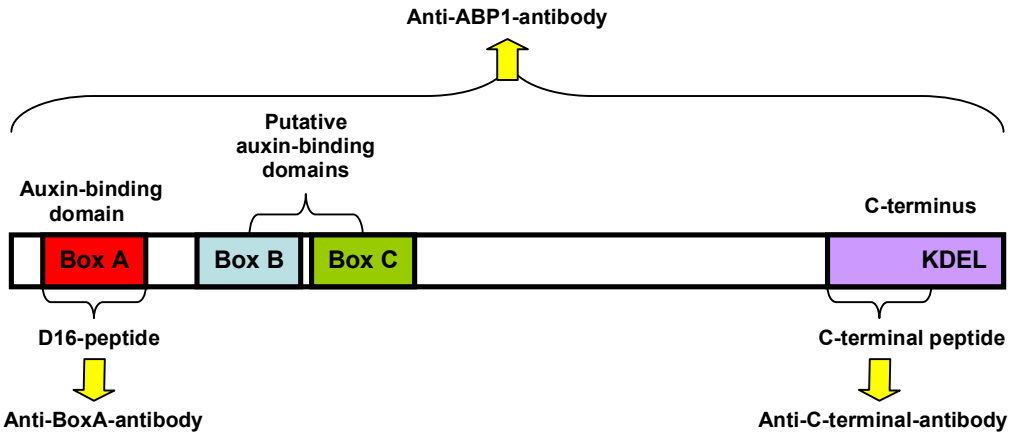


Fig. 5 Schematic structure of ABP1 with source of peptides and antibodies used in auxin-signaling studies

The boxes A, B and C represent three conserved domains which are believed to be involved in the auxin binding. The function of C-terminus is to transmit signal to the trans-PM protein. Synthetic C-terminal peptides and Anti-BoxA-antibodies have auxin agonist activities, and Anti-ABP1-antibodies inhibit auxin action in protoplasts. The scheme was drawn with reference to Christian et al. (2006b).

On the other hand, cells with no detectable ABP1 show higher rate of auxin metabolism what may indicate its involvement in the mediating of auxin availability (Chen et al. 2006).

The role of ABP1 during embryogenesis in Arabidopsis is essential for the organized cell elongation and division (Chen et al. 2001). Conditional repression of ABP1 using an inducible cellular immunization approach and inducible antisense construct enabled to make progress in the searching for a role of ABP1 during postembryonic development in Arabidopsis. Several studies revealed that ABP1 is required also for the normal postembryonic development. Specifically, ABP1 was shown to be essential for the leaf morphogenesis, early leaf initiation (Braun et al. 2008) and root growth (Tromas et al. 2009). Braun et al. (2008) proposed a model for the ABP1 action during leaf initiation and development, where ABP1 controls the cell division and cell expansion depending on the local levels of auxin (Fig. 6). Two works mentioned above (Braun et al. 2008, Tromas et al. 2009) brought another very interesting observation that ABP1-regulated expression of *Aux/IAA* genes is involved in the TIR1-mediated auxin signal transduction pathway. Very recently, characterization of the Arabidopsis heterozygous *abp1/ABP1* insertion mutant revealed that ABP1 is important for PAT and the modulation of transcription of early auxin-regulated genes (*Aux/IAA*s, *SAURs*, *GH3* and *ABP1*) (Effendi et al. 2011). Supporting evidence for the ABP1 function in the regulation of PAT comes from the work of Robert et al. (2010), who found that ABP1 mediates the auxin-induced inhibition of endocytosis, which controls PIN transporters localization, thus PAT. Moreover, ABP1 was found to be required for the spatial coordination of cell expansion in Arabidopsis, which also involves PIN auxin efflux carriers (Xu et al. 2010). The mechanism of ABP1-signaling pathway is still far away from being fully understood, however the model of the ABP1 and TIR1 signaling pathways was recently proposed (Scherer 2011, Scherer et al. 2012). This model proposes ABP1 to regulate TIR1-signalling pathway by controlling of the auxin accumulation in the cytosol through the PIN transporters.

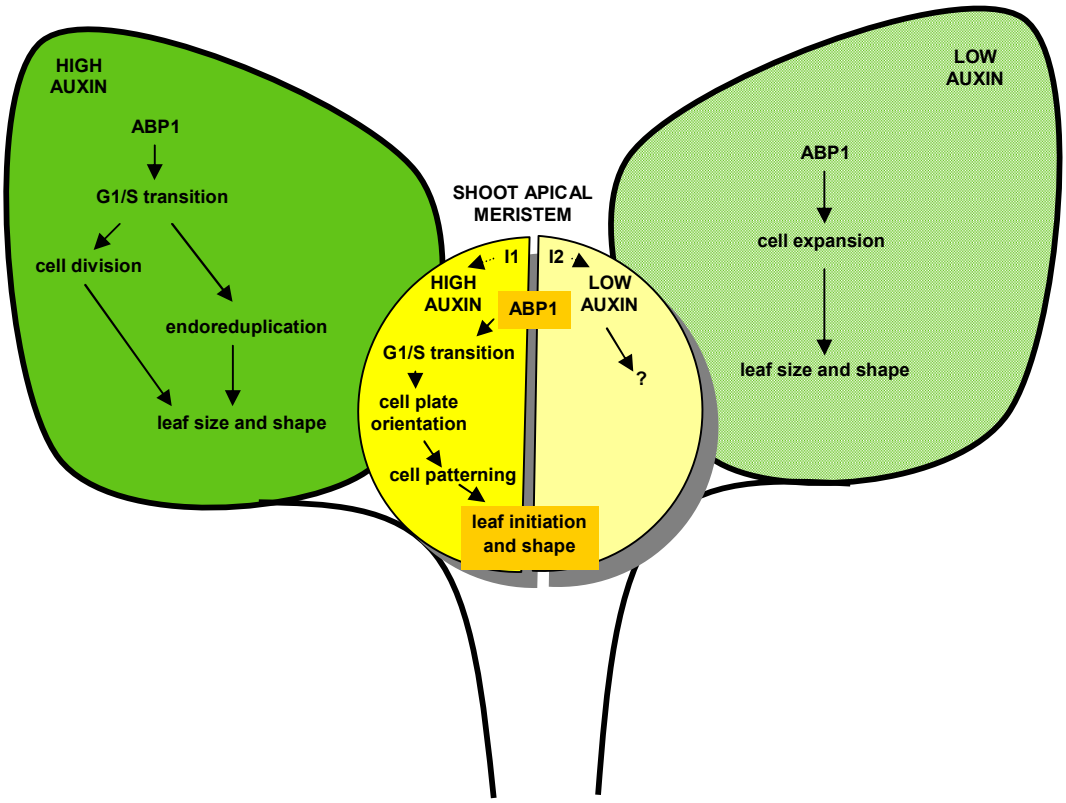


Fig. 6 Model of context-dependent role of ABP1 in shoot

ABP1 can mediate differential cellular responses depending on the local auxin concentration and developmental stage of target tissue. ABP1 is involved in processes determining the leaf size and shape through the controlling of cell cycle (David et al. 2007, Braun et al. 2008) in high auxin level conditions and through the controlling of cell expansion in low auxin level conditions (Braun et al. 2008). In the shoot apical meristem, ABP1 plays role during the cell plate formation in the I1 position (where a high level of auxin is expected), thus being important for the leaf initiation and shape (Braun et al. 2008). The scheme was re-drawn based on the reference to Braun et al. (2008).

1.1.4.3 SKP2A - mediated signaling

Very recently, a novel auxin receptor SKP2A was identified. SKP2A was shown to bind auxin and consequently regulate the cell proliferation (Jurado et al. 2010). SKP2A is a F-box protein involved in the ubiquitin-dependent degradation of proteins in a proteasome (described above), that promotes degradation of at least two transcription factors - DPB and E2FC involved in cell division (del Pozo et al. 2006). The binding of auxin to SKP2A was shown to be required for the DPB and E2FC degradation (Jurado et al. 2010).

The function of auxin in the regulation of growth and development of plants is very important. However, development of a plant organism is a complex process regulated by much more factors – endogenous as well as exogenous. Plant hormones

represent endogenous regulators such as auxin and the stimuli coming from the environment represent exogenous regulators. Developmental pattern of plants depends greatly on the environmental factors, from which light is the most significant one.

1.2 LIGHT

Seedlings exhibit different phenotypes when growing under the light (photomorphogenesis) and in the dark (skotomorphogenesis). Skotomorphogenesis is a developmental program that determines the plant growth and development in response to growth in darkness. The shoot of an etiolated seedling grows rapidly at the expense of cotyledon and roots development. Chloroplasts are undifferentiated, leaves unexpanded and root system weakly developed, but exaggerated elongation of the shoot affords opportunity to seek the light. When the seedling once reaches the light, all incoming events are under the control of a light-dependent developmental program – photomorphogenesis. This switch-over from skotomorphogenic to photomorphogenic program is called de-etiolation. During this period, the inhibition of shoot elongation, chloroplast maturation and cotyledon expansion is initiated. A seedling is adapting for the new environment and becomes an autotrophic organism. All light induced processes in the plant organism are mediated by specific receptors, which are able to perceive the light and transfer the signal into series of biochemical reactions that eventuate in specific physiological responses. The most active regions of light spectrum, which largely influence the plant growth and development, are the red/far-red (R/FR) light with absorption maximum at ~660 nm/ ~730 nm and the blue light (BL) with absorption maxima at ~370 nm, ~450 and 480 nm.

1.2.1 Red light-absorbing photoreceptors

The R/FR photoreceptors called phytochromes form the most studied light receptors family. Phytochromes mediate light signals that are crucial for the control of seedling germination, development, reproduction, dormancy, shade avoidance, and nastic (sleep) movements (Mathews 2006).

Phytochrome consists of a protein bearing a covalently attached specific chromophore. The chromophore phytochromobilin is a linear tetrapyrrole that changes its configuration upon absorption of the R and FR light (~660 nm/ ~730 nm). The change in the chromophore configuration causes changes of protein structure and biological activity. A phytochrome exists in two forms: red light (RL)-absorbing

phytochrome – Pr (P_{660}) and FR-absorbing phytochrome – Pfr (P_{730}). Absorption of RL converts Pr to Pfr form while the absorption of FR reverse the Pfr form to Pr. Pfr is an active form of phytochrome in the most of photomorphogenic responses (Batschauer 1999). A functional phytochrome occurs in plants as a dimer. Phytochromes are synthesized in the dark in Pr form. Each phytochrome response has a specific range of required light fluence, by which they can be divided into the three categories: very low-fluence responses (VLFRs), low-fluence responses (LFRs), and high irradiance responses (HIRs). VLFRs are R/FR irreversible and include for example the germination of Arabidopsis seeds (Botto et al. 1996, Shinomura et al. 1996). A typical character of LFRs is their R/FR reversibility. The first evidence of the R/FR reversibility was provided in 1952 by Borthwick et al., who demonstrated that lettuce seeds germinate when treated by RL and this response is lost by the FR treatment. The third class of phytochrome responses – HIRs is R/FR irreversible. Typical HIR response is synthesis of anthocyanin (Rabino et al. 1977) and the inhibition of stem or hypocotyl growth (Shinomura et al. 2000).

In Arabidopsis, phytochromes are encoded by a small multigene family, which contains five genes (*PHYA*, *PHYB*, *PHYC*, *PHYD* and *PHYE*). On the contrary, the phytochrome gene family in all monocots studied to date contains only three genes – *PHYA, PHYB/D* and *PHYC* (Mathews and Sharrock 1996, 1997).

1.2.2 Blue light-absorbing photoreceptors

BL is also a very important signal that regulates seedling development. The sensing of BL in higher plants is provided by several classes of photoreceptors: cryptochromes, phototropins, members of Zeitlupe (ZTL) family and zeaxanthin.

Cryptochromes play an important role during the photomorphogenesis. Notably, their function is essential for the regulation of the circadian clock and for the control of flowering and de-etiolation (Lin and Shalitin 2003). In the Arabidopsis genome, three genes encoding CRYPTOCHROME 1 (CRY1), CRY2 and CRY3 were identified. CRY1 (coding by *HY4* gene) is responsible for the BL-stimulated inhibition of the hypocotyl elongation (Liscum and Hangarter 1991). CRY2 mediates the BL-dependent inhibition of elongation of hypocotyl, and stimulates cotyledons opening and expansion under the low light intensities. Since expression of *CRY2* is rapidly decreasing in a light-dependent manner, CRY2 functions primarily under the low light conditions during the early development of seedlings (Lin et al. 1998). The third cryptochrome in

Arabidopsis – CRY3 (cry DASH) is closely related to the *Synechocystis* cryptochrome and its function is still not known (Kleine et al. 2003, Brudler et al. 2003). However, its structural characteristics show localization within the cell, which is distinct from the other plant cryptochromes. While CRY1 was shown to be localized in the nucleus and cytoplasm and CRY2 only in the nucleus, CRY3 is targeted into chloroplasts and mitochondria. Moreover, CRY3 lacks C-terminal domain that is involved in the BL-signaling in CRY1 and CRY2 (Yang et al. 2000, Kleine et al. 2003).

Phototropins are involved in the photomorphogenic response called phototropism and in the BL-induced chloroplasts and stomata movements (Sakai et al. 2001, Inoue et al. 2010). Phototropism is classically determined by an asymmetric growth and bending toward the light. In Arabidopsis, two phototropins were identified so far. *PHOTOTROPIN 1* (*PHOT1*) gene, originally named *NPH1* (*NON-PHOTOTROPIC HYPOCOTYL 1*), was for the first time identified in the Arabidopsis mutant that failed in phototropism (Huala et al. 1997). The second, *PHOT2* gene, was identified by genome sequence analysis as homolog of *PHOT1*, initially named *NPH1-like 1* (Briggs and Christie 2002). The BL-stimulated movements of chloroplasts serve as adaptation to light quantity. When the light intensity is very weak, chloroplasts accumulate at the upper and lower surfaces of cells and maximize light absorption. This accumulation response is mediated by both PHOT1 and PHOT2. However, only PHOT2 is involved in avoidance response of chloroplasts under the strong light when chloroplasts move away from the light, what helps to reduce photodamages (Kagawa et al. 2001, Kasahara et al. 2002, Kagawa and Wada 2002). BL also regulates the opening of stomata, what is very important for the photosynthesis and water use efficiency and consequently for the yield (Assmann and Wang, 2001). This BL-induced opening of stomata is known to be mediated through the phototropins. Activated phototropin initiates a signaling cascade that leads to the activation of H^+ATPases and pumping H^+ out of the cell, what results in increased inside-negative electrical potential, opening the K^+ channels and consequently to stomata opening (Inoue et al. 2010, Chen et al. 2012). Phototropins as well as ZTL family proteins use light oxygen voltage (LOV) photosensory domains to perceive BL. Arabidopsis ZTL family comprise LOV Kelch Protein 2 (LKP2) and flavin-binding Kelch F-box1 (FKF1). ZTL proteins are known to play role in the regulation of the circadian clock and a photoperiodic control of the flowering in Arabidopsis (Imaizumi et al. 2003, Demarsy and Fankhauser 2009). Zeaxanthin is BL

photoreceptor in guard cells that mediates BL-stimulated stomata opening (Zeiger and Zhu 1998).

1.3 INTERACTION OF THE LIGHT AND AUXIN SIGNALING PATHWAYS

The first comprehensive view on the interaction of light and auxin signaling pathways was provided by "the Cholodny-Went theory of tropism" in 1937 (Went and Thiman 1937). This theory proposes that tropic stimulus, i.e. light in our point of view, induces differential lateral auxin transport that leads to the unequal distribution of auxin, and hence different growth on the two sides of a curving organ.

1.3.1 Phototropism

Phototropic response of coleoptile was beheld much earlier than the Cholodny-Went theory was formulated. At the end of 19th century, Charles and Francis Darwins observed that coleoptile of canary grass illuminated on one side grows toward the source of light and that the tip of coleoptile perceives the stimulus, whereas the response - bending occurs in the distinct part, more basally localized portion of coleoptile. Auxin produced at the tip of coleoptile is transported laterally toward the shaded side after the perception of unilateral light stimulus and then transported basipetally to the elongation zone, where it stimulates cell elongation (Briggs 1963, Baskin et al. 1986). This theory was supported also by the molecular genetic studies. Blakeslee et al. (2004) demonstrated that the localization of PIN1, an auxin efflux facilitator, at the basal part of cortical cells is disrupted after phototropic stimulation and this response was not observed in *phot1* mutant. One supporting molecular evidence comes also from monocots, in particular from rice. CPT1 (COLEOPTILE PHOTOTROPISM 1) that is orthologous to Arabidopsis NPH3, a component of the phototropin mediated BL signaling pathway, was shown to be crucial for the unilateral BL-stimulated asymmetric distribution of auxin (Haga et al. 2005).

1.3.2 Photomorphogenesis

Photomorphogenesis, the light driven developmental program, is also closely related to the auxin action. Light has been shown to affect auxin biosynthesis, homeostasis, transport and signaling/response.

Following the observations which showed that RL reduces the amount of diffusible auxin obtained from coleoptile tips, it has been proposed that RL-induced inhibition of mesocotyl elongation is at least partially a result of the reduced supplies of auxin from the coleoptile (van Overbeek 1936, Huisinga 1976, Iino 1982a). RL was found to inhibit biosynthesis of IAA from Trp in the maize coleoptile tip (Iino 1982b, Koshiba et al. 1995, Nishimura et al. 2006). However, Iino (1982a) also observed that RL can inhibit mesocotyl growth in seedlings from which the coleoptile was removed. This inhibition (cca 25%) is lower than RL-induced inhibition of mesocotyl in intact seedling (70-80%). It does not involve changes in the IAA content and it is not relieved by exogenous auxin. Using Arabidopsis mutants defective in light receptors genes, it was shown that *PHYA*, *PHYB*, *CRY1* and *CRY2* mediate RL and BL-induced reduction of IAA accumulation in aerial parts of seedlings (Nagashima et al. 2008).

Light affects also auxin homeostasis through the regulation of its conjugation. Cytochrome P450 monooxygenase CYP83B1, the enzyme that plays a role in formation of indole-glucosinolates, thus inactivation of IAA, was shown to be positively regulated by *PHYB* (Hoecker et al. 2004). Expression of some members of *GH3* family that encodes enzymes catalyzing IAA conjugation to amino acids is also RL-regulated process (Tepperman et al. 2001, Tanaka et al. 2002).

Massive reduction of mesocotyl growth after irradiation by RL is connected with auxin transport. Jones et al. (1991) found that capacity but not velocity of basipetal auxin transport in maize shoots is decreased after low fluence R irradiation. They performed in situ localization of transported auxin analog, tritiated 5-azidoindole-3-acetic acid ([3H],5-N3IAA), that revealed that the epidermis of irradiated organs (coleoptile and mesocotyl) contains less IAA than the dark control, while cortical tissues do not display such great differences. RL-targeted decrease of IAA in epidermis harmonizes with the function of this tissue – controlling the growth rate (Kutschera and Niklas 2007). Light-regulated auxin transport has also been described for dicotyledonous plants. It was shown that the inhibitor of polar auxin transport 1-naphthylphthalamic acid (NPA) has no effect on elongation of hypocotyl in etiolated Arabidopsis seedlings but strongly reduces the elongation of hypocotyls grown in light. Moreover, mutants in various photoreceptors are less sensitive to the NPA action (Jensen et al. 1998). Recently, it was found that light increases PAT in the hypocotyls of dark-grown Arabidopsis and tomato seedlings, and phytochrome seems to be involved in this response (Liu et al. 2011). It was also demonstrated that *PHYB* is

involved in controlling of auxin transport from shoot to the root and by this way controls the growth of lateral roots (Salisbury et al. 2007). Few works reported the evidences about light and auxin efflux carrier cooperation during de-etiolation. Activated phytochromes reduce the accumulation of *PIN3* and *PIN7* mRNA (Salisbury et al. 2007). PHYA, PHYB, CRY1 and CRY2 downregulate the expression of *PGP19* and accumulation of the respective protein within the upper portion of hypocotyl (Nagashima et al. 2008). Light is affecting the localization of PIN2 transporter by targeting it to PM, while in the dark PIN2 displays vacuolar targeting (Laxmi et al. 2008). Further evidence comes from works focusing on the Arabidopsis calossin-like protein named BIG (*tir3/doc1/asa1/umb3* gene). BIG was shown to be required for a normal PAT and to affect photomorphogenic responses (Gil et al. 2001, Kanyuka et al. 2003). Therefore, light regulates the transport of auxin through the modulation of function and expression of auxin transport carriers.

Auxin signaling pathways may be regulated by light as well. Normal turnover of AUX/IAAs, the components of TIR1-mediated signaling pathway, seems to be important for photomorphogenic development because many *AUX/IAA* mutants reveal de-etiolation in dark (Nemhauser and Chory 2000). It was proven that *SHORT HYPOCOTYL* (*SHY2)/IAA3* gene is a negative regulator of auxin signaling and its expression is regulated by light (Tian et al. 2002). Moreover, recombinant oat PHYA can phosphorylate recombinant AUX/IAA proteins from Arabidopsis (SHY2/IAA3, AXR3/IAA17, IAA1, IAA9) and pea (Ps-IAA4) *in vitro* (Colón-Carmona et al. 2000). Additional evidence comes from microarray analysis and semiquantitative RT-PCR in *hy5* (*long hypocotyl 5*) mutant. HY5 is transcriptional factor promoting photomorphogenesis (Osterlund et al. 2000). Genes coding for two auxin signaling regulators *AUXIN RESISTANT 2* (*AXR2*)*/IAA7* and *SOLITARY ROOT* (*SLR*)*/IAA4* contain in their promoter a putative HY5 binding site, what indicates that light may regulate expression of these genes. Moreover, expression of these two genes in the *hy5* mutant is reduced (Cluis et al. 2004). However, the strongest connection between light and auxin signaling is found in plants exposed to low R:FR ratio; this light conditions result in a specific growth behavior called shade avoidance syndrome (SAS).

1.3.3 Shade avoidance

The ability to sense shading by neighboring plants is important property of a plant organism that is provided by phytochromes. In such conditions, plants exhibit

shade avoidance responses, i.e. enhanced elongation growth of stem, reduced expansion of leaves, reduced branching and accelerated flowering (reviewed in Smith and Whitelam 1997). Seedlings sense the decreased ratio of red to far-red wavelength (low R:FR) and adapt their growth to escape from the shade and reach optimal light conditions. Decreasing R:FR ratio originates from an increasing portion of FR that converts more Pfr to Pr, thus Pfr/Pr_{total} ratio decreases too, and it correlates with the strength of a response. It was shown that PHYB plays a major role in the mediation of SAS (Somers et al. 1991, López-Juez et al. 1992, Devlin et al. 1992).

Number of evidences support auxin function in SAS. The most visible shade avoidance response is an enhanced elongation and auxin is apparently involved in this process. Kurepin et al. (2007) reported that reducing of R:FR ratio leads to changes in IAA content in youngest internodes of sunflower seedlings. Identification and characterization of the Arabidopsis *sav3* (*shade avoidance response 3*) mutant revealed that the IPA biosynthetic pathway (Fig. 1) is required for SAS in plants. *SAV3* gene encodes aminotrasferase that catalyzes the formation of IPA from Trp, and the *sav3* mutant fail to induce SAS except the early flowering response (Tao et al. 2008). Upon exposure to the low R:FR and low BL, auxin action (demonstrated by DR5 reporter gene) is increased and NPA-inhibited auxin transport blocks elongation growth induced by these light cues (Pierik et al. 2009). DNA microarrays that were used to analyze global changes in the gene expression induced by the low R:FR ratio identified a large number of auxin-related genes (Devlin et al. 2003). Interaction between shade avoidance and auxin responses is proposed to occur through the PAR1 (PHYTOCHROME RAPIDLY REGULATED) and PAR2 proteins that are negative regulators of SAS and act as direct transcriptional repressors of two auxin-responsive genes, *SAUR15* and *SAUR69* (Roig-Villanova et al. 2007). The intense research in this field is devoted to the HD-Zip class-II subfamily of transcriptional factors. Several genes of this family, e.g. *ATHB4* and *HAT2*, are implicated in low R:FR induced events connected with auxin. ATHB4 was shown to repress *SAUR15* and *SAUR68* expression and its enhanced activity causes the reduction of auxin sensitivity, affecting the responses to the low R/FR ratio (Sorin et al. 2009). Another member of that family – HAT2 was found to be upregulated by the low R:FR as a consequence of the auxin signaling pathway triggered in such light conditions (Sawa et al. 2002, Ciarbelli et al. 2008). Changes induced by the low R:FR include the inhibition of leaf growth, where auxin was shown to be involved. Auxin induces cytokinin degradation in leaf primordia

through the activation of AtCKX6, a cytokinin oxidase associated with cytokinin degradation, thus inhibiting the leaf growth (Werner et al. 2003, Carabelli et al. 2007). Data demonstrating that *AtCKX6* gene is rapidly induced by the low R:FR and down-regulated by NPA strongly supports an existence of this regulatory mechanism (Werner et al. 2006, Carabelli et al. 2007). Recently, a linker between the phytochrome action and changes in the plant architecture in low R:FR conditions was found. PIF7 (phytochrome interacting factor 7) was shown to be a mediator between a PHYB and auxin biosynthetic genes, which mediates the low R:FR-induced increase in auxin production and consequently changes in plant growth (Li et al., 2012).

Agricultural practices in the crop cultivation strive for a high-dense planting, however in such a condition plants shade each other, what leads to the initiation of shade-avoidance responses and reduction of overall yield. It has been proposed that attenuation of certain shade avoidance responses, but maintenance of other phytochrome-mediated responses was probably affected during the artificial selection of crops, e.g. *Zea mays* (Markelz et al. 2003).

1.4 *ZEA MAYS* SUBSP. *MAYS* L.

Zea is a rather small genus within the grasses family comprised of five species exclusively found in the Central America. The most important species from the whole genus is *Zea mays* L. The infraspecific classification recognised four subspecies within *Zea mays*: *Zea mays* L. subsp. *huehuetenangensis* (H. H. Iltis & Doebley) Doebley, subsp. *mexicana* (Schrad.) H. H, Iltis subsp. *parviglumis* (H. H. Iltis & Doebley) and the most important – subsp. *mays* (Matsuoka 2002). Recent studies brought biological evidences that maize was domesticated more than 8,700 years ago from its wild progenitor Balsas teosinte (*Zea mays* subsp. *parviglumis*), which is native to Mexico´s Central Balsas River Valley. Thus teosinte is the nearest relative of all cultural maize plants grown today (Matsuoka 2005, Ranere et al. 2009). As stated, *Zea mays* subsp. *mays* belongs to the family *Poaceae* Barnhart and share the basic morphology structure with this grass family. It is a tall, monoecious annual grass-like plant with conspicuous nodes and internodes on the stem, producing large, narrow alternate leaves. *Zea* gives only unisexual flowers. Male flowers form long terminal staminate spikelets spreading into terminal panicles (tassels). Female pistillate flowers seat on thickened woody axis (cob) in the leaf axils. The whole female inflorescence is enclosed and protected by several foliaceous bracts. From the tip of this structure (ear) only the long styles (silks)

are protruding. Maize is wind pollinated and both self and cross pollination may occur (Vožda et al. 1962). Maize can form lateral branches from the lower nodes, which may develop into the tillers, but developed tillers occur rarely. However, forming the tillers is a characteristic of maize progenitor teosinte, as an adaptation to certain environmental conditions. Using the QTL (Quantitative Trait Loci) analysis, the gene locus responsible for this difference in the plant architecture (Fig. 7) – *teosinte branched* (*tb1*) was identified. It is proposed that during the evolution of maize, a change in this locus resulted in the short branches under all environments (Doebley et al. 1995, Doebley et al. 1997).

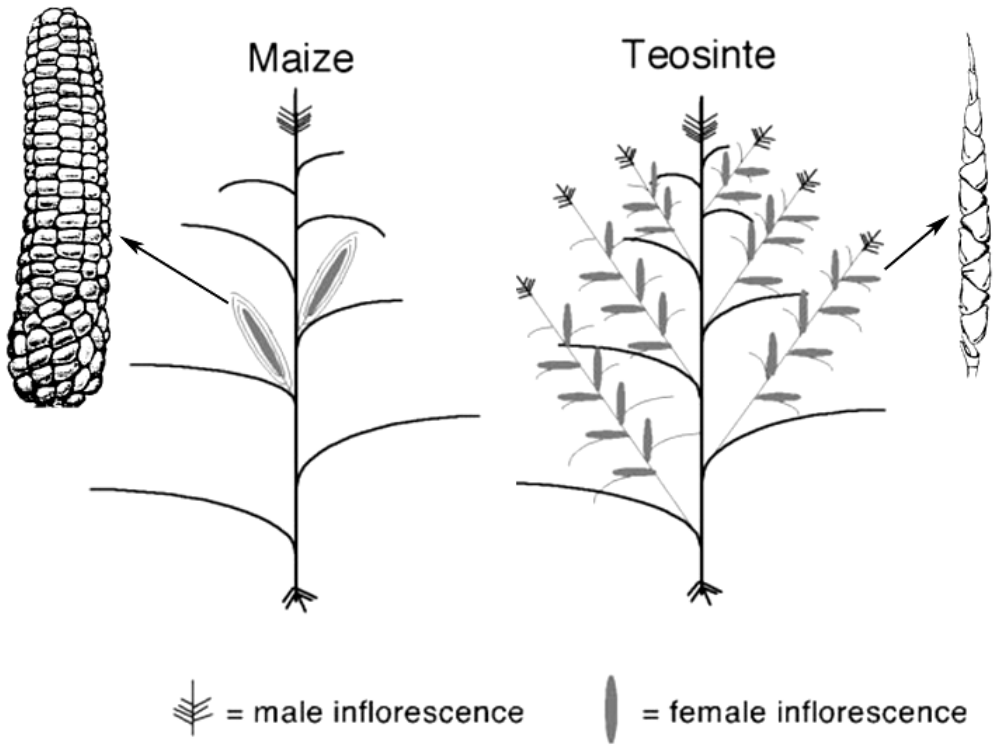


Fig. 7 Diagram of maize and teosinte habitus and their ears at maturity
(adapted from http://ucsdnews.ucsd.edu/newsrel/science/mcmaize.asp)

1.4.1 Development of the maize seedling

Growth stages of maize are divided into two broad categories: vegetative and reproductive. Maize kernel is a one-seed fruit consisting of pericarp, endosperm and embryo (germ). A major part of the kernel is created by endosperm that provides energy for a growing seedling. The germ contains scutellum and embryo axis. The scutellum mediates absorption and digestion of starch from the endosperm. Embryo consists of the shoot and root primordial. Shoot primordial contains 5 embryonic

leaves, the stem apical meristem and coleoptile, which covers the growing shoot. The root primordial holds the sheath called the coleorhiza that protects the first root (radicle) and the lateral root initials.

The first visible sign of the germination is an emerging radicle. When the coleoptile is visible, the shoot elongates and the germination is completed. Maize germination is hypogeal, thus a seed stays in the soil. The first internode of a young seedling is called mesocotyl (Fig. 8). An elongating mesocotyl pushes a coleoptile up to the soil surface. When a coleoptile reaches the light, the elongation of both mesocotyl and coleoptile decreases and stops soon. Light stimulates expansion of leaves that break through the coleoptile tissue. A young seedling generates embryonic seminal roots that help to anchor the plant in the soil and provide nutrients and water (Fig. 8) (Vožda et al. 1962). Maize root system is composed of primary root, embryonic adventitious roots – called seminal, and post-embryonic adventitious roots – shoot derived crown roots, which arise from the stem tissue underground and prop roots, which arise from the stem tissue above ground. Lateral roots may be initiated on all types of roots (McSteen 2010).

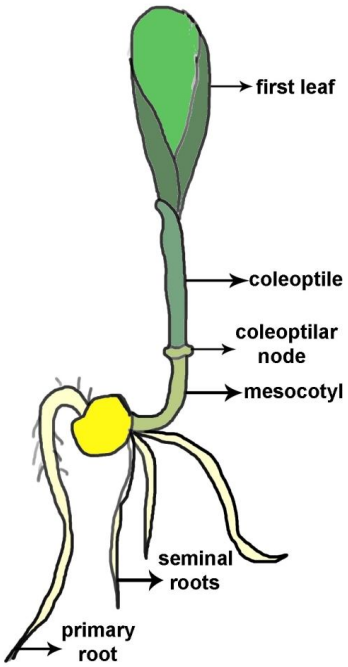


Fig. 8 Graphic figuration of the young light-grown maize seedling

When the first leaf collar is visible, the main source of energy starts to derive from photosynthesis. A leaf consists of the leaf blade, collar and sheath (Fig. 9).

An appearance of the collar indicates that leaf is fully developed. Male inflorescences, tassels formation, are initiated approximately 3-4 weeks after emergence, while female inflorescences, ear shoots, are initiated several days later and 6-8 or more nodes below the tassels.When the male flowers are developed, the stamens start to produce the pollen grains. Ovules are produced in female spikelet-like inflorescences ears. Each female spikelet possesses one ovary with just one fertile ovule. The upmost part of elongated style called silk is covered with moist viscous hairs capable of tracing the pollen grains. When the anthesis and the silks occur, the vegetative stage is finished and reproductive stage of growth is initiated. After the pollen grain is captured on the sticky stigma, it starts to germinate within few minutes. Approximately 12-28 hours are necessary for the pollen tube to grow through the style till the spermal cells reach the egg and fertilization may start. After successful fertilization, the zygote arises and in the subsequent various cell divisions the embryo is ground (Vožda et al. 1962).

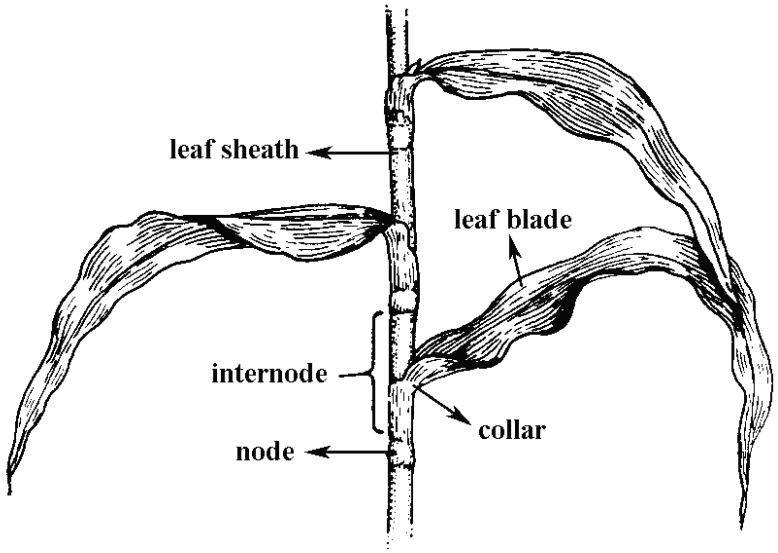


Fig. 9 Partial graphic figuration of the maize shoot
(adapted from http://www.arthursclipart.org)

1.4.2 Pioneer Hi-Bred maize hybrids

In this work, five maize hybrids were used that had been commercially released by Pioneer Hi-Bred International, Intl., Des Moines, Iowa, USA (Tab. 1 in Material and Methods). Hybrids commercially released by Pioneer Hi-Bred International from 1930s to 1990s were characterized by a gradual increase in the grain yield even under high planting density. Indeed, the yield plateau of modern hybrids was reached at a higher plant density than for old hybrids (Duvick et al. 2004, Hammer et al. 2009). In 1975,

Mock and Pearce proposed the maize ideotype that should maximally utilize environment for its production. They characterized environmental conditions and morphological and physiological traits of plants that will produce optimally in such an environment. One of postulated morphological characteristics was a stiff, vertically oriented leaf above the ear. Characterization of adult plants of the Pioneer hybrids 307, 3306 and 3394 showed that the modern hybrid 3394 has more erect foliage in low/high density planting (LD/HD) and when growing in the greenhouse. Nevertheless, more upright leaves above the ear and horizontal below the ear, as has been suggested for maize ideotype, were not observed (Ford et al. 2008). The same trend in leaf positioning was described earlier for juvenile plants of these three hybrids (Fellner et al. 2003). Measurement of leaves of 307, 3306 and 3394 hybrids at adult stage revealed that the modern hybrid 3394 has shorter leaves with smaller area, reduced curvature and smaller auricle angle. No significant difference in the leaf midrib morphology or anatomy between 3306 and 3394 hybrids was found, thus it has been suggested that the difference in leaf curvature may consist of differences in the leaf length, area, weight and auricle angle. During field experiments at LD and HD planting, which were made with the older hybrid 3306 and modern hybrid 3394, both hybrids reacted to HD conditions by the increased leaf length, decreased leaf area, decreased leaf weight and reduced curvature of leaves. However, the modern hybrid 3394 exhibited more reduced leaf area and leaf curvature than the hybrid 3306 in HD planting conditions (Ford et al. 2008). Fellner et al. (2003, 2006) proposed a hypothesis that differential yield production of the old hybrids 307 and 3306 compared to the modern hybrid 3394 in crowded conditions that are characterized by the low R:FR ratio, may be explained by differential responses to R and/or FR signals, which may affect the auxin distribution or sensitivity. Several experiments supported this hypothesis. Light was shown to increase leaf declination and NPA could abolish this effect of light in the hybrid 307, but not in 3394. Analysis of the effect of light and auxin on the membrane potential of epidermal or cortical mesocotyl cells brought another evidence for the hypothesis postulated by Fellner et al. (2003). Both, light (WL) and auxin (50 μM IAA) caused hyperpolarization of PM in 307 cells but had no effect on the membrane potential of 3394 cells (Fellner et al. 2006). Differential sensitivity to auxin (NAA), antiauxin (PCIB – *p*-chlorophenoxyisobutyric acid) and inhibitors of PAT (NPA) was confirmed. NAA treatment caused greater inhibition of mesocotyl and coleoptile elongation in the older hybrids 307 and 3306 than in the modern hybrid. Similarly, NPA also reduced

elongation of mesocotyl in the older hybrids, but had no effect on the mesocotyl growth of 3394 seedlings. Additionally, NPA treatment caused changes in the leaf vascular morphology of the older hybrid 307, whereas no changes were observed in 3394 leaves. Differences were observed also between etiolated seedlings of the old hybrids and the modern hybrid. Etiolated seedlings in the modern line are shorter than in the older hybrids, they have shorter coleoptile and also mesocotyl. Moreover, modern hybrid showed reduced responsiveness to the inhibitory effect of light (RL, FR, WL) on elongation in comparison to the hybrid 307. However, content of free IAA in etiolated, R and FR-grown coleoptiles, as well as intensity of PAT was similar in the both hybrids. This led to the suggestion that light may reduce the seedling receptivity to auxin and fewer auxin receptors could be present in etiolated seedlings of the modern hybrid 3394 than in the older hybrids (307, 3306). After all this findings it was proposed to focus the next research on putative auxin receptors – auxin-binding proteins, because maize *abp* mutants (single mutants *abp1* and *abp4*, double mutant *abp1abp4*) differ in their leaf angle (Fellner et al. 2006).

2 AIMS OF THE THESIS

The main aims of the thesis were as follows:

1. To examine cross-sensitivity of young seedlings of the old and modern Pioneer hybrids to auxin and light and test the hypothesis that the erect leaf-developing modern hybrid 3394 and the old hybrids differ basically in auxin and light-related properties.

2. To test the hypothesis that maize auxin-binding protein 1 represents a linkage element between the light and auxin signaling pathways that was altered during the breeding of the modern hybrid 3394.

3. To contribute to the understanding of a role of the auxin-binding protein 1 and 4 during the development of maize seedling.

3 MATERIAL AND METHODS

3.1 PLANT MATERIAL

Kernels of five Pioneer hybrids of *Zea mays* L.: 307, 317, 3306, 3366, 3394 (provided by Pioneer Hi Bred, Intl., Des Moines, Iowa, USA) (Tab.1), and three maize *auxin-binding protein* (*abp*) mutants and their corresponding WT were used for experiments. The hybrids 307, 317, 3306 and 3366 are referred to as old hybrids, whereas the hybrid 3394 is referred to as a modern hybrid. The pedigree information is available in Smith et al. (2004).

Tab. 1 Maize hybrids used in this work

Hybrid	Year of commercial release	Type of cross
307	1936	double cross
317	1937	double cross
3306	1963	single cross
3366	1972	single cross
3394	1991	single cross

Mutants used in this work are loss-of-function single mutants *abp1*, *abp4* and double mutant *abp1abp4*, which contain Robertson´s *Mutator* transposable elements in *ABP1* or/and *ABP4* genes (Im et al. 2000). All mutant seeds were a gift from Alan M. Jones from the University of North Carolina at Chapel Hill, USA.

3.2 GROWTH CONDITIONS

For sterile cultures, kernels were rinsed in 60% (v/v) commercial Savo solution (Bochemie, Czech Republic) (~3% sodium hypochlorite) supplemented with a drop of Tween 20 (Sigma-Aldrich, Czech Republic) for 30 min, and then extensively rinsed with sterile distilled water. Kernels germinated on 0.7% (w/v) agar medium in Magenta GA7 boxes, 77x77x196 mm (Sigma, USA), 9 seeds per box. The basal medium (BM) contained Murashige and Skoog salts (Sigma-Aldrich, Czech Republic; Murashige and Skoog 1962), 1% (w/v) sucrose and 1 mM Mes (2-(N-morpholino)-ethanesulfonic acid); pH was adjusted to 6.1 by KOH before autoclaving. For particular experiments, the BM was further supplemented with various concentrations of auxin (NAA: 1-

naphthalene acetic acid). Kernels in the Magenta boxes were placed in a growth chamber (Microclima 1000E; Snijders Scientific B.V., The Netherlands), and incubated at 23°C under continuous blue light (BL) with maximum irradiance at 460 nm, continuous red light (RL) with maximum irradiance at 660 nm, or in the dark or continuous white light (WL). BL and RL were provided by blue (Philips TLD-36W/18-Blue, Phillips, USA) and red fluorescent tubes (Philips TLD-36W/15-Red, Phillips, USA), respectively. The total photon fluence rates of BL and RL were identical, *i.e.* 10 $\mu mol.m^{-2}.s^{-1}$. WL-grown plants were incubated in the Percival PGC-10 growth chamber (Percival Scientific, USA) under the continuous WL with total fluence rate 150 $\mu mol.m^{-2}.s^{-1}$. The fluence rates were measured with a portable spectroradiometer (model LI-1800; Li-Cor, USA) calibrated at the Department of Biophysics, Palacký University in Olomouc.

For the greenhouse experiments, plants were grown in soil (Potground H, Klasmann Deilmann, Germany) in small pots (190x190 mm; one seed per pot; 1 cm deep sowing) and regularly watered. In summer, the plants grew in natural light conditions at the temperature of 15°C and higher. In winter, the plants grew under natural light supplemented with the light from high-pressure sodium lamps PlantaStar E40/ES 400 W (Osram, Germany) to create 16-hour photoperiod. The temperature was controlled in the range 15 – 27°C.

3.3 MEASUREMENTS OF SEEDLING´S GROWTH RESPONSES

3.3.1 Measurement of leaf declination, leaf length and width, and determination of leaf area

For the study of the leaf angle development, plants were grown in the greenhouse in soil as described above. The leaf angle that is characterized as the declination of a leaf from the vertical axis was measured at the leaf base with a protractor. The protractor was placed upside down along the midrib of the leaf blade closest to the vertical axis. The size of the leaf angle was indicated by a freely swinging rod (Fig. 10). Measurements were done 4 days after the appearance of well-developed leaf. The leaf length was measured from the auricle termination to the tip of the leaf blade. The leaf width was measured in the widest part of the leaf blade. Lengths were measured with a ruler to the nearest millimeter. The leaf area was calculated using the

following formula: *leaf length* x *leaf width* x *α*, where the coefficient α determined by Stewart and Dwyer (1999) has a value 0.743. For each hybrid, 21 to 24 plants from four biologically independent experiments were measured.

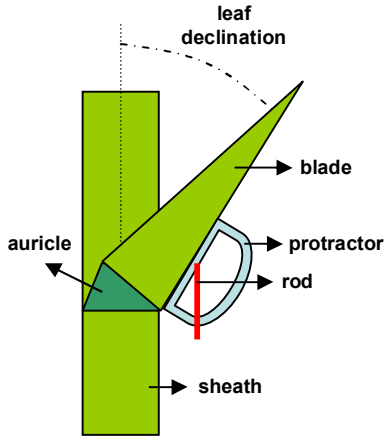


Fig. 10 Scheme of the leaf angle measurement

3.3.2 Measurement of the length of coleoptiles, mesocotyls, primary and seminal roots

The size of various organs was measured with a ruler to the nearest mm in 4-day-old intact seedlings developed on the BM in the dark, continuous BL or RL. The mesocotyl length was measured from the scutellar to the coleoptilar node and the coleoptile length was measured from the coleoptilar node to the tip of the coleoptile. Only seedlings that germinated on the same day were measured. Five independent experiments per treatment were performed and results represent average of a total number of 20 to 37 measured plants. Changes in growth (*i.e.* inhibition or stimulation) caused by an effector (NAA, light) in individual genotype were expressed in percents based on the following formula:

$$X = 100(A - B) / A,$$

where "X" is the change in growth (in %), "A" and "B" stand for growth (in mm) in the absence and presence of the effector, respectively.

3.4 EXTRACTION AND QUANTIFICATION OF ENDOGENOUS AUXIN

For analysis of endogenous free IAA accumulation in coleoptiles and mesocotyls, 4-day-old seedlings of hybrids and mutants grown on the BM in the dark, RL, BL or WL as described above were used. Coleoptiles, mesocotyls and primary roots were excised from several seedlings, placed into prechilled aluminum foil envelopes, immediately frozen in liquid nitrogen and then stored in a deep-freezer at -80°C. Endogenous auxin was extracted, purified from 10 mg sample by immunoaffinity extraction and quantified by high performance liquid chromatography coupled to tandem mass detection as described in Pěnčík et al. (2009). Extraction, purification and measurements of the free IAA were done at the Laboratory of Growth Regulators, Palacký University in Olomouc & Institute of Experimental Botany AS ČR by Aleš Pěnčík and Jakub Rolčík. Three experiments were performed at the same time.

3.5 *ABP1* GENE EXPRESSION ANALYSIS

The expression of *ABP1* gene (acc. no. L08425) was studied in coleoptiles and mesocotyls of 4-day-old seedlings grown on the BM in dark, continuous BL or RL. Total RNA was extracted from both organs using RNeasy Plant Minikit (Qiagen, Germany) according to the manufacturer's instructions. Genomic DNA was removed by 30 min DNaseI treatment (Sigma-Aldrich, Czech Republic) at 37°C and total RNA was purified using phenol/chloroform/isoamylalcohol (25:24:1). Synthesis of first-strand cDNA was performed from 1 μg of total RNA primed with random primers using SuperScript™ III Reverse Transcriptase (Invitrogen, USA). Quantitative RT-PCR analyses were made on Mx3000P™ Real-Time PCR System (Stratagene, USA). The reaction mixture contained 50 nM of each primer, 12.5 μl of Absolute™ QPCR SYBR Green ROX Mix (Thermo Scientific, USA) and 2 μl of 50x diluted cDNA in a total volume of 25 μl. For amplification of the *ABP1* gene, the following primers were used: F: 5'-AGGTGGAAGTGTGGCTTCAG-3', and R: 5'-ATCCCATCAAGAGCGTACCC-3'. The *18S* rRNA gene of maize (gene ID: 4055912) was used as the reference gene and amplified using the following primers: F: 5'-ACGAACAACTGCGAAAGC-3', R: 5'-CGGCATCGTTTATGGTTG-3'. Amplifications were carried out as follows: 95°C for 10 min and then 50 cycles of 95°C for 30s and 60°C for 1 min. A melting curve analysis was performed at the end of each

PCR reaction to confirm the product quality. All data were normalized with respect to the *18S* amplicon. The $\Delta\Delta C_T$ method and differences in the cycle numbers during linear amplification phase between samples were used for the determination of relative gene expression. The effect of light on the *ABP1* expression and the comparison of *ABP1* expression between the hybrids in certain light conditions were analyzed separately. When the effect of light on the *ABP1* expression was analyzed, data were expressed relative to that estimated for the dark-grown seedlings. For comparison of individual hybrids, data were expressed relative to those estimated for the oldest hybrid – 307. Final values represent averages of two biological repeats done in triplicates.

3.6 ANALYSIS OF *ABP1* SEQUENCES

Mesocotyls of 307 and 3394 hybrids grown in the dark on the BM were used for the analysis. The total RNA was extracted as described above. DNaseI treatment was performed using RQ1 RNA-free DNase (Promega, USA) for 30 min at 37°C. cDNA synthesis was performed as described above. The PCR reactions contained 1 µM of each primer, 100 µM of dNTP (each), 1X GoTaq DNA polymerase buffer, 1U of GoTaq DNA polymerase (Promega) and 2 µl of cDNA template; volume was filled up to 20 µl with ultrapure water. Amplification was carried out in a DNA Engine Peltier Thermal cycler (BioRad, USA). For *ABP1* gene amplification, the initial denaturing step at 94°C for 3 min was followed by 30 cycles: 94°C for 30 sec, 60°C for 30sec, 72°C for 45 sec. A final elongation step was performed at 72°C for 5 min. Sequences of primer combinations used in the study of full length *ABP1* were: F: 5'-TGTCGGGAGCAGGCAATGGC-3' and R: 5'-GCAGGAAACACTTGTGACCTAGAG-3'. PCR products were purified from agarose gel according the instructions of the manufacturer of the NucleoSpin® Extract II kit (Macherey-Nagel, Germany) and introduced into the pGEM-Teasy vector following the kit instructions (Promega, USA). Plasmids were introduced into *E. coli* DH5α competent cells and purified according the protocol of the NucleoSpin® Plasmid QuickPure (Macherey-Nagel). Sequences of plasmids were obtained from Macrogen (South Korea). BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) analyses were performed in order to compare sequences of genes isolated from both genotypes.

3.7 QUANTIFICATION OF ABP1 PROTEIN

The analysis of ABP1 content was done in coleoptiles and mesocotyls of 4-day-old seedlings grown on the BM in the dark, continuous BL or RL. Tissues were grounded in liquid nitrogen with a mortar and pestle and stored in -80°C before protein extraction. Proteins were extracted from the powdered tissues with extraction solution (9 M urea, 4.5% SDS (w/v), pH was adjusted to 6.8 using HCl) containing 7.5% (v/v) β-mercaptoethanol. The samples were incubated 5 min at 60°C, vortexed, incubated again 5 min at 60°C, and centrifuged 10 min at 10,000 *g*. The supernatant was transferred to a new eppendorf tube and used for further analysis. The concentration of proteins was determined from absorption at 280 nm in 20x diluted samples by NanoDrop 2000 (Thermo Scientific, USA). Aliquots of 200 µg were loaded and electrophoresed on a 12% SDS-PAGE gel. Proteins were transferred on a nitrocellulose membrane (Trans-Blot® Transfer Medium, Bio-Rad, Czech Republic) using OWL semi-dry electroblotter (Thermo Scientific, USA). The rabbit polyclonal antibody raised against ABP1 (Napier et al. 1988) diluted 1:2000 and Anti-Rabbit IgG – Peroxidase (Sigma-Aldrich, Czech Republic) diluted 1:5000 were used as primary and secondary antibody, respectively. Nonspecific binding sites were blocked by washing the membranes in phosphate buffered saline containing 0.1% (v/v) Tween 20 (Sigma-Aldrich, Czech Republic) (PBST) supplemented with 5% non-fat dry milk (w/v) for 1 h. Afterwards, the membranes were washed twice for 10 min in PBST. The membranes were then incubated with primary and secondary antibody in the blocking solution (3% non-fat milk (w/v) in PBS), each antibody 1 hour, always followed by washing the membranes twice in PBST for 10 min. The peroxidase activity was detected using the Amersham ECL™ Western Blotting Detection Reagents on the Amersham Hyperfilm™ ECL (GE Healhtcare, UK). Films were scanned (CanonScan 8600F) and analyzed by ImageJ (Abramoff et al. 2004). Data were expressed as percentage of the total area of all signals present on the gel and represents two biological repeats done in duplicates, which show the abundance of ABP1 in hybrids grown in the assigned light conditions or the effect of light on the ABP1 accumulation.

3.8 STATISTICAL ANALYSIS

A statistical significance of the differences between treatments was assessed using the Student's t-test (MS Office Excel), or ANOVA (NCSS 2007). Except tests for the gene expression analysis, the significance level was 0.05 and the results were significantly different when the P-value was lower than the assigned significance level. For the analysis of the gene expression data the significance level was chosen to be 0.1.

4 RESULTS

It was previously reported that relative to the old maize hybrid 307, the modern hybrid 3394 develops more erect leaves and differs in the R-, FR- and WL-induced as well as auxin-induced responses (Fellner et al. 2003, 2006, Ford et al 2008). In this work three additional old hybrids were examined, thus their leaf declinations, light- and auxin-induced responses were measured and the results are described in the first part of this chapter. So this part describes a partial phenotypic characterization of five maize hybrids – 307, 317, 3306, 3366 and 3394 grown in the greenhouse and their growth responses to exogenous auxin (NAA) in different light conditions (in growth chambers). Since the plant growth is very closely related to the auxin action, and therefore to the metabolism of endogenous auxin, the accumulation of free IAA in separated organs was measured. The results from measurements of free IAA content in hybrids, which show the effect of light on the free IAA accumulation, are described in the second part of this chapter. The main aim of the third part of this chapter was to investigate the role of putative auxin receptor – ABP1 in the interaction of light and auxin signaling pathways in hybrids´ seedlings by using the molecular approach. ABP1 is the major and the best known ABP present in maize. Since it was found that maize *abp* mutants are affected in the development of leaf declination (Fellner et al. 2006), the analysis of *ABP1* gene expression, gene sequence and the protein content were performed and the results were compared between the old and modern hybrids. For better understanding of a role of auxin-binding proteins in the developing maize seedling, maize *abp* mutants were explored in the fourth part of this chapter. Because the modern hybrid 3394 showed an altered interaction of the BL and auxin signaling pathway, the growth responses and accumulation of free IAA in the *abp* mutants were monitored only in BL-grown plants.

4.1 HYBRID GROWTH RESPONSES AND PHENOTYPIC CHARACTERIZATION

4.1.1 Phenotypic characterization of maize hybrids

Phenotypic characterization of hybrids used for this study was done with the respect to the leaf development on plants grown in the greenhouse until the stage when the 4th leaf (counted from the base of the plant) was fully developed.

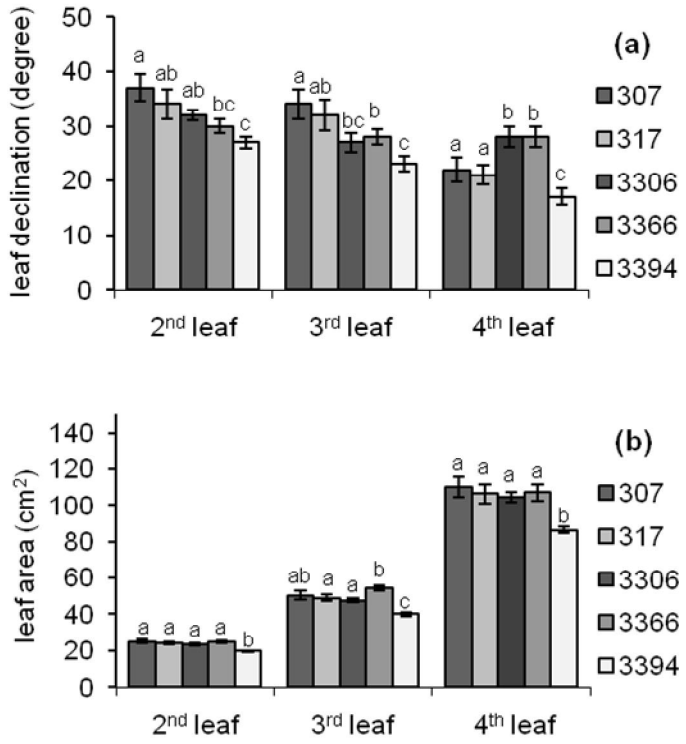


Fig. 11 **Declination (a) and leaf area (b) of the 2nd, 3rd and 4th leaf of five maize hybrids grown in the greenhouse**
Measurements were done 4 days after the appearance of well-developed leaf in all examined hybrids. Data represent averages ± SE obtained in 4 independent experiments. In each experiment, 5 to 7 seedlings were measured. Different lowercase letters above the bars indicate significant difference within the data obtained for 2nd, 3rd and 4th leaf (t-test, $P<0.05$).

The leaf angle, leaf width and leaf length were measured and leaf surface calculated for the 2nd, 3rd and 4th leaf. Regardless of the rank of the leaf, the leaf angle of the modern hybrid 3394 was always smaller than that of any old hybrid tested (Fig. 11a). Blade surface of the modern hybrid was also significantly smaller than that for the old hybrids, which did not essentially differ from each other (Fig. 11b).

4.1.2 BL- and RL-induced growth responses of hybrid seedlings

To examine the effect of light on the seedling growth, plants were grown on the BM in the dark, continuous BL or RL. Seeds germinated two days after sowing, independently of the light conditions. Etiolated 4-day-old seedlings of all old hybrids developed significantly longer coleoptiles than seedlings of the modern hybrid 3394 (Fig. 12a). Light, stimulated coleoptile growth in all hybrids, except for the oldest one, 307 (Fig. 12a). As all hybrids exposed either to BL or RL have finally reached almost the same length of coleoptiles, the stimulation was more pronounced in the modern hybrid 3394 than in the others (50% in BL, 60% in RL in the modern hybrid 3394, in contrast to 20% in BL and 13% in RL in the old hybrid 317) (Fig. 12a).

Like coleoptiles, etiolated mesocotyls of the 3394 hybrid were significantly shorter than those in the old hybrids (Fig. 12b), being about one third shorter than the mesocotyls of the oldest hybrid 307. Elongation of mesocotyl was strongly reduced by BL, and even more by RL. Nevertheless, both BL and RL were less efficient to inhibit mesocotyl growth in the modern hybrid 3394 (39% and 59%, respectively) than in the old hybrids (60-70% and 70-80%, respectively).

Primary roots of etiolated seedlings of all hybrids except the hybrid 317 have grown to similar length (Fig. 12c). The effect of light on the elongation of primary roots was variable: light reduced the root length of the hybrids 317 (both BL and RL) and 3366 (BL), but induced the root elongation of 3306 and 3394 hybrids (RL), while not affecting the roots of 307 (Fig. 12c).

Length of etiolated seminal roots of all five hybrids was also very variable (Fig. 12e). The longest seminal roots were produced by the hybrid 317 and the shortest by the hybrids 3306 and 3394. However, all dark-grown hybrids developed similar number of seminal roots (Fig. 12e). The length and number of seminal roots was also affected by light (BL as well as RL), except for the oldest hybrid 307 (Fig. 12d,e). Two hybrids (old hybrid 317 and modern hybrid 3394) developed shorter seminal roots when grown in BL and RL, the old hybrid 3366 had shorter roots only in BL, while 3306 hybrid exhibited even longer roots in RL (Fig. 12d). Light also affected the number of seminal roots in the hybrids 317, 3306, 3366, and 3394. Whereas both BL and RL stimulated seminal root formation in the old hybrid 3306 and in the modern hybrid 3394, two other hybrids (317 and 3366) made more seminal roots only when growing in RL (Fig. 12e).

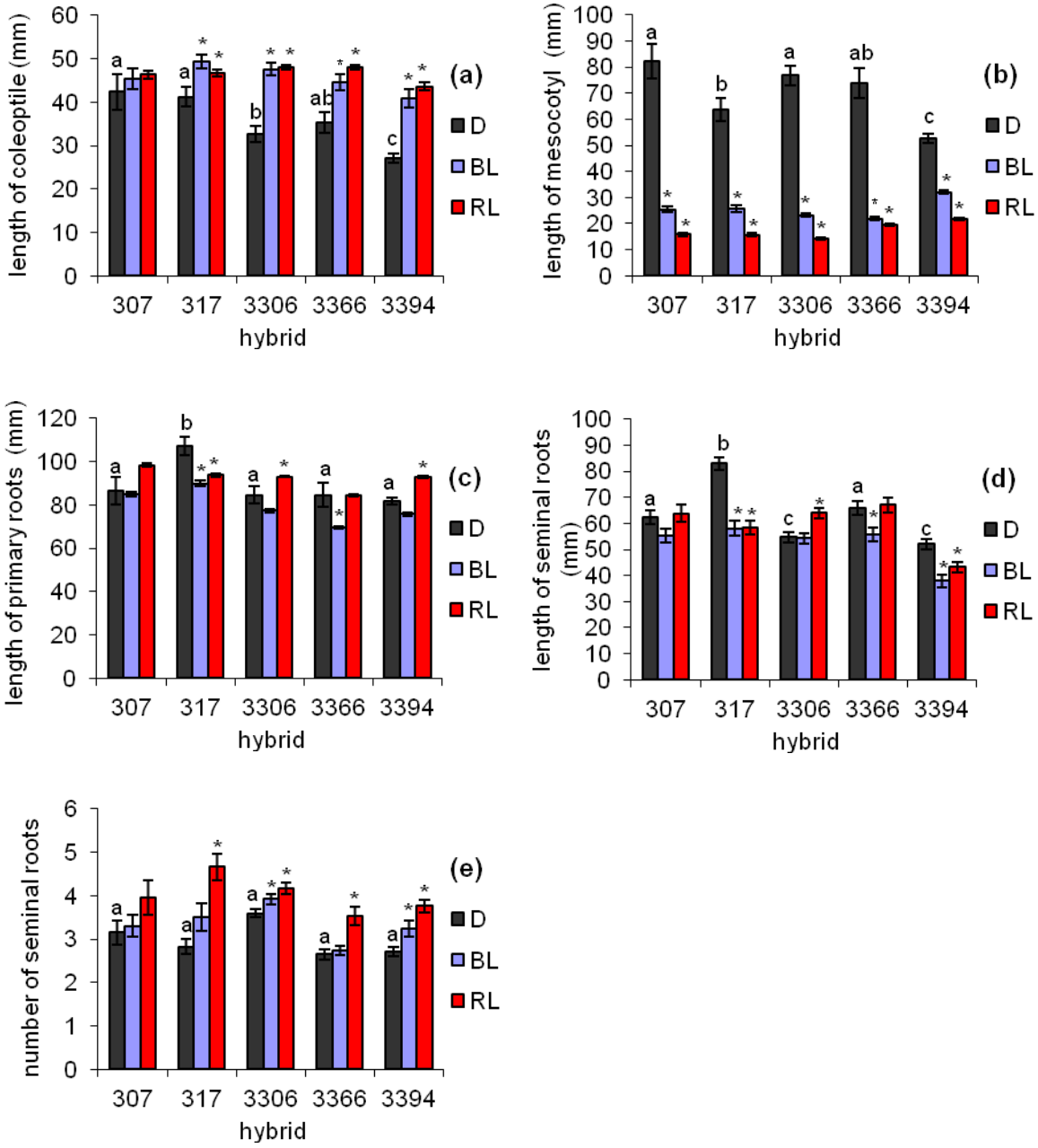


Fig. 12 **Comparison of the length of coleoptiles (a), mesocotyls (b), primary root (c), seminal roots (d) and the number of seminal roots (e) of studied maize hybrids grown in the various light conditions**

Data represent averages ± SE obtained in 5 independent experiments, together 20-37 plants. Different lowercase letters above the bars indicate significant differences within the dark-grown seedlings (ANOVA, $P<0.05$). * - significantly different from values obtained for dark-grown plants (t-test, $P<0.05$).

4.1.3 Hybrid growth responses to exogenous auxin.

The effect of exogenous auxin (NAA) on the growth of hybrids was examined on 4-day-old seedlings grown on the BM in the dark, continuous BL or RL. To compare the effect of NAA on the growth of hybrids, or the growth in different light conditions, the two responses were observed – change in the sensitivity (an ability of certain concentration of NAA to induce some response) and change in responsiveness (an extent of the response to the same concentration of NAA is different).

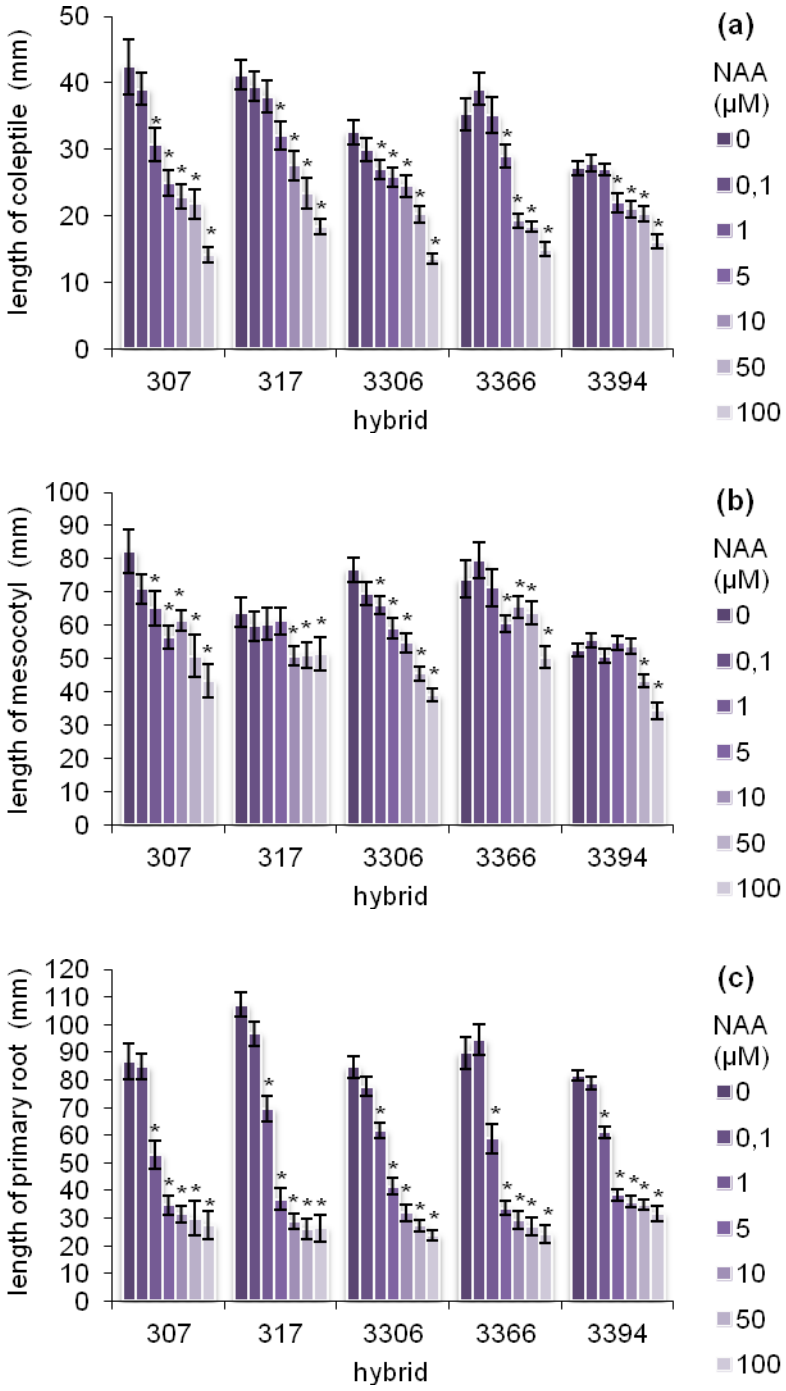
4.1.3.1 Responses of etiolated seedlings.

When seedlings were grown in the dark, the coleoptile growth of the old hybrid 307 and 3306 was inhibited by 1 μM NAA, while the coleoptile growth of the hybrids 317, 3366 and 3394 was inhibited only at higher concentrations of NAA such as 5 μM (Fig. 13a). However, the growth of etiolated coleoptile of the modern hybrid 3394 was inhibited by exogenous auxin much less than the growth of the old hybrids. For example, 50 μM NAA inhibited elongation of coleoptiles of the old hybrids by 40% or more, whereas coleoptile growth in 3394 hybrid was inhibited by only 25% (Fig. 13a).

Reduced responsiveness and also sensitivity to auxin-induced inhibition of elongation of etiolated 3394 seedling was even more obvious on the mesocotyl level. The most sensitive were the old hybrids 307 and 3306, whose mesocotyls growth was reduced already by 1 μM NAA (Fig. 13b). Growth of the mesocotyl of 317 and 3366 hybrids was inhibited by higher concentrations of NAA, 5 and 10 μM, respectively. In the case of 3394 hybrid, mesocotyl elongation was inhibited only by much higher concentrations used (50 μM and above). Thus, auxin at the concentration of 10 μM inhibited mesocotyl growth in all old hybrids by 20 to 40%, whereas it exhibited no effect on mesocotyl elongation in the modern hybrid 3394 (Fig. 13b).

The elongation of primary roots of etiolated seedlings was inhibited much more than elongation of aerial organs. For example, in the hybrid 307 that showed the highest values of growth inhibition of aerial organs the growth of primary roots at the concentration 10 μM was inhibited by 64%, whereas the coleoptile and mesocotyl growth was inhibited by 46% and 25%, respectively. The lowest concentration of NAA in the medium that effectively inhibited elongation of roots in all genotypes was 1 μM (Fig. 13c). Primary roots of the modern hybrid 3394 were slightly, but not distinctly, less responsive to the inhibitory effect of exogenous auxin. Indeed, at the concentration of 10 μM NAA, the growth of dark-grown primary roots in old hybrids was inhibited

by 62% (hybrid 3306) or 73% (hybrid 317), while the elongation of primary roots of the modern hybrid was reduced by approx. 56%.



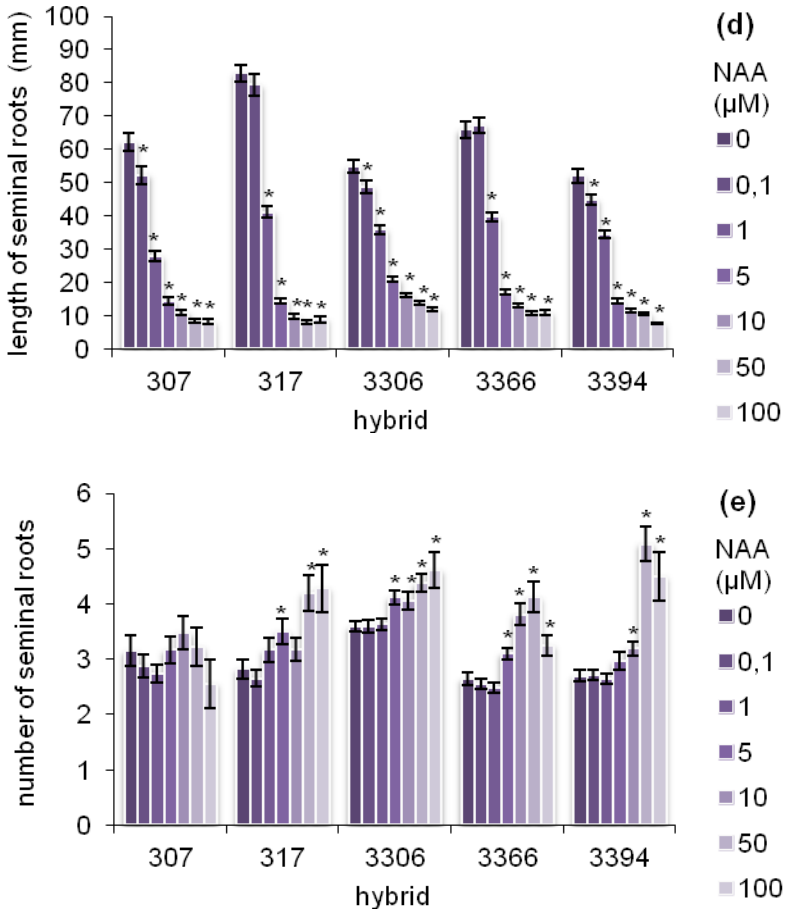


Fig. 13 **Effect of exogenous auxin (NAA) on elongation of the coleoptile (a), mesocotyl (b), primary root (c), seminal roots (d) and production of seminal roots (e) in 4-day-old etiolated maize hybrids**
Data represent averages ± SE obtained in 5 independent experiments, together 20-37 plants. * - significantly different from values obtained for control plants grown in absence of NAA (t-test, $P<0.05$).

Similarly to the primary roots, growth of seminal roots of all dark-grown hybrids was greatly inhibited by exogenous auxin to the similar extent in all hybrids. At 10 μM NAA, the most potent inhibition of growth was observed in the hybrid 317 (88%) and the least in the hybrid 3306 (71%). However, seminal roots of hybrids 307, 3306 and 3394 were highly sensitive to the NAA application, since their growth was already reduced at the lowest NAA concentration tested, 0.1 μM (Fig. 13d). Exogenous auxin stimulated production of seminal roots in etiolated seedlings of all hybrids except the oldest one 307 (Fig. 13d). The lowest concentration of NAA that efficiently increased the number of seminal roots in the old hybrids was 5 μM, while in the modern hybrid it was 10 μM.

4.1.3.2 Responses of light-grown seedlings

The elongation of maize seedlings grown under the continuous BL or RL was also inhibited by exogenous auxin. The Fig. 14 shows a comparison of NAA-induced inhibition of coleoptile elongation in different light conditions, thus demonstrating the interaction of light and auxin signaling pathways in individual hybrids.

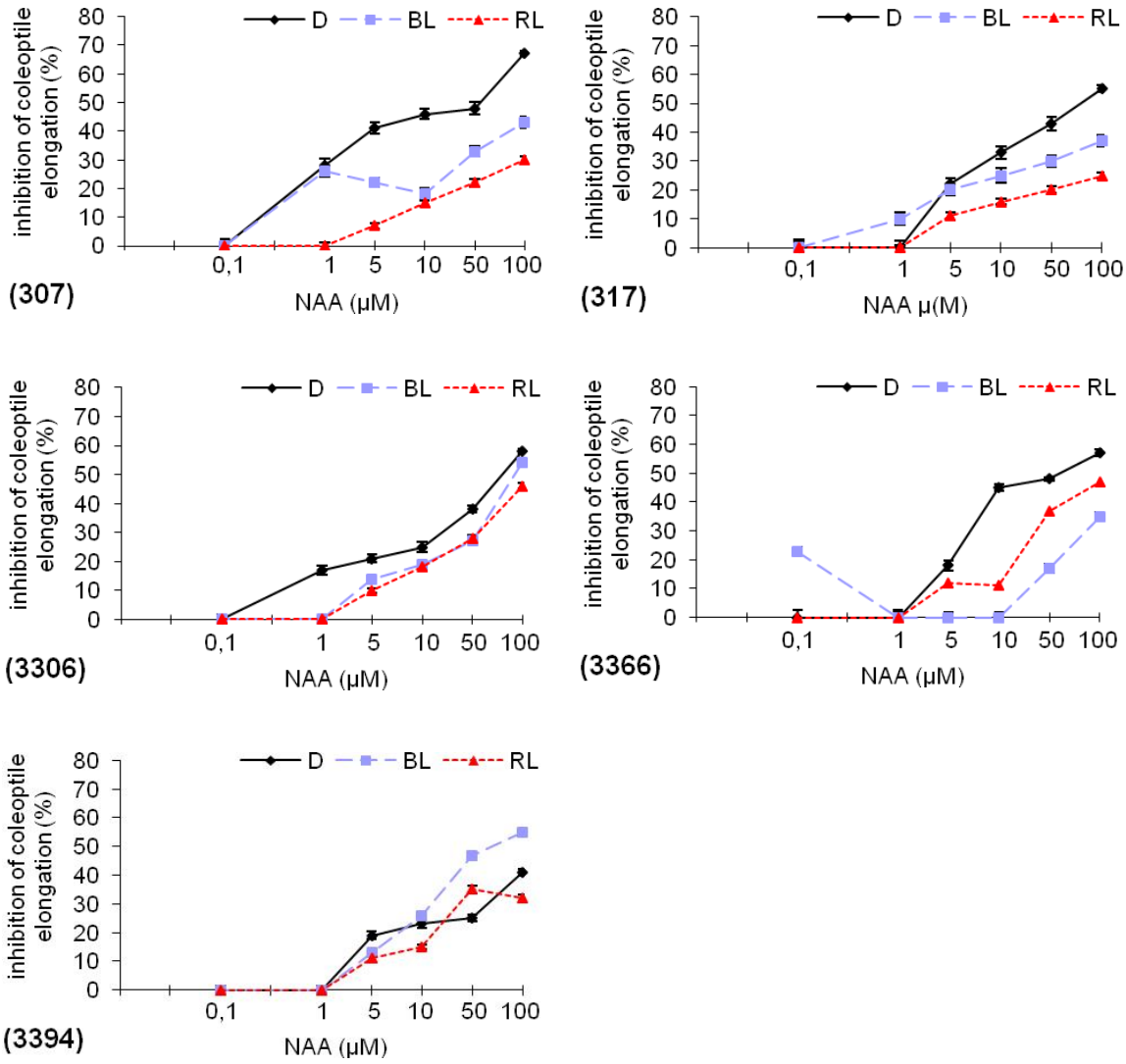


Fig. 14 **NAA-induced inhibition of coleoptile elongation in individual hybrids under different light conditions**
The hybrid, whose data are presented in the graph, is marked on the bottom left edge of each graph. Data represent averages ± SE of calculated percentage of inhibition obtained in 5 independent experiments, together 20-37 plants. All presented data are significantly different from the data obtained for control seedlings (grown without NAA in the medium in the dark, BL or RL respectively) (t-test, $P<0.05$). The graphs of the absolute lengths that were measured are included in appendix.

When seedlings of the old hybrids were grown in BL, the growth of coleoptiles was inhibited by NAA to less extent than in plants grown in the dark. Surprisingly, the 3394 seedlings grown in BL were more responsive to NAA than those of the old hybrids. Indeed at the concentration of 50 μM, NAA inhibited the coleoptile growth of 3394 hybrid by 47% whereas in 307 and 3306 hybrid by 33% and 27%, respectively. Moreover, BL increased the responsiveness of 3394 hybrid to NAA. For instance, at the concentration of 50 μM NAA, the elongation of 3394 coleoptile was inhibited by 47%, while in the dark it was only 25%. Like BL, continuous RL also significantly decreased the inhibitory effect of exogenous auxin on the coleoptile elongation. However, this effect was found to be more striking in the hybrids selected in 1930s (307, 317), whereas the hybrids from 1960s (3306, 3366) and the modern hybrid 3394 were less affected (Fig. 14).

The comparison of interactions of light and auxin signaling pathways in the mesocotyl tissues of studied hybrids is presented in the Fig. 15. Continuous BL did not essentially influence the mesocotyl sensitivity to exogenous auxin in the three oldest hybrids, 307, 317 and 3306. However, the reducing effect of BL on the mesocotyl responsiveness to auxin was clearly obvious in the hybrid 3366. On the contrary, BL markedly increased the sensitivity as well as responsiveness of mesocotyls to exogenous auxin in the hybrid 3394. The growth of mesocotyls of the modern hybrid 3394 under BL was inhibited already at the lowest concentration of NAA applied (0.1 μM). With the exception of hybrid 3366, mesocotyls in all hybrids grown in continuous RL were almost (in hybrid 3306) or completely (in hybrids 307, 317 and 3394) insensitive to the inhibitory effect of NAA over whole concentration range tested. Interestingly, in the hybrid 3366 grown in RL was the mesocotyl growth stimulated by NAA (Fig. 15).

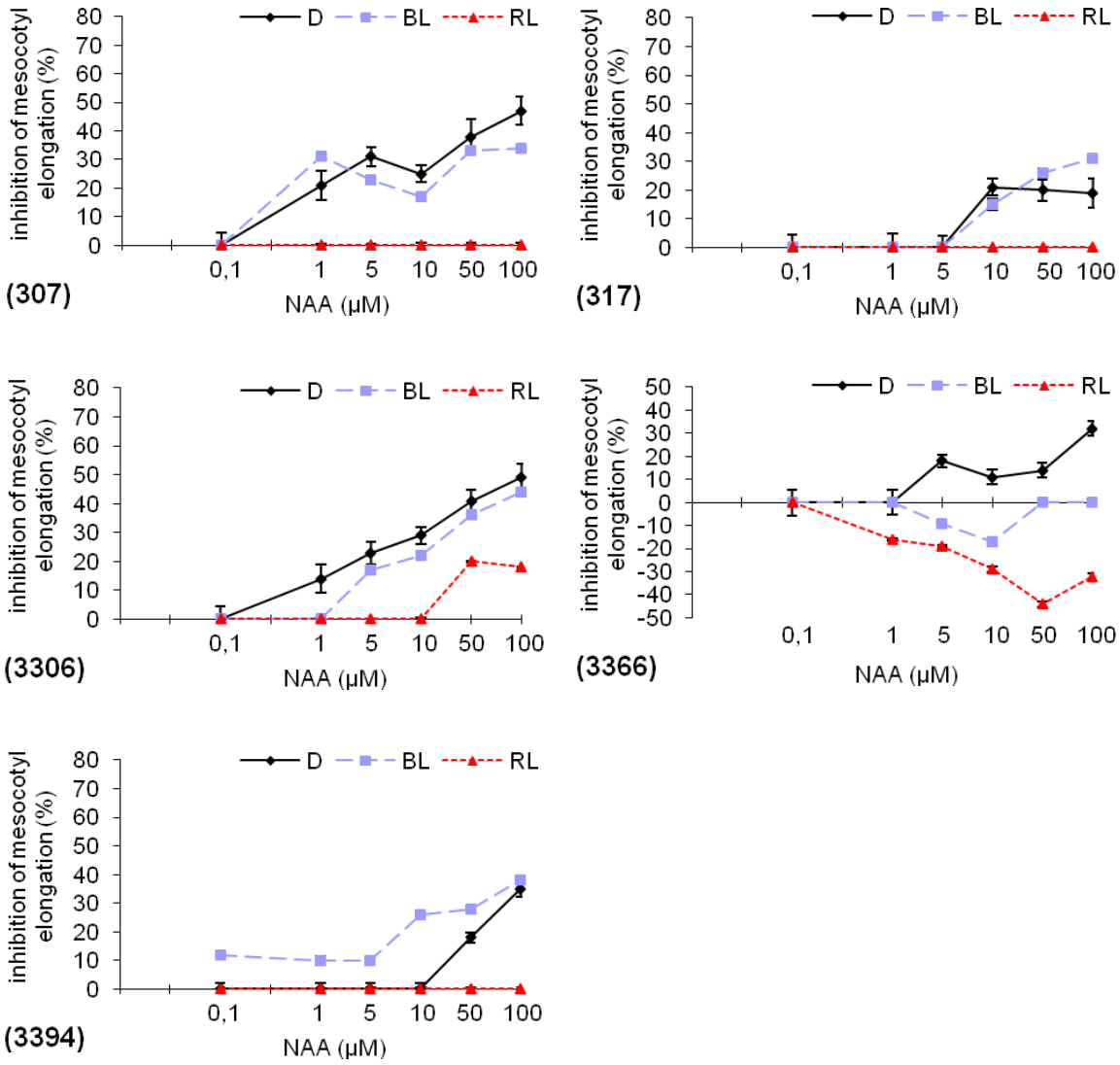


Fig. 15 **NAA-induced inhibition of mesocotyl elongation in individual hybrids under different light conditions**

A hybrid, whose data are presented in the graph, is marked on the bottom left edge of each graph. Data represent averages ± SE of calculated percentage of inhibition/stimulation obtained in 5 independent experiments, together 20-37 plants. All presented data are significantly different from the data obtained for control seedlings (grown without NAA in the medium in the dark, BL or RL respectively) (t-test, $P<0.05$). The graphs of the absolute lengths that were measured are included in appendix.

In contrast to coleoptiles and mesocotyls, light did not greatly influence responsiveness of primary roots to exogenously applied auxin. However, RL increased the sensitivity to NAA in all hybrids except the oldest one 307, and the growth of primary roots in these hybrids was inhibited already by the 0.1 µM NAA. Moreover, the elongation of roots of light-grown seedlings of hybrid 3394 and 3306 was reduced by NAA slightly (3306) or markedly (3394) more than in the dark (Fig. 16).

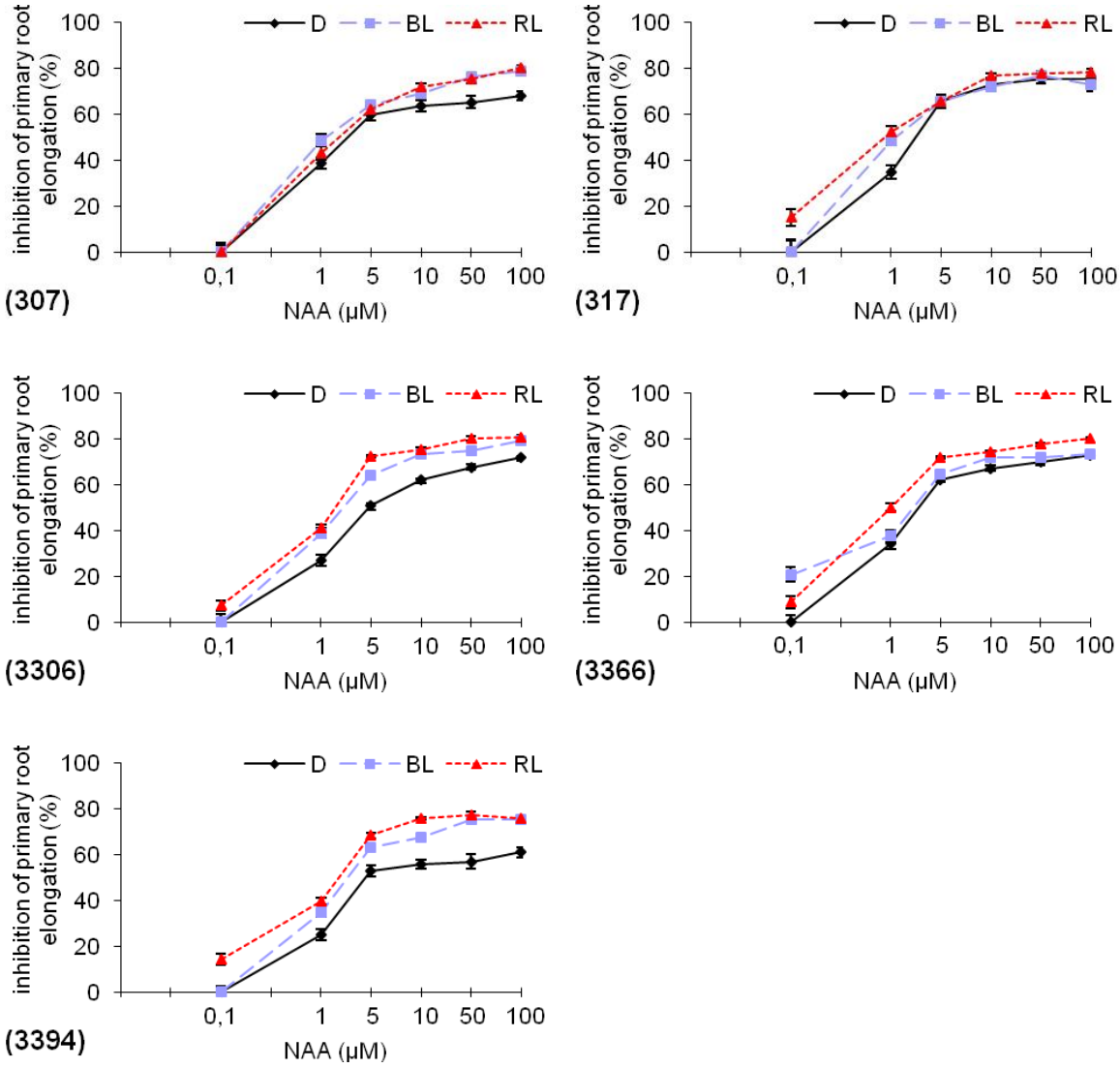


Fig. 16 **NAA-induced inhibition of primary root elongation in individual hybrids under different light conditions**
A hybrid, whose data are presented in the graph, is marked on the bottom left edge of each graph. Data represent averages ± SE of calculated percentage of inhibition obtained in 5 independent experiments, together 20-37 plants. All presented data are significantly different from the data obtained for control seedlings (grown without NAA in the medium in the dark, BL or RL respectively) (t-test, $P<0.05$). The graphs of the absolute lengths that were measured are included in appendix.

For example, roots of etiolated 3306 hybrid were about 62% shorter when grown on the medium supplemented with 10 μM NAA, whereas in BL and RL they were about 75% shorter. 10 μM NAA inhibited elongation of primary roots in etiolated hybrid 3394 by 56%, while BL-grown roots were inhibited by 68% and RL-grown roots by 76%. In other hybrids (307, 317, and 3366) light did not affect the responsiveness of primary roots to the exogenous auxin (Fig. 16).

The effect of light on the auxin-induced inhibition of growth of seminal roots was variable. Both BL and RL reduced sensitivity to NAA in the old hybrid 3306 and modern hybrid 3394, whereas in the old hybrids 307 and 3366 this response was observed only in RL-light grown plants. Surprisingly, BL increased the sensitivity to exogenous auxin of seminal roots of the hybrid 3366 (Fig. 17). Generally, light did not affect the responsiveness to NAA in the modern hybrid 3394 and old hybrids selected in 1930´s (307, 317). However, elongation of seminal roots of the hybrids selected in 1960´s was slightly (3366) or markedly (3306) more inhibited in light-grown seedlings than in dark-grown ones (Fig. 17).

Effect of light on the NAA-induced production of seminal roots was very variable and differed from a hybrid to hybrid. While exogenous auxin did not affect the production of seminal roots in etiolated seedlings of the oldest hybrid 307, the formation of seminal roots in BL and RL-grown seedlings was greatly stimulated by NAA (Fig. 18). BL suppressed NAA-induced production of seminal roots in hybrids 317 and 3306, whereas it enhanced auxin responsiveness in the hybrid 3366 and increased the sensitivity to auxin in the hybrid 3394. RL suppressed or reduced NAA-induced production of seminal roots in the hybrid 317 or 3394, but increased its stimulatory effect in hybrids 3306 and 3366 (Fig. 18).

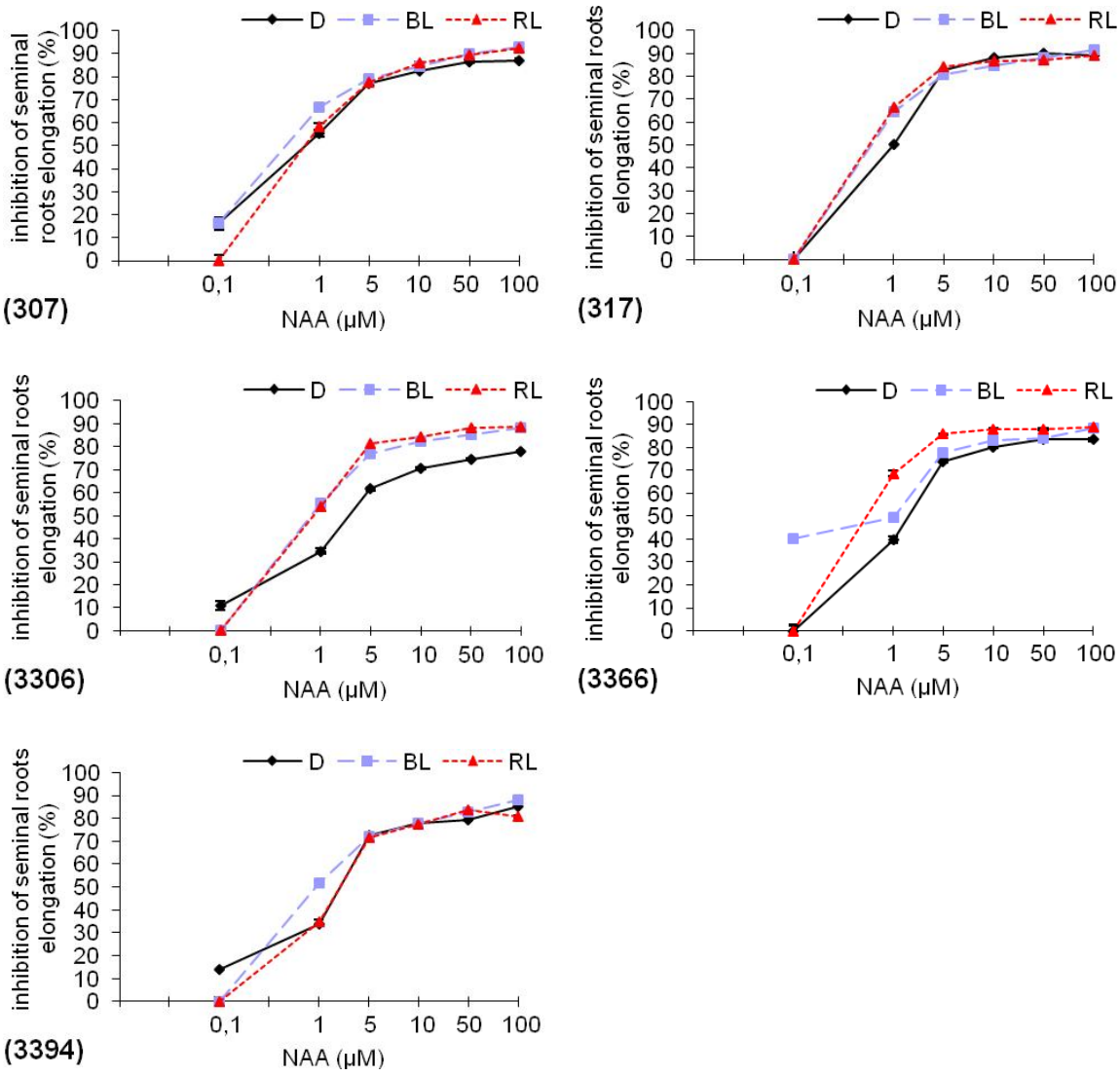


Fig. 17 **NAA-induced inhibition of seminal roots elongation in individual hybrids under different light conditions**

A hybrid, whose data are presented in the graph, is marked on the bottom left edge of each graph. Data represent averages ± SE of calculated percentage of inhibition obtained in 5 independent experiments, together 20-37 plants. All presented data are significantly different from the data obtained for control seedlings (grown without NAA in the medium in the dark, BL or RL respectively) (t-test, $P<0.05$). The graphs of the absolute lengths that were measured are included in appendix.

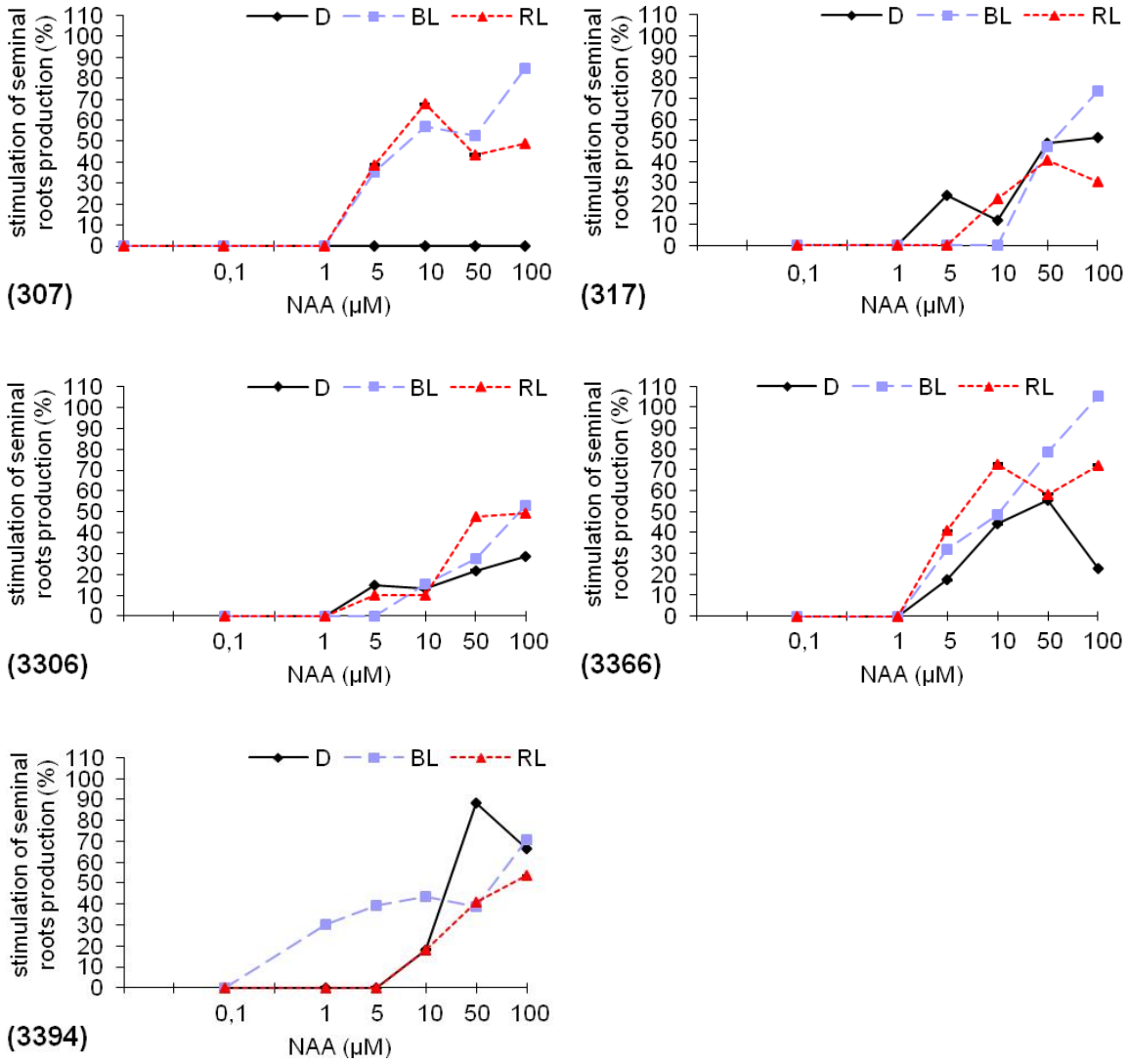


Fig. 18 **Effect of light on NAA-induced production of seminal roots in 4-day-old maize hybrids**

A hybrid, whose data are presented in the graph, is marked on the bottom left edge of each graph. Error Data represent averages ± SE of calculated percentage of stimulation obtained in 5 independent experiments, together 20-37 plants. All presented data are significantly different from the data obtained for control seedlings (grown without NAA in the medium the in dark, BL or RL respectively) (t-test, $P<0.05$). The graphs of the absolute lengths that were measured are included in appendix.

4.2 ANALYSIS OF THE ACCUMULATION OF FREE IAA IN STUDIED HYBRIDS

The quantification of the free IAA was performed in 4-day-old seedlings grown on the BM in the dark, continuous BL or RL. The highest amount of free IAA was detected in etiolated seedlings (Tab. 2). Coleoptiles of etiolated seedlings contained the highest free IAA levels, whereas in mesocotyls IAA content was approximately 50% and in primary roots 27% lower than in coleoptiles. Generally, BL reduced free IAA accumulation only in coleoptiles (44%), but RL reduced IAA accumulation also in mesocotyls and primary roots (coleoptile 62%, mesocotyl 25%, and primary root 14%) (Tab. 2).

Tab. 2 Average values (for all hybrids) of free IAA amounts detected in 4-day-old maize seedlings

Organ	Free IAA content [pmol/g fresh weight (FW) ± SE]		
	Dark	BL	RL
Coleoptile	145.0 ± 9.3	81.7 ± 4.0*	55.2 ± 2.6*
Mesocotyl	73.2 ± 4.7	69.1 ± 3.3	54.6 ± 3.1*
Primary root	105.8 ± 6.1	103.5 ± 4.7	90.7 ± 3.2*
Total	**324.0 ± 20,8**	**254.4 ± 10.1**	**200.5 ± 11.9**

* - significant difference between the dark-grown and BL or RL-grown plants (t-test, $P<0.05$). Data represent averages ± SE from three experiments performed at the same time.

The accumulation of free IAA was the highest in the oldest hybrid 307 and gradually decreased towards the modern hybrid 3394, however no statistically significant difference was found (Fig. 19a). Coleoptiles of all hybrids grown under BL contained a similar amount of free IAA. Compared to the ones grown in dark, free IAA content was significantly decreased in the old hybrids, but not in the modern hybrid (Fig. 19a). RL-grown coleoptiles of all hybrids contained significantly less free IAA than etiolated organs and no major differences were found for all hybrids studied (Fig. 19a).

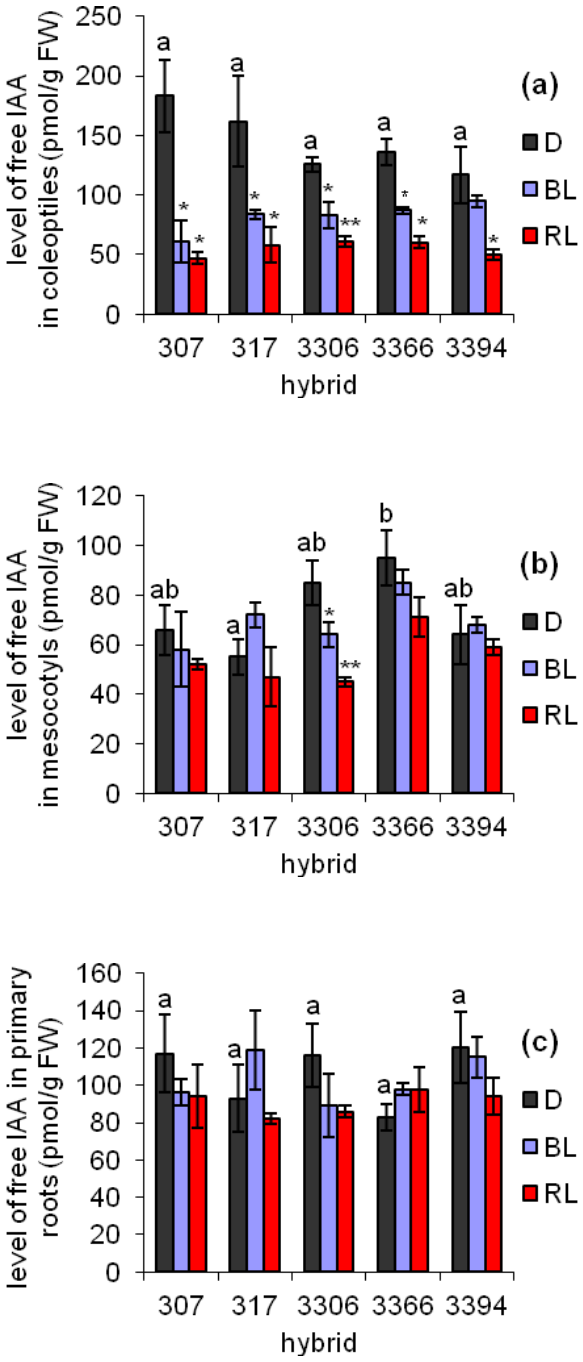


Fig. 19 **Amount of endogenous free IAA in coleoptiles (a), mesocotyls (b) and primary roots (c) of 4-days-old maize hybrids grown in the dark, BL and RL**

Data represent averages ± SE obtained from three experiments performed at the same time. Different lowercase letters above the bars indicate significant difference within the dark-grown seedlings (ANOVA, $P<0.05$). * - significantly different from values obtained for dark-grown plants (t-test, $P<0.05$); ** - significantly different from values marked by *(t-test, $P<0.05$).

The accumulation of free IAA in etiolated mesocotyls of the modern hybrid was similar to that observed in all old hybrids (Fig. 19b). The only difference was found

between old hybrids 317 and 3366, where mesocotyls in the hybrid 317 contained about 42% less free IAA than mesocotyls in the hybrid 3366.

Light (BL and RL) significantly lowered the IAA contents only in mesocotyls of the old hybrid 3306 (Fig. 19b), but not in other hybrids.

In primary roots of etiolated seedlings of all studied hybrids, similar amounts of free IAA were determined and they were not affected by light (Fig. 19c).

Additionally, free IAA accumulation was quantified also in WL-grown seedlings of all hybrids tested. Fig. 20 shows levels of IAA in separated organs of dark- and WL-grown plants. When comparing to etiolated seedlings, WL significantly decreased amount of free IAA in coleoptiles of all hybrids. However, the ability of WL to reduce IAA accumulation was found to be continually decreasing from the oldest one towards the modern hybrid 3394 (Fig. 20a). Specifically, WL-grown coleoptiles of the oldest hybrid 307 contained about 69% less free IAA than dark-grown coleoptiles, whereas in the modern hybrid IAA accumulation was inhibited by only 43%.

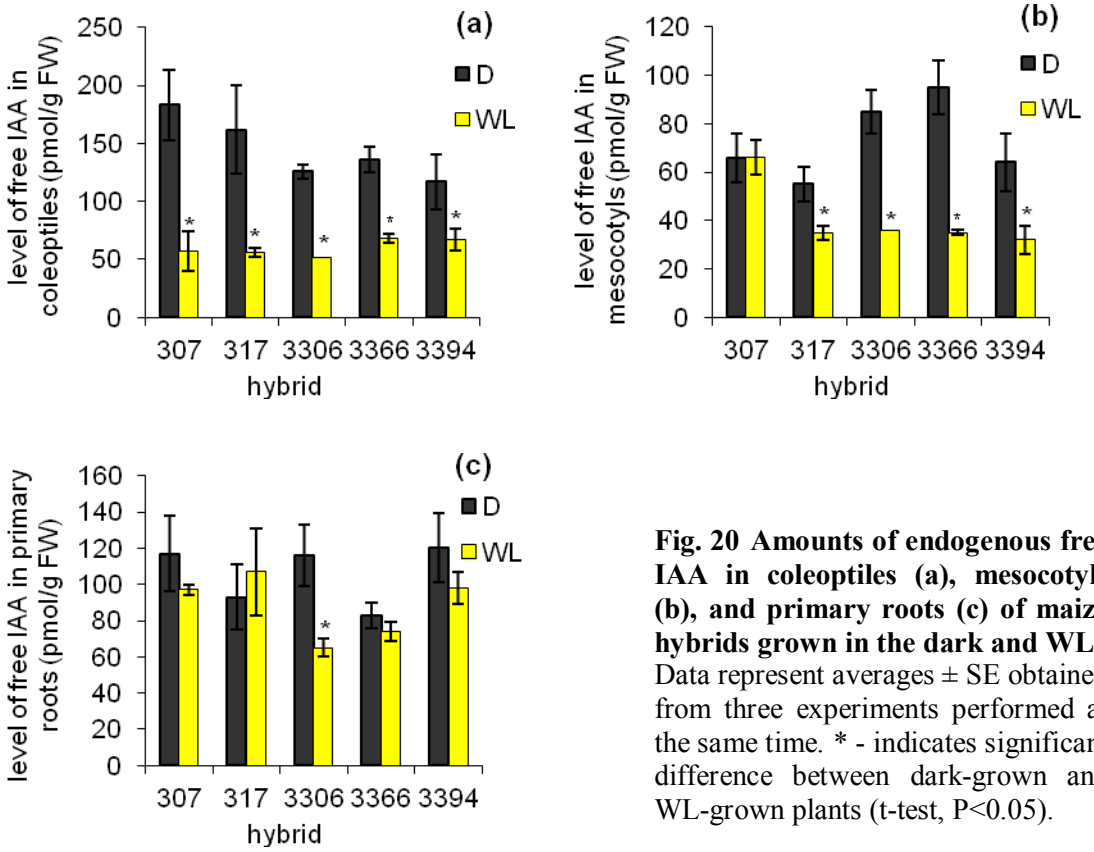


Fig. 20 Amounts of endogenous free IAA in coleoptiles (a), mesocotyls (b), and primary roots (c) of maize hybrids grown in the dark and WL
Data represent averages ± SE obtained from three experiments performed at the same time. * - indicates significant difference between dark-grown and WL-grown plants (t-test, $P<0.05$).

Interestingly, WL essentially reduced levels of free IAA in mesocotyls of all hybrids except the oldest one 307 (Fig. 20b), whereas BL or RL were not efficient enough to reduce IAA accumulation, except for the hybrid 3306 (Fig. 19b). In the WL-grown primary roots, the content of free IAA was found significantly lower only in the hybrid 3306. Primary roots of WL-grown seedlings of other hybrids contained similar amounts of IAA as dark-grown ones (Fig. 20c).

4.3 ANALYSIS OF THE *ABP1* GENE EXPRESSION, PROTEIN ACCUMULATION AND *ABP1* SEQUENCE IN THE STUDIED HYBRIDS

The expression of *ABP1* was studied in coleoptiles and mesocotyls by quantitative Real-Time PCR and results were normalized using the reference gene *18S*. The analyses were performed in 4-day-old seedlings grown on the BM in the dark, continuous BL or RL.

4.3.1 The effect of light on the *ABP1* transcript accumulation

To compare the expression profiles of *ABP1* in hybrids under specific light conditions, the expression data were related to the data obtained for the oldest hybrid 307 (Fig. 21a and 22a). For the assessment of the effect of light on the *ABP1* expression, the expression data were related to the values obtained for dark-grown seedlings (Fig. 21b and 22b).

The comparison of *ABP1* expression among all hybrids in assigned light conditions showed that relative amounts of *ABP1* transcript in coleoptiles of all hybrids grown in the dark or BL did not differ from each other (Fig 21a). In RL, the *ABP1* expression in coleoptiles of the hybrid 3394 was found to be significantly higher than in coleoptiles of the oldest hybrid 307, but it did not differ from the data obtained for other old hybrids 317, 3306 and 3366 (Fig 21a). The effect of light on the *ABP1* expression was calculated separately and is shown in the Fig. 21b. In either hybrid, BL did not affect the expression of *ABP1* gene in coleoptiles compared to etiolated plants. RL increased the accumulation of *ABP1* transcripts in coleoptiles of the old hybrid 317, whereas it did not affect significantly the *ABP1* expression in other hybrids (Fig. 21b).

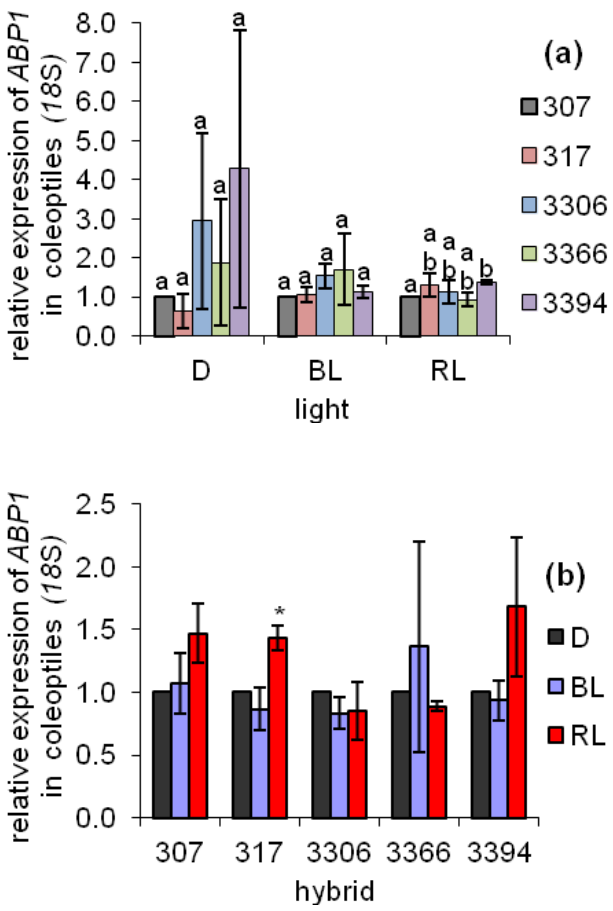


Fig. 21 **Relative expressions of *ABP1* gene in coleoptiles. (a) The comparison of relative expression among the hybrids grown in assigned light conditions. (b) The effect of light on the relative expression in individual hybrids.**
Expression profiles were estimated by qRT-PCR. Data are related to the expression values obtained for the hybrid 307 (a) or to the expression values obtained for etiolated plants (b). Data represent averages ± SE obtained from two independent experiments each with three technical replicates (n = 6). Different lowercase letters above the bars indicate significant difference within the dark-, BL- or RL-grown seedlings (t-test, P<0.1); * - significantly different from values obtained for dark-grown plants (t-test, P<0.1).

Expression profiles of *ABP1* gene in mesocotyls are shown in Fig. 22. The data show that in dark-grown mesocotyls, *ABP1* is expressed much less in the hybrid 3306 and 3394 than in the hybrid 307. In BL, the level of *ABP1* transcripts in mesocotyls was similar in all hybrids tested, but in RL the *ABP1* expression in the hybrid 317 was significantly lower than in the hybrid 307 (Fig. 22a).

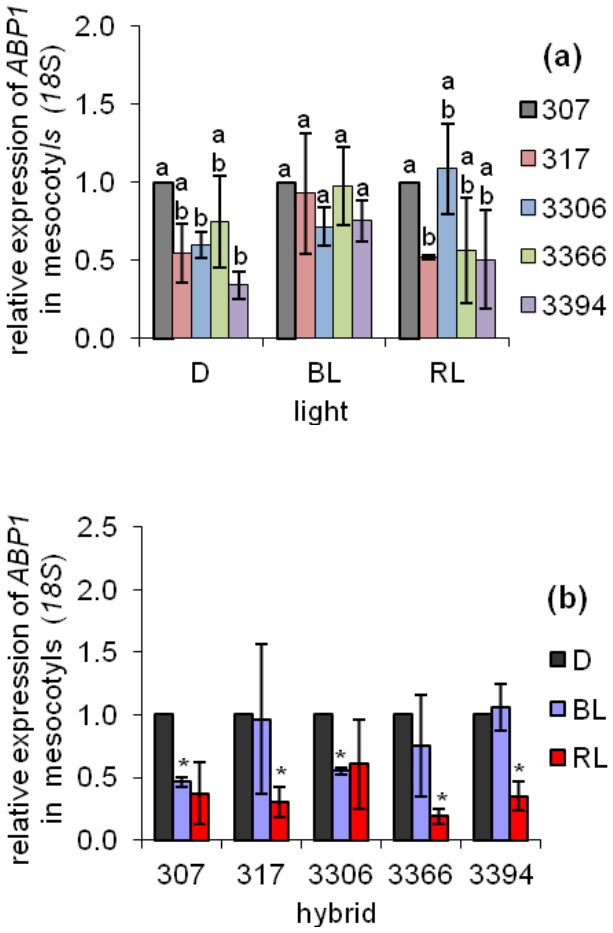


Fig. 22 **Relative expressions of *ABP1* gene in mesocotyls. (a) The comparison of relative expression among the hybrids grown in assigned light conditions. (b) The effect of light on the relative expression in individual hybrids.**
Expression profiles were estimated by qRT-PCR. Data are related to the expression values obtained for the hybrid 307 (a) or to the expression values obtained for etiolated plants (b). Data represent averages ± SE obtained from two independent experiments each with three technical replicates (n = 6). Different lowercase letters above the bars indicate significant difference within the dark-, BL- or RL-grown seedlings (t-test, P<0.1); * - significantly different from values obtained for dark-grown plants (t-test, P<0.1).

In general, light affected *ABP1* expression in mesocotyls much more than in coleoptiles. Light-induced regulation of *ABP1* transcription that depended on the light spectrum was found in each hybrid. BL reduced *ABP1* expression in mesocotyls of old hybrids 307 and 3306, whereas RL reduced its expression in old hybrids 317 and 3366 and in the modern hybrid 3394 (Fig. 22b).

4.3.2 The effect of NAA on the *ABP1* transcript accumulation

Because the mesocotyl was found to be the most light-sensitive organ of the maize seedling and the interaction of BL and auxin signaling pathways was found to be altered in the modern hybrid 3394, the expression of *ABP1* gene and its involvement in this interaction was studied in this tissue.

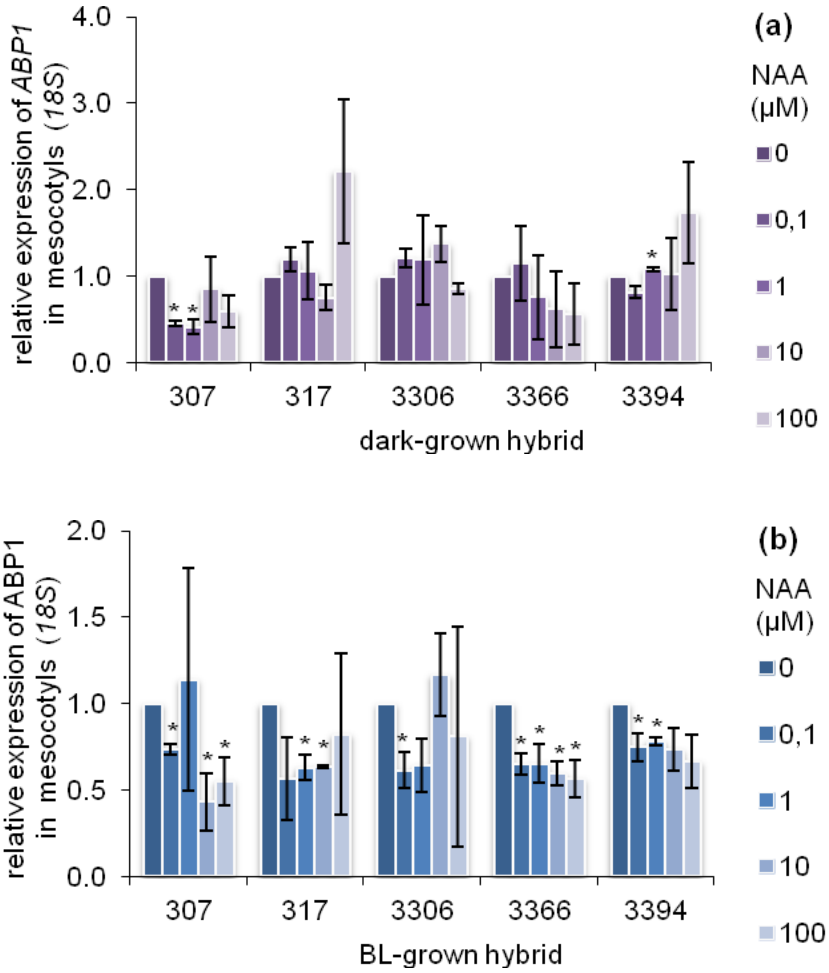


Fig. 23 **Relative expressions of *ABP1* gene in mesocotyls of plants grown in the dark (a) and BL (b) at various concentration of NAA**
Expression profiles were estimated by qRT-PCR. Data are related to the expression values obtained for control plants grown on the BM with no NAA addition.
Data represent averages ± SE obtained from two independent experiments each with three technical replicates (n = 6). * - significantly different from control values (t-test, P<0.1).

As shown in Fig. 23a, exogenous auxin did not affect the expression of *ABP1* in dark-grown mesocotyls of hybrids 317, 3306 and 3366. In the oldest hybrid 307, the

accumulation of *ABP1* transcript was down-regulated at 0.1 and 1 μM NAA, whereas in the modern hybrid 3394 it was up-regulated at 1 μM NAA (Fig. 23a). In the BL-grown seedlings, exogenous auxin affected *ABP1* transcription much more than in rdark-grown plants. The *ABP1* expression was reduced in mesocotyls of all hybrids grown in BL at various concentrations of NAA (Fig. 23b).

4.3.3 Accumulation of ABP1 protein.

ABP1 content was determined in coleoptiles and mesocotyls of all studied hybrids grown on the BM in the dark, BL or RL. Amount of ABP1 protein was compared among hybrids grown in the defined light conditions (Fig. 25a and 26a). The effect of light on ABP1 accumulation was analyzed separately and is shown in the Fig. 25b and 26b.

When comparing the amount of ABP1 in all hybrids that grew in studied light conditions, coleoptiles always accumulated the similar amount of the protein (Fig. 25a). In coleoptiles of the two old hybrids 307 and 3366, light (BL as well as RL) did not influence the protein accumulation, whereas in old hybrids 317 and 3306 RL had a positive effect on the accumulation of ABP1. BL did not affect the content of ABP1 in coleoptiles of all old hybrids, whereas it decreased the amount of ABP1 in the modern hybrid 3394 (Fig. 25b).

In mesocotyls of etiolated and BL-grown plants, the amounts of ABP1 were comparable in all hybrids (Fig. 26a). However in RL-grown plants, a decreased content of ABP1 in mesocotyls of the old hybrid 3366 and the modern hybrid 3394 was found (Fig. 26a). Generally, light (BL, RL) had a positive effect on the accumulation of ABP1 protein in mesocotyls. Except for the hybrid 3366, the amount of ABP1 was higher in all RL-grown mesocotyls than in dark-grown ones (Fig. 26b). BL had a stimulatory effect on the ABP1 accumulation only in hybrids 317 and 3306 (Fig. 26b).

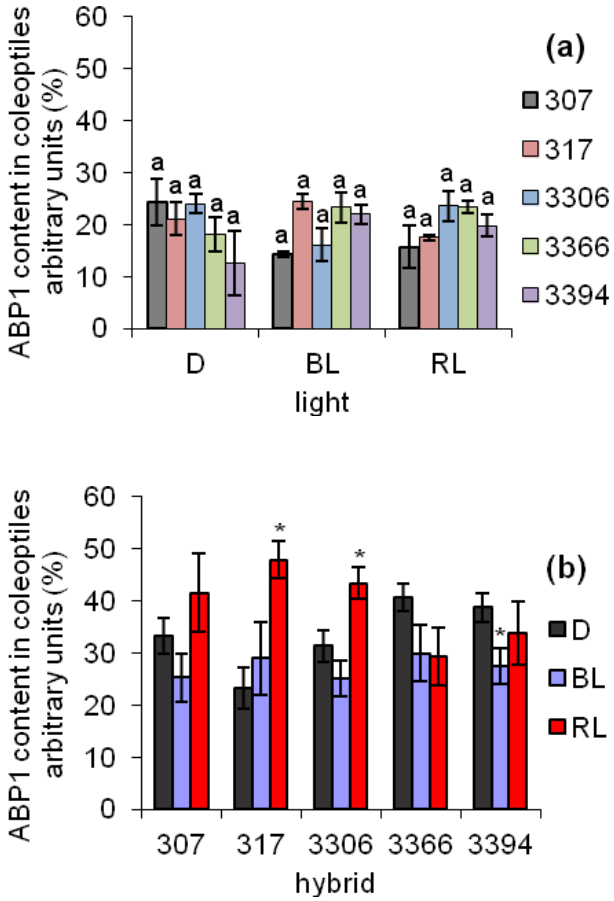


Fig. 25 **Accumulation of ABP1 in coleoptiles. (a) The comparison among the hybrids grown in the assigned light conditions. (b) The effect of light on the protein accumulation in the individual hybrids.**
Data are expressed as percentage of the total area of all signals present on the gel. The signal area was determined by ImageJ software. Results represent averages ± SE obtained from two independent experiments each with two technical replicates (n = 4). Different lowercase letters above the bars indicate significant difference within the dark-, BL- or RL-grown seedlings (ANOVA, $P<0.05$). * - significantly different from the values obtained for dark-grown plants (t-test, $P<0.05$).

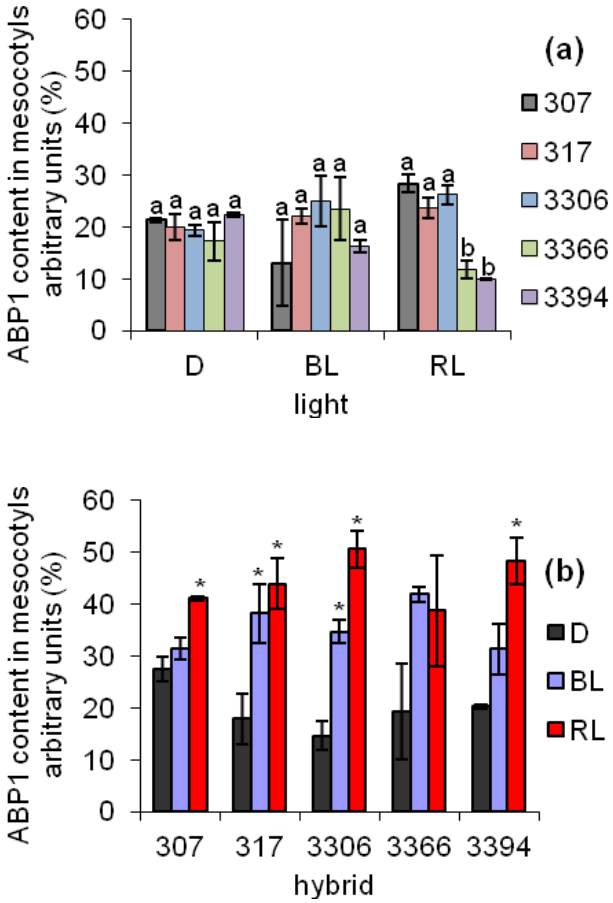


Fig. 26 **Accumulation of ABP1 in mesocotyls. (a) The comparison among the hybrids grown in the assigned light conditions. (b) The effect of light on the protein accumulation in the individual hybrids.**
Data are expressed as percentage of the total area of all signals present on the gel. The signal area was determined by ImageJ software. Results represent averages ± SE obtained from two independent experiments each with two technical replicates (n = 4). Different lowercase letters above the bars indicate significant difference within the dark-, BL- or RL-grown seedlings (ANOVA, $P<0.05$). * - significantly different from values obtained for dark-grown plants (t-test, $P<0.05$).

4.3.4 Sequence analysis of *ABP1* gene in the old hybrid 307 and the modern hybrid 3394

After determination of *ABP1* transcript and protein accumulation, cDNA of *ABP1* gene was cloned by reverse transcription PCR from corresponding mRNA and its sequence compared between the oldest hybrid 307 and the modern hybrid 3394. This analysis

revealed no difference in the *ABP1* gene sequence (sequences not shown) and consequently in the sequence of the corresponding translated proteins between the hybrids (Fig. 27).

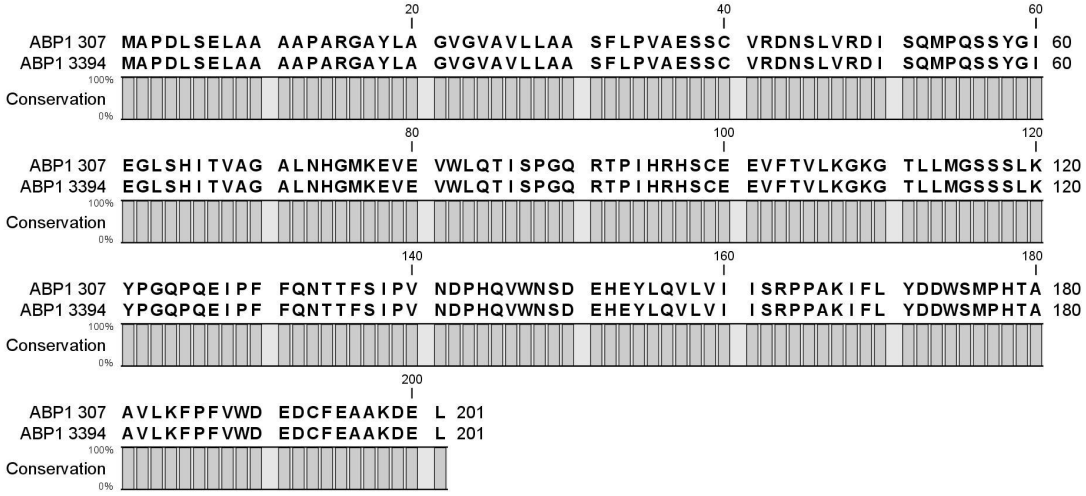


Fig. 27 **Comparison of ABP1 amino acid sequences between the oldest hybrid 307 and the modern hybrid 3394**

4.4 THE ANALYSIS OF THE MAIZE AUXIN-BINDING PROTEIN MUTANTS

In the search for the role of auxin-binding proteins in the BL signaling pathway, further experiments were done by using the auxin-binding protein mutants: the single mutants *abp1* and *abp4* and the double mutant *abp1abp4*, together with a corresponding wild type (WT). Plants were grown in the same conditions as hybrid plants in previous experiments and analyzed at the same age.

4.4.1 BL-induced growth responses of maize *abp* mutants

When plants were grown in the dark, seedlings of the *abp1* mutant possessed the longest shoot (coleoptile + mesocotyl) and shoots of the double mutant *abp1abp4* were the shortest. The length of shoots of the single mutants and WT plants was not found to be significantly different, but double mutant developed significantly shorter shoots than WT as well as single mutants seedlings. In the continuous BL, the longest shoots were observed in WT plants, and the shortest again in the double mutant. The difference between the length of WT shoots and *abp1abp4* shoots was 28%.

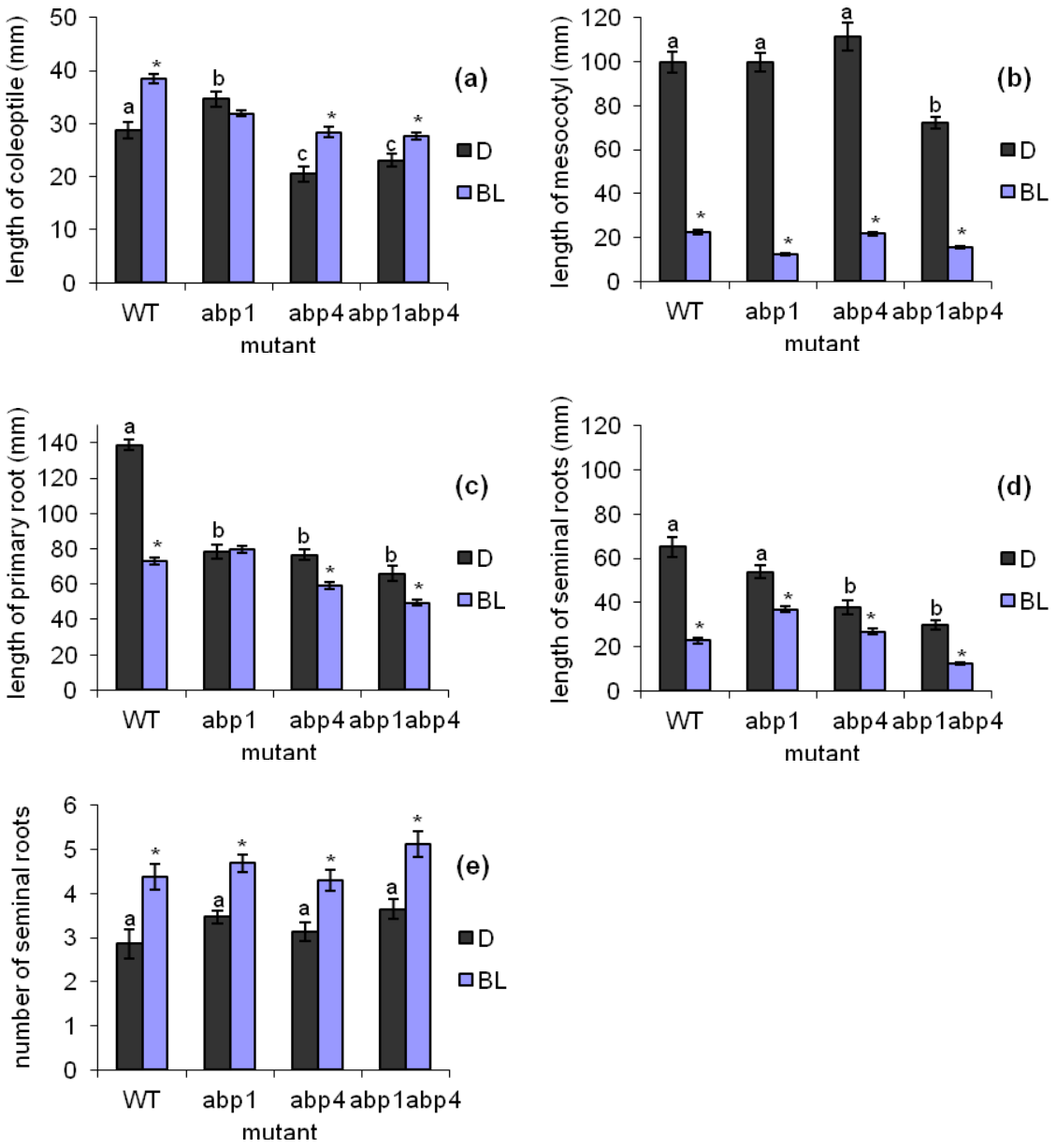


Fig. 28 **Lengths of coleoptiles (a), mesocotyls (b), primary root (c), seminal roots (d) and the number of seminal roots (e) of maize *abp* mutants compared to WT grown in the dark and BL**

Data represent averages ± SE obtained in 5 independent experiments, together 20-37 plants. Different lowercase letters above the bars indicate significant difference within the dark-grown seedlings (ANOVA, $P<0.05$). * - significant difference between BL-grown and dark-grown seedlings of one genotype (t-test, $P<0.05$).

The *abp1* mutant developed the longest coleoptiles, whereas the shortest coleoptiles were observed in the single mutant *abp4* and double mutant *abp1abp*. BL stimulated the

coleoptile elongation in the WT, *abp4* and *abp1abp4* mutant, but not in *abp1* mutant (Fig. 28a). In the dark, double mutant developed markedly shorter mesocotyls than those of the other studied genotypes, which reached the similar length. BL greatly inhibited the elongation of mesocotyls in all genotypes (Fig. 28b). The percentage of the inhibition was similar in all genotypes: WT and *abp1abp4* 78%, *abp1* 88%, *abp4* 81%.

Primary roots of the mutants grown in the dark were all about half shorter than those of WT plants. In BL, roots of WT seedlings were about 47% shorter than in the dark, whereas in the *abp4* and *abp1abp4* mutants they were about 25% shorter. The length of *abp1* primary roots was similar to that observed in etiolated plants (Fig. 28c).

Etiolated seedlings of the *abp4* and *abp1abp4* mutants developed shorter seminal roots than WT and *abp1* seedlings, however all genotypes produced a similar number of seminal roots (Fig. 28d,e). BL inhibited the elongation of seminal roots in all genotypes, especially in WT seedlings (65%), whereas the least inhibition was found in the *abp4* mutant (29%) (Fig. 28d). The effect of BL on the production of seminal roots was found similar in all genotypes: BL stimulated their formation from 35% in the *abp1* mutant to 53% in WT (Fig. 28e)

4.4.2 The effect of BL on the accumulation of free IAA in maize *abp* mutants

The analysis of the free IAA accumulation in coleoptiles, mesocotyls and primary roots was performed in 4-day-old seedlings grown on the BM in the dark or BL.

Coleoptiles of etiolated seedlings of WT and the single mutants contained similar amounts of free IAA, however in the double mutant the IAA content was much higher (Fig. 29a). BL reduced free IAA accumulation in the coleoptiles of all genotypes, except for the mutant *abp4*. The strongest BL-induced reduction of IAA accumulation was observed in the coleoptiles of the double mutant *abp1abp4* (82%), while in the *abp1* mutant it was 51%, and in WT 60% (Fig. 29a).

When seedlings were grown in the dark, mesocotyls of *abp1* mutant contained significantly more free IAA than those of the double mutant, whereas mesocotyls of WT and *abp4* plants contained a similar amount of IAA than those in *abp1* and in the double mutant. BL was found to trigger different changes in IAA level in the mesocotyl tissues of studied genotypes.

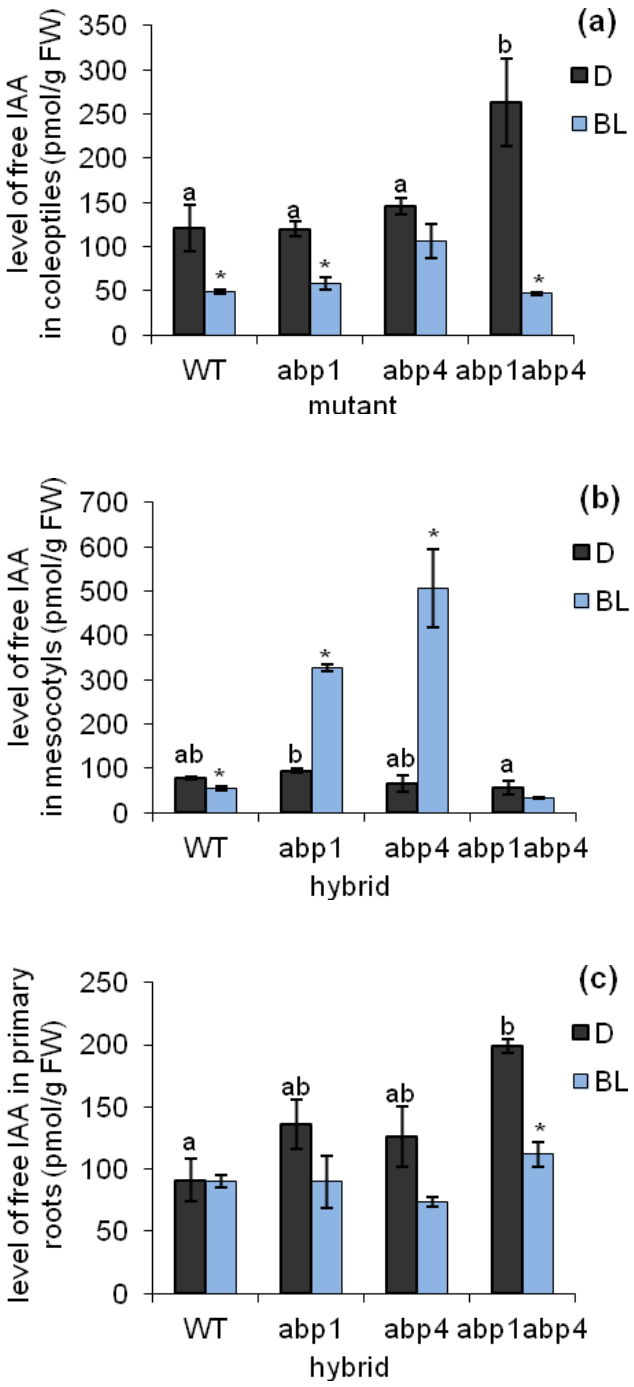


Fig. 29 **Amount of endogenous free IAA in coleoptiles (a), mesocotyls (b) and primary roots (c) of 4-days-old *abp* mutant seedlings grown in the dark and BL**
Data represent averages ± SE obtained from three experiments performed at the same time. Different lowercase letters above the bars indicate significant difference within the dark-grown seedlings (ANOVA, $P<0.05$). * - significantly different from values obtained for dark-grown plants (t-test, $P<0.05$).

BL reduced IAA content in WT mesocotyls, strongly increased IAA in *abp1* and *abp4* mesocotyls, whereas it did not affect IAA accumulation in *abp1abp4* mesocotyls (Fig. 29b). In BL-grown *abp1* mesocotyls, free IAA content was more than 3 times higher than in dark-grown ones, and in the *abp4* mesocotyls it was more than 7 times higher.

Primary roots of single mutants and WT grown in the dark contained similar amounts of free IAA, whereas the double mutant accumulated more IAA in roots than other genotypes (Fig. 29c). BL decreased the IAA accumulation in primary roots of the double mutant, while the roots of other genotypes grown in BL contained similar amounts of free IAA as those grown in the dark (Fig. 29c)

Generally, etiolated *abp1abp4* seedlings (a whole seedling, except the seminal roots) contained the highest amount of free IAA, whereas WT seedlings contained the lowest amount of free IAA, which did not greatly differ from the values obtained for *abp1* and *abp4* seedlings (Tab. 3). The effect of BL on the accumulation of free IAA was found to be varied, depending on the genotype. BL-grown seedling of WT and double mutant *abp1abp4* contained less free IAA than etiolated seedlings. By contrast, BL increased the free IAA accumulation in seedlings of *abp1* and *abp4* mutants (Tab. 3).

Tab. 3 **Average values of free IAA amounts detected in whole 4-day-old seedlings** Values represent the sum of the average values obtained for coleoptiles, mesocotyls and primary roots of individual genotypes grown in assigned light conditions.

Genotype	**Free IAA content [pmol/g fresh weight (FW) ± SE]**		**IAA increase (+) or decrease (-) [%]**
	Dark	**BL**	**BL**
WT	289	195	- 33
abp1	351	476	+ 37
abp4	338	685	+ 103
abp1abp4	518	192	- 63

5 DISCUSSION

With a world production of 818 millions of tones, maize (*Zea mays* subsp. *mays*) is the second most important crop in the world (source: FAO, 2010; http://faostat.fao.org). Consequently, several breeding programs have been established, making the grain yield one of the targets of a selection. Fellner et al. (2003, 2006) proposed that the differences in grain yield between the old and modern Pioneer Hi-Bred hybrids in high density planting may be explained by different responses to R and/or FR light, which in turn affect auxin distribution and/or sensitivity, and consequently affect leaf declination. The goal of the presented Ph.D. thesis was formulated on the basis of this proposition. Therefore, a cross-sensitivity of young seedlings of the old and modern Pioneer hybrids to auxin and light was examined and the hypothesis that the modern hybrid 3394 and the old hybrids differ basically in auxin-related properties, which involve ABP and/or light signaling pathway(s) was tested.

5.1 PHENOTYPIC TRAITS IN OLDER AND MODERN HYBRIDS

5.1.1 Development of leaf angle

The analysis of leaf angle development of juvenile plants confirmed that all examined old hybrids that were grown in the greenhouse have leaves less erect than the modern hybrid 3394. It is likely that during the breeding process, which was focused on increasing the productivity, the leaf declination was affected (see also Ford et al. 2008). Relationship between the productivity and leaf angle was suggested more than forty years ago. Indeed, Pendleton et al. (1968) observed that a backcross-derived isogenic single cross hybrid carrying the Ig_2 gene responsible for erect leaves produced 40% more grain than control plants with horizontally oriented leaves. Moreover, when they mechanically manipulated leaf positioning of Pioneer hybrid 3306 (used also in this study) into more upright, the yield of plants with all leaves tied was 6.5% higher, and the yield of plants with upright leaves only above the ear was 14.2% greater than the yield of non manipulated control plants. This kind of leave arrangement allows better distribution of light energy to the lower layer of the canopy and apparently contributes to the higher grain yield (Pendleton et al. 1968, Duncan 1971, Long et al. 2006). The fact that erect positioning of

leaves increase the biomass production and the grain yield is well known from the studies made on rice, which showed that upright leaves reduce damages on photosystem II caused by excess light and allow a better penetration of light toward the basal leaves (Murchie et al. 1999). In rice leaf, the positioning was found to be regulated by brassinosteroids and several rice "brassionosteroid" mutants showed erect leaf phenotypes and higher yield (Morinaka et al. 2006, Li et al. 2009). However, erect leaves do not enhance yield in barley, thus breeding for erect leaf angle does not seem to be an effective tool for improving yield in all cereal crops (Tungland et al. 1987).

5.1.2 Development of etiolated seedlings

The analysis of the seedling growth responses revealed several differences between the old and modern maize hybrids. When compared to all old hybrids, aerial organs of etiolated seedlings of the modern hybrid 3394 showed a reduced growth and sensitivity to NAA, what is in agreement with Fellner et al. (2003, 2006). These results suggest that the long-term selection process of modern hybrid with erect leaves probably affected the auxin responsiveness in the hybrid 3394 shoots, but not in roots. In an effort to find the explanation for this trait, quantification of free IAA and estimation of ABP1 protein accumulation in the separated organs was performed. Since no significant difference was found in the accumulation of free IAA among the studied hybrids, the short stature of the hybrid 3394 seedlings could be the result of a decreased responsiveness to auxin. However, the decreased auxin responsiveness does not seem to be mediated by ABP1-dependent signaling pathway, because its amount examined by Western blot did not differ among the old and modern hybrids in both aerial organs – coleoptiles and mesocotyls. Fellner et al. (2006) reported that the old hybrid 307 and the modern hybrid 3394 differ in the expression of *ABP4* gene in mesocotyls, therefore, it is tempting to consider that reduced elongation and responsiveness of dark-grown 3394 mesocotyls to exogenous auxin is associated with ABP4. The role of ABP1 and ABP4 proteins during the development of etiolated maize seedlings was further investigated using *abp1* and *abp4* single and double mutants. However, only etiolated plants of the double mutant *abp1abp4* exhibited reduced growth of coleoptiles and mesocotyls, whereas all *abp* mutants produced significantly shorter primary root than WT plants. Thus, functional *ABP1* or *ABP4* seems to be required for normal

development of primary root, but not for the shoot of etiolated seedlings. Because etiolated roots of maize hybrids were found to be much more responsive to the NAA than their shoots, a high sensitivity of roots to auxin may explain the more striking role of ABPs in this organ. The maize gene family coding for auxin-binding proteins consist of at least five genes and their functions and/or interactions are not known so far (Schwob et al. 1993). From the obtained results, it becomes clear that knocking out a single *ABP* gene does not greatly affect the shoot development of etiolated seedling, hence the reduced responsiveness and sensitivity to auxin observed in hybrid 3394 shoots seems to be under the control of a complex mechanism requiring more mediators and their interactions. Auxin-binding proteins are not the only auxin receptors present in maize; the responsiveness to auxin may be modulated for example through the *tir*-like gene, which expression was shown in maize shoot apical meristem (Zhang et al. 2007).

5.2 PHOTOMORPHOGENIC GROWTH RESPONSES OF MAIZE HYBRIDS

In the presented study, coleoptiles of light-grown seedlings (BL or RL) were longer than those of etiolated plants in almost all hybrids tested (except the hybrid 307). On the other hand, light greatly inhibited the growth of mesocotyls. The inhibitory effect of light on mesocotyls is a well known process, which represents a component of photomorphogenic developmental program (e.g. Vanderhoef et al. 1979, Markelz et al. 2003). However, the effect of light on the elongation of coleoptiles is not comprehensive and depends on the stage of development of the organ. A coleoptile is the organ, which elongation may be stimulated by light at the early stage of growth, but on the contrary, it can be inhibited in its growth at the later stage (Thomson 1950). Light induced promotion of coleoptile elongation and simultaneously the inhibition of mesocotyl growth was already observed in oat (Blaauw et al. 1968, Schneider 1941) and maize (Warner et al. 1981). Data obtained in the presented work show that BL- as well as RL-grown coleoptiles of all hybrids reached similar length. Interestingly, the light-grown mesocotyls (either BL or RL) of all old hybrids were significantly shorter than the mesocotyls of the modern hybrid 3394. Hence, it is obvious that 3394 mesocotyl is less sensitive to the inhibitory effect of light on elongation, and probably this was one of the traits targeted during the breeding selection. It

is known that light signaling pathways represent a promising target for the crop improvement (Ballaré 1999, Markelz et al. 2003), thus it is tempting to speculate that the breeding strategy used for Pioneer hybrids caused alterations of photomorphogenic responses, which contributed to the increase in yield.

Although photomorphogenic growth changes are obvious particularly in the aerial part of the seedling, light is controlling also the development of the roots (Feldman 1984). However, reports describing the effect of light on the root elongation are contradictory. White light was shown to inhibit root elongation e.g. in rice (Ohno and Fujiwara 1967), pea (Torrey 1952), wheat and maize (Wilkins et al. 1974, Pilet and Ney 1978). Ohno and Fujiwara (1967) reported that BL inhibits both cell elongation and cell division in rice primary roots, but RL inhibited only the cell elongation. On the other hand, BL as well as RL were found to stimulate the elongation of primary root in Arabidopsis and the shoot localized cryptochrome CRY1 was shown to function as a receptor involved in this mechanism (Canamero et al. 2006). Furthermore, the biochemical explanation of light-induced root elongation in Arabidopsis has been recently provided by Dyachok et al. (2011). The authors found that ARP2/3-SCAR complex is required for the light-induced promotion of root elongation, and the light signal is perceived by root photoreceptors. In this study, the effect of light on the elongation of maize hybrids' roots was variable. BL was found to inhibit the growth of primary roots in two hybrids (317 and 3366) and the growth of seminal roots in three hybrids (317, 3366, and 3394). RL inhibited growth of primary roots in hybrid 317, but stimulated the growth in hybrids 3306 and 3394 and had no effect in two other hybrids (307, 3366). Taken together, for the primary roots as well as seminal roots, no trends that could be directly related to the breeding strategy were observed; the effect of light on the root elongation seems to be highly dependent not only on the plant species but also on the variety within a single genus. Similar conclusion can be made about the effect of light on the production of seminal roots. Both BL and RL stimulated seminal roots production in the hybrids 3306 and 3394, whereas the hybrids 317 and 3366 produced more seminal roots only in RL. Thus the modern hybrid does not differ from the old hybrids in this response; and the results indicate that during the selection process the root system was not specifically affected. This conclusion is further supported by other data discussed below.

5.3 LIGHT AND AUXIN INTERACTION DURING DEVELOPMENT OF MAIZE HYBRIDS

Photomorphogenesis is a process closely related to auxin action. Interaction of auxin and light signaling pathways is known to play a role in the development of vegetative tillers and shade-avoidance responses in grasses (Kebrom and Brutnell 2007). Domestication of maize eventuated in suppression of vegetative tillers (Doebley et al. 1995, 1997), a typical shade-avoidance response occurring in grasses that is mediated by phytochrome photoreceptors, which provides a striking evidence about an artificial manipulation of the auxin-light signaling (Kebrom and Brutnell 2007). In this study, light and auxin interaction was investigated in maize hybrids grown in BL and RL at various concentrations of exogenous auxin.

BL enhanced the responsiveness (an extend of the response to the same concentration of NAA) of coleoptile to exogenous auxin in the modern hybrid, whereas the opposite effect was observed in the old hybrids. This effect was even more striking in mesocotyls, where BL enhanced not only the responsiveness, but also sensitivity (an ability of certain concentration of NAA to induce some response) to auxin in the modern hybrid 3394. Similarly to BL, RL reduced the sensitivity or responsiveness to auxin in coleoptiles of the old hybrids, but not in the modern hybrid. Mesocotyls of the 1930s' hybrids (307, 317) as well as mesocotyls of the hybrid 3394 grown in RL completely lost their sensitivity to the exogenous auxin, whereas it was partially retained in the hybrids from 1960s (3306, 3366). From the presented data, it appears that the responsiveness of maize seedling to the exogenous auxin is a light-dependent process, like it was described for the moss (*Physcomitrella patens* (Hedw.) Bruch & Schimp.) (Imaizumi et al. 2002), oat (*Avena sativa* L.) mesocotyl sections (Kondo et al. 1969) and pea plants (*Pisum sativum* L.) (Galston and Baker 1953). Moreover, both BL and RL signaling pathways share transcription factors HY5 and HYH, which regulate the process of de-etiolation along with regulation of sensitivity to auxin through the regulation of auxin signaling pathway (reviewed in Halliday et al. 2009, Stewart and Nemhauser 2010). Apparently, the impact of light on the sensitivity to auxin became one of the events affected during the breeding process. According to data obtained in this work, the effect of BL on auxin sensing had

reversed from suppression in the old hybrids to enhancement in the modern hybrid. Interaction of RL and auxin signaling pathway in the modern hybrid 3394 was altered only in the coleoptile, but not in mesocotyl tissues. Therefore, the modification of BL and auxin interaction seems to be highly profitable for the maize yield improvement, and consequently, for the enhancement of the tolerance to crowded conditions.

The effect of light on the root sensitivity to NAA did not differ between the old hybrids and the modern hybrid. However, an interesting observation was that RL increased the sensitivity to NAA in the primary roots of all hybrids except for the oldest one – 307. Moreover, in the hybrids 3306 and 3394, light enhanced the responsiveness of primary roots to the NAA. The topic of the interaction of light and auxin signaling pathways during the root development has not been studied in details yet; a solitary report by Eliasson and Palén (1972) shows that WL enhances the inhibitory effect of 2,4-D (synthetic auxin) and NAA on the elongation of roots in pea, but not in wheat. The interaction of auxin and light signaling pathway in the process of initiation of seminal roots is also not well understood up to now. In rice, the role of auxin in this process is known to be important at several levels – biosynthesis, transport and signaling (McSteen 2010). Exogenous auxin was shown to induce crown root initiation (rice analogs of maize seminal roots), where *CRL1* (*CROWN ROOTLESS1*) gene was shown to be a positive regulator (Inukai et al. 2005). A maize ortholog of *CRL1* is *RCTL* (*ROOTLESS CONCERNING CROWN AND SEMINAL ROOTS*) gene, which shows an auxin-inducible expression, being crucial for the embryonic seminal and post-embryonic shoot-borne roots initiation (Taramino et al. 2007, Hetz et al. 1996). In this work, exogenous auxin induced production of seminal roots in all studied hybrids when grown in light, but etiolated seedlings of the oldest hybrid 307 were completely non sensitive to NAA. Light was found to affect the NAA-induced production of seminal roots also in other hybrids, especially in the modern hybrid 3394, which showed greatly increased sensitivity to auxin in BL. Therefore, it might be concluded that light and auxin signaling pathways cooperate in the regulation of seminal root initiation in maize, but this cooperative mechanism was not systematically affected during the breeding of examined hybrids. The cooperation of light and auxin signaling in the regulation of adventitious rooting was already described in Arabidopsis, and a major regulator of this process seems to be the auxin response factor ARF17, which negatively regulates adventitious rooting

through the *GH3* genes in a light-dependent manner (Sorin et al. 2005). It is possible that a similar mechanism controls the seminal roots initiation in monocots.

5.4 THE ROLE OF FREE IAA IN THE LIGHT-REGULATED DEVELOPMENT OF MAIZE HYBRIDS

The mechanism of inhibitory effect of light on the mesocotyl elongation became the subject of investigation more than 70 years ago. Since it was found that RL reduces diffusible auxin obtained from a coleoptile, it was postulated that RL inhibits the elongation of mesocotyl by reducing free IAA supply from coleoptiles (Huisinga 1976, Iino 1982a,b, Iino and Carr 1982a,b). A decreased content of free IAA was detected also in apical parts of RL-irradiated mesocotyls, where RL-induced alterations in lateral distribution of auxin within the mesocotyl were confirmed (Iino 1982b, Jones et al. 1991). The RL-induced reduction of free IAA content in the maize coleoptile tip was demonstrated to be caused by the inhibition of IAA biosynthesis from tryptophan (Iino 1982a, Koshiba et al. 1995, Nishimura et al. 2006). However, not all the works done in this field can support this hypothesis (e.g. Schneider 1941, Mer 1951, Kondo et al. 1969).

In this study, light reduced the free IAA content in maize coleoptiles. Especially in RL, decreased levels of free IAA were detected in all hybrids tested, which is consistent with other reports (e.g. Iino 1982a, Koshiba et al. 1995, Nishimura et al. 2006). However, in BL, accumulation of free IAA was reduced only in the coleoptiles of old hybrids, but not in the modern hybrid 3394. This observation thus provides another supporting evidence for the altered BL signaling pathway in the modern hybrid 3394. Since a coleoptile growth was stimulated by light almost in all hybrids and the level of free IAA was oppositely decreased by light, it may be concluded that the elongation of light-grown coleoptiles is not a result of free IAA content changes, what corresponds with the investigations made by Iino (1982a). In the mesocotyl tissue, the BL- and RL-induced reduction of free IAA content was detected only in the hybrid 3306 but not in the other ones. The effects of BL or RL on the elongation of mesocotyls cannot be explained solely by changes in free IAA content, because it does not seem to be a trait common to all varieties of maize. However, the possibility cannot be excluded that light affects the lateral distribution of auxin, since it decreases its content in the epidermal tissue that controls the growth rate of the shoot (Jones

et al. 1991, Barker-Bridgers et al. 1998). Apparently, the light-suppressed sensitivity and responsiveness to auxin (discussed above) takes a part in the inhibition of mesocotyl elongation of light-grown maize plants (Galston and Baker 1953). Additionally, WL greatly reduced free IAA levels in all hybrids' coleoptiles as expected, but surprisingly reduced free IAA amounts also in the mesocotyls of all hybrids except the hybrid 307. It is therefore likely that the regulation of IAA metabolism in this organ is under a complex control of BL and RL.

Accumulation of free IAA in primary roots was similar for all hybrids grown in the dark, BL or RL. Similarly to the coleoptiles and mesocotyls, there was not found the correlation between the growth and free IAA content in the roots, thus the elongation rate of these organs does not seem to be controlled solely by auxin. WL reduced IAA accumulation only in the roots of hybrid 3066, in which light was found to be more efficient to regulate auxin metabolism also in shoots than in the other hybrids.

5.5 THE ROLE OF ABP1 IN THE LIGHT-REGULATED DEVELOPMENT OF MAIZE HYBRIDS

In an effort to reveal the role of ABP1 protein during the light-driven development of maize hybrids, the analysis of the amount of ABP1 protein as well as the analysis of corresponding gene expression was performed.

Obtained data showed no clear correlation between *ABP1* gene expression and protein content. However, these results are not surprising since the post-transcriptional regulation of ABP1 content was already mentioned and the role of ABP4 in this process evoked by Im et al. (2000). Comparison of ABP1 protein content among the hybrids did not correlate with their differential light or auxin-induced growth responses. Therefore, a direct role of ABP1 in the mediating light-induced and simultaneously auxin-dependent processes during the maize seedling development was not confirmed. Functional ABP1 protein was shown to be involved in number of auxin-mediated processes, such as cell expansion, cell division or cell cycle in a context-dependent manner (David et al. 2007, Braun et al. 2008, Tromas et al. 2009). Conditional inactivation of ABP1 in tobacco plants led to a decrease in transcript levels for several *Aux/IAA* genes, which are known as active repressors of auxin-induced gene expression (Braun et al. 2008, Tiwari et al. 2001). It is likely that the presence

of higher ABP1 activity found in light-grown mesocotyls leads to the increase of *Aux/IAA* expression and consequently to the downregulation of the plant sensitivity to auxin and reduced growth. Although the function of ABP1 is generally considered to be positively involved in the elongation, a detailed investigation of this possible regulatory mechanism of photomorphogenesis may uncover new facts in the ABP1–auxin receptor story that has not been resolved during more than 30 years of research. An interesting observation about the reduction of auxin-induced accumulation of ABP1 transcripts in BL-grown mesocotyls (when compared to dark-grown ones) brings the first evidence about the interaction of auxin and BL signaling pathway being possibly mediated through the *ABP1*, but it does not help to elucidate the role of the protein during the development of maize hybrids.

Because no clear correlation between ABP1 protein content, free IAA content and growth responses influenced by BL, RL or NAA was found, the modification of auxin sensing during the breeding process of maize hybrids, observed in this study, does not seem to be directly related to ABP1 protein. However, the light-induced suppression of sensitivity to auxin in rice is known to be mediated through the jasmonate signaling pathway (reviewed in Nick 2006), therefore it is possible that a similar regulatory mechanisms may occur in maize.

5.6 THE ROLE OF ABP1 AND ABP4 DURING THE BL-REGULATED GROWTH OF MAIZE SEEDLINGS

As mentioned above, the complicating element in determination of ABP1 function in the development of maize seedlings is the presence of other auxin-binding proteins. Therefore, the maize *abp* mutants represent a promising research tool. Three maize *abp* mutants prepared by Im et al. (2000) were explored in this work in regards to their growth responses and free IAA accumulation in the dark and BL.

Single mutation in *abp1* or *abp4* gene did not greatly influence the growth of etiolated shoots, but the double mutant *abp1abp4* produced about one quarter shorter shoots than WT plants. Thus, it indicates that maize ABP1 and ABP4 are positively involved in the elongation growth of etiolated shoot tissues, but their functional redundancy makes their action hardly distinguishable. Both ABP1 and/or ABP4 proteins seem to be involved more in the root growth regulation machinery than in shoot development, since all etiolated

mutants produced significantly shorter primary roots than WT. The functional redundancy is obvious also in this organ, because primary root of the double mutant *abp1abp4* possessed the same length as the roots of single mutants *abp1* and *abp4*. The effect of BL on the development of mutant seedlings was altered only in the *abp1* single mutant, whose coleoptile and primary root were insensitive to BL-induced growth changes, in contrast to the other mutants and WT. Similarly to hybrids, the accumulation of free IAA in the mutants did not correlate with the growth of the individual organs. However, ABPs were found to be involved in the free IAA homeostasis, what provides a novel insight into their role in developing maize seedlings. Single ABP1 or ABP4 itself seems to down-regulate accumulation of free IAA in the etiolated WT coleoptiles, but not in mesocotyls and primary roots. The most surprising observation brought the analysis of free IAA content in BL-grown seedlings, especially in the mesocotyl. While BL decreased the IAA content in WT and did not affect IAA accumulation in the double mutant, mesocotyls of single mutants contained 3 – 7 times more IAA than those grown in dark. Thus, it is possible that ABP1 and ABP4 interaction promotes the BL-induced reduction of free IAA accumulation in WT mesocotyls, but when both genes/proteins are non-functional other maize ABPs complement their action, although less effectively. Moreover, it is likely that the presence of ABP1 or ABP4 prevents the action of other ABPs in mediating the BL effect on free IAA accumulation and this regulatory mechanism is tissue-dependent. Furthermore, ABP4 seems to be involved in the BL-induced changes in free IAA content in coleoptiles, and both ABP1 and ABP4 regulate auxin homeostasis in the BL-grown roots. The role of ABP1 in the mediation of auxin availability was already observed in tobacco cell cultures (Chen et al. 2006), and very recently ABP1 was found to be involved also in the auxin transport (Robert et al. 2010, Effendi et al. 2011).

6 Conclusions

In this work, four old Pioneer hybrids (307, 317, 3306 and 3366) and one modern hybrid (3394) were analyzed in an effort to reveal physiological changes acquired during the selection of the modern hybrid, which has been selected for the higher yield in HD planting conditions. A phenotypic analysis of the juvenile seedlings revealed that this hybrid develops more erect leaves with a smaller leaf area than the seedlings of all old hybrids. Results from the analysis of auxin- and light-induced growth responses of the hybrids indicate that the selection of modern hybrid altered light and auxin signaling pathways. When comparing to the old hybrids, a shorter stature of the etiolated hybrid 3394 shoots is very likely the result of a decreased responsiveness to auxin, which does not seem to be mediated by ABP1. Especially, an interaction of BL and auxin signaling pathways was found to be altered in the modern hybrid, which in contrast to the old hybrids showed an increased sensitivity and responsiveness to auxin under BL. These results suggest that the modification of light and auxin signaling pathways might represent a promising target for the crop improvement.

This study contributed also to the understanding of the role of light, auxin, their interactions and the role of ABP1 and ABP4 proteins during the development of young maize seedlings. Auxin action on the elongation of maize shoot and root was found to be regulated by light. Furthermore, light and auxin signaling pathways cooperated in the regulation of the seminal roots initiation. The search for the role of ABP1 and ABP4 proteins was carried out using the maize *abp* mutants. Obtained data suggest that ABP1 or ABP4 (separately) are required especially for the normal development of primary roots, but not for the shoots of etiolated maize seedling. Functional ABP1 seems to be involved in the BL-induced growth responses of maize coleoptiles and primary roots, and single ABP1 or ABP4 are important for the tissue-dependent regulation of free IAA homeostasis in BL-grown seedlings. In hybrids, BL decreased the auxin-stimulated expression of *ABP1* gene in mesocotyls, but the protein level seems to be regulated differently by an unknown post-transcriptional mechanism. The function and action of maize ABPs is still far from being understood, and I hope that this work will pave the way for the further research, which is needful for the elucidation of a role of these proteins that seems to be involved in various essential processes.

7 Závěr

Ve snaze odhalit fyziologické změny získané při šlechtení moderního hybridu 3394 byly v této práci analyzovány čtyři dřívější hybridy firmy Pioneer (307, 317, 3306 a 3366) a jeden moderní hybrid (3394), který byl šlechtěn na vyšší produkci v podmínkách husté výsadby. Fenotypová analýza mladých rostlin prokázala, že moderní hybrid má menší a více vzpřímené listy než všechny dřívější zkoumané hybridy. Výsledky analýzy auxinem- a světlem-indukovaných růstových reakcí ukazují, že v průběhu selekce moderního hybridu došlo ke změně v signálních drahách světla a auxinu. V porovnání se staršími hybridy, byl růst etiolovaných rostlin hybridu 3394 výrazně redukovaný, a je pravděpodobné, že jde o důsledek snížené citlivosti k auxinu, která se ale nezdá být zprostředkována ABP1. Bylo zjištěno, že u moderního hybridu došlo k změně zvláště v interakci signálních drah modrého světla a auxinu, protože na rozdíl od starších hybridů, modré světlo zvýšilo citlivost k auxinu v jeho nadzemních orgánech. Tyto výsledky naznačují, že změny v signálních dráhach světla a auxinu a jejich interakce mohou představovat slibný cíl pro šlechtění rostlin.

Tato práce přispívá také k pochopení úlohy světla, auxinu, jejich vzájemné interakce a úlohy ABP1 a ABP4 při vývoji mladých klíčenců kukuřice. Bylo zjištěno, že modré a červené světlo reguluje auxinem-indukované růstové reakce ve všech orgánech mladých rostlin. Signální dráhy světla a auxinu interagují také při regulaci produkce seminálních kořenů kukuřice. Pro objasnění úlohy ABP1 a ABP4 proteinů při vývoji klíčenců kukuřice byly použity *abp* mutanty, a to „single“ mutanti *abp1* a *abp4* a „double“ mutant *abp1abp4*. Získaná data ukazují, že jednotlivě ABP1 nebo ABP4 jsou nezbytné pro normální vývoj primárních kořenů, ale ne výhonků etiolovaných rostlin. Funkční ABP1 se zdá být zapojen do růstových odpovědí koleoptylů a primárních kořenů indukovaných modrým světlem. ABP1 nebo ABP4 jednotlivě jsou důležité pro regulaci obsahu endogeního auxinu v rostlinách rostoucích na modrém světle, ale tato funkce závisí na typu rostlinného pletiva. U hybridů, modré světlo snižuje auxinem stimulovanou expresi *ABP1* genu v mezokotylech, ale obsah proteinu je regulován jiným neznámým post-transkripčním mechanismem. Funkce a činnost ABP u kukuřice ještě nejsou zdaleka známy, a proto doufám, že tato práce připraví půdu pro další výzkum, který je potřebný pro objasnění role těchto proteinů, které se zdají být zapojeny do základních procesů při vývoji a růstu rostlin.

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9 Abbreviations

(q)RT-PCR – (quantitative) reverse transcription – polymerase chain reaction

ABP – auxin binding protein

BL – blue light

BM – basal medium

CRY – cryptochrome

D – dark

ER – endoplasmic reticulum

FR – far red

HD – high density

HIRs – high irradiance responses

IAA – indole-3-acetic acid

IPA – indole-3-pyruvic acid

LD – low density

LFRs – low-fluence responses

LOV - light oxygen voltage

NAA – 1-naphthalene acetic acid

NPA – 1-naphthylphthalamic acid

PAT – polar auxin transport

Pfr – far red light absorbing phytochrome

PHOT – phototropin

PHY – phytochrome

PM – plasma membrane

Pr – red light absorbing phytochrome

R(L) – red (light)

SAS – shade avoidance syndrome

Trp – tryptophan

VLFRs – very low-fluence responses

WL – white light

WT – wild type

ZTL – zeitlupe

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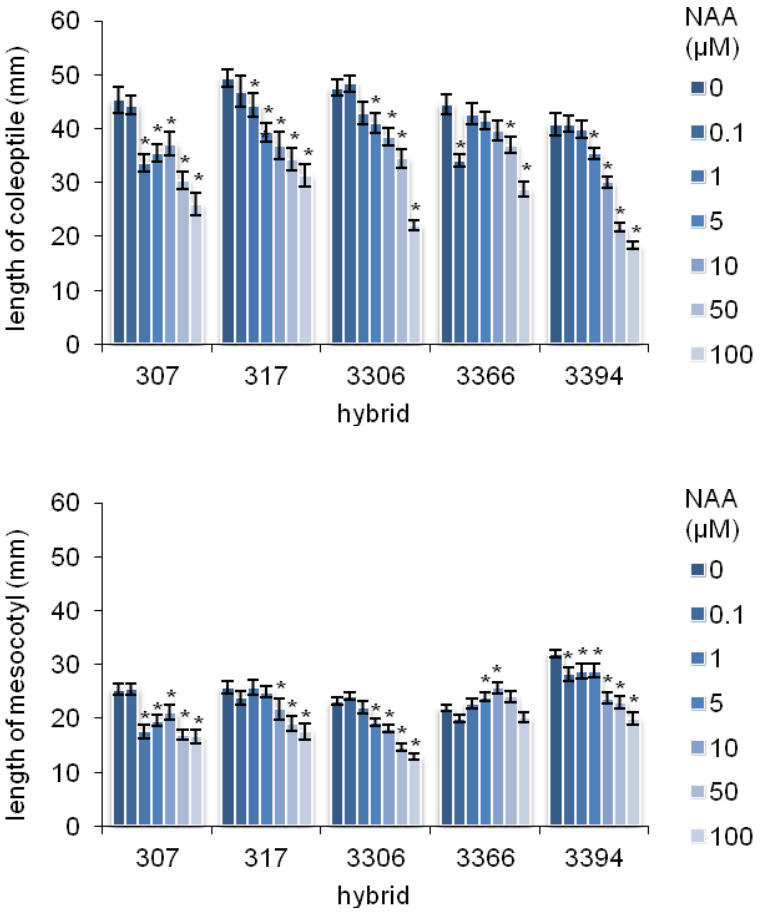
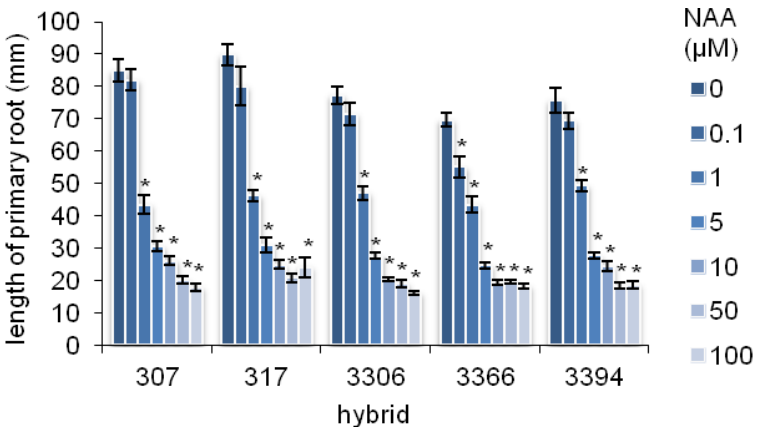
13 APPENDIX

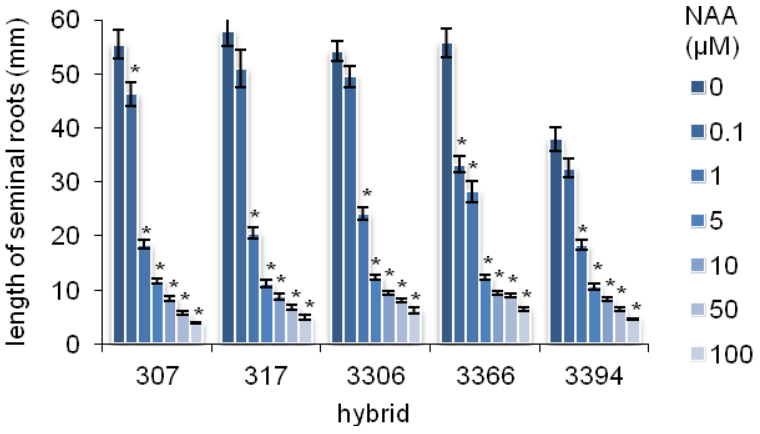
Supplemental Figure 1

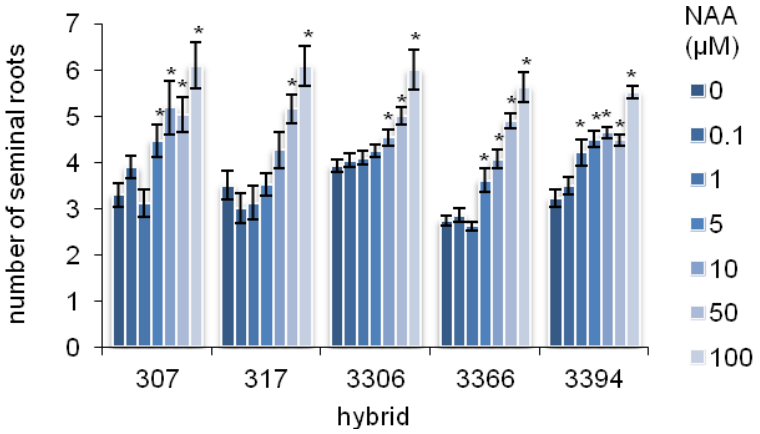
The graphs of absolute lenghts of organs of 4-day-old maize seedlings that were grown in BL in presence or absence of exogennous axin – NAA

Data represent averages ± SE obtained in 5 independent experiments, together 20-37 plants. * - significantly different from values obtained for control plants grown in absence of NAA (t-test, $P<0.05$).




length of primary root (mm)
100
90
80
70
60
50
40
30
20
10
0
307
317
3306
3366
3394
hybrid
NAA (μM)
0
0.1
1
5
10
50
100

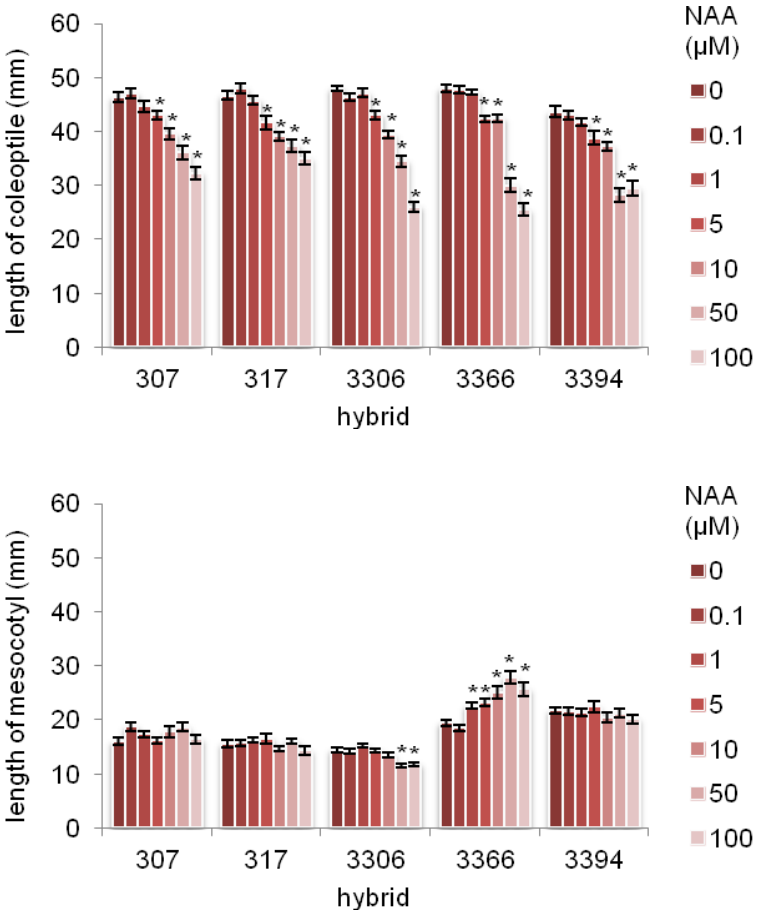

length of seminal roots (mm)
60
50
40
30
20
10
0
307
317
3306
3366
3394
hybrid
NAA (μM)
0
0.1
1
5
10
50
100

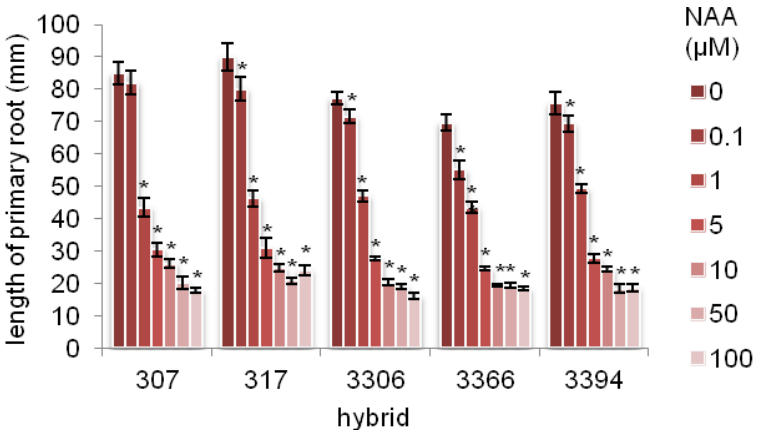

number of seminal roots
7
6
5
4
3
2
1
0
307
317
3306
3366
3394
hybrid
NAA (μM)
0
0.1
1
5
10
50
100

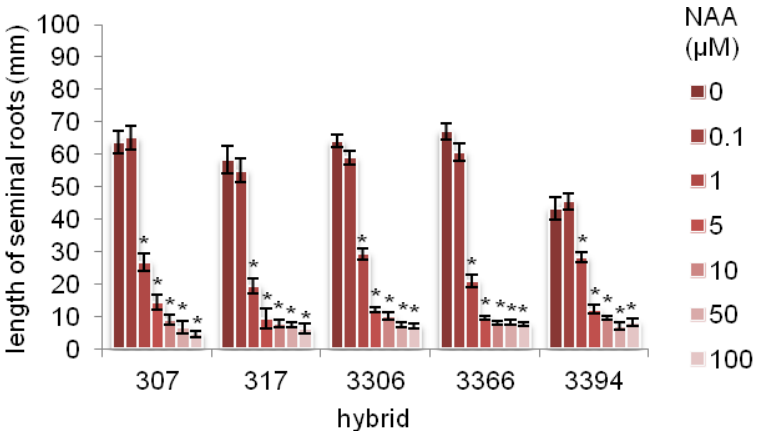
Supplemental Figure 3

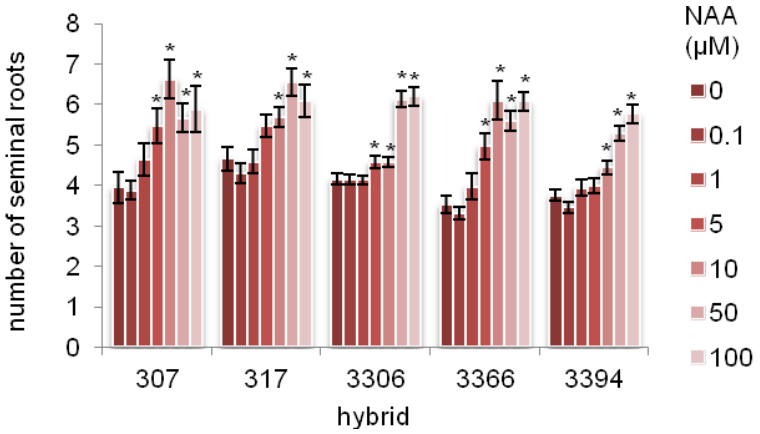
The graphs of absolute lenghts of organs of 4-day-old maize seedlings that were grown in RL in presence or absence of exogennous axin – NAA

Data represent averages ± SE obtained in 5 independent experiments, together 20-37 plants. * - significantly different from values obtained for control plants grown in absence of NAA (t-test, $P<0.05$).




length of primary root (mm)
100
90
80
70
60
50
40
30
20
10
0
307
317
3306
3366
3394
hybrid
NAA (μM)
0
0.1
1
5
10
50
100


length of seminal roots (mm)
100
90
80
70
60
50
40
30
20
10
0
307
317
3306
3366
3394
hybrid
NAA (μM)
0
0.1
1
5
10
50
100


number of seminal roots
8
7
6
5
4
3
2
1
0
307
317
3306
3366
3394
hybrid
NAA (μM)
0
0.1
1
5
10
50
100