PALACKY UNIVERSITY IN OLOMOUC
Faculty of Science
Department of Botany

The role of boron and light signaling pathways in cell transporters activity during plant growth

Ph.D. thesis

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Field of study: Botany

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Olomouc 2019

UNIVERZITA PALACKÉHO V OLOMOUCI
Přírodovědecká fakulta
Katedra botaniky

Úloha boru a světelných signálních drah v aktivitě buněčných transportérů boru během růstu rostlin

Disertační práce

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Olomouc 2019

DECLARATION

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

In Olomouc ……

Signature

ACKNOWLEDGEMENTS

This work would not be possible without the kind help and valuable insight from my supervisor Prof. Martin Fellner who introduced me to the problematics of auxin and boron research and patiently discussed my results and their interpretation throughout my study. Additionally, I would like to thank Prof. Miroslav Strnad for valuable comments leading to the improvement of my thesis. The research was supported by the following grants: 1P05ME792, MRTN-CT-2006-035833 and MSM6198959215 (all to MF).

BIBLIOGRAFIC IDENTIFICATION

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Title: The role of boron and light signaling pathways in cell transporters activity during plant growth

Type of thesis: Ph.D. thesis

Department: Department of Botany, Palacky University in Olomouc

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

The year of presentation: 2019

Abstract

The main goal of my PhD research was to understand the mechanism how light, in interaction with boron and auxin, regulates the growth and development of plants. I hypothesized that the triple combination of factors such as light, boron/auxin concentration and differences in cell wall composition could bring new insight into plant response to abiotic conditions.

In the first part of my thesis, I studied involvement of regulators of cell wall biosynthesis in boron transport. I analyzed *Arabidopsis* mutants with defects in genes involved in synthesis of plant cell wall for their growth responsiveness to elevated boron supply under different light conditions. I studied the cell wall mutants with defect in monosaccharide biosynthesis (*mur1-1 to 1-10*), cellulose-deficient mutants from radially swollen class (*rsw1-10, rsw2-1; rsw3-1*) and cellulose synthase mutant *procruste* (*prc1-1*). Mutants *prc1-1* and *rsw1-10* showed decreased cell elongation, especially in dark-grown roots and hypocotyls. The results revealed that in the dark, the above-mentioned cell wall mutants showed ability of hypocotyl elongation at the concentrations, which are usually highly toxic for wild type plants. Based on these results, I further focused on functional connections of boron transporters BOR1 and NIP5;1 in response to different light conditions on the transcriptional level. The results revealed that the stimulation of growth was associated with reduction of genes for boron transporters. Different expression of *BOR1* and *NIP5;1* in *prc1-1* and *rsw1-10* and wild type plants supported the existence of mechanism by which plants can tolerate toxic effect of high boron concentrations on plant growth.

In the second part, I studied a role of auxin binding proteins (ABPs) in light signaling during development of *Zea mays* mesocotyls. I investigated expression of red light photoreceptors phytochrome A (PHYA) and phytochrome B (PHYB) in loss-of-function mutants *abp1* and *abp4*. Further, I studied whether exogenous auxin NAA can influence

expression of these phytochromes and whether it may be part of ABP1 signaling pathway. Data from my research confirmed that the knock-out of ABP1 and ABP4 resulted in reduced expression of PHYB in dark-grown mesocotyls. Additionally, ABP1 and ABP4 mediated the inhibitory effect of exogenous auxin on level of PHYB transcript.

Key words: *Arabidopsis thaliana*, auxin, auxin binding protein, boron, cell wall, hypocotyl, light, mesocotyl, phytochrome, *Zea mays*

Language: English

Number of pages: 100

BIBLIOGRAFICKÁ IDENTIFIKACE

Jméno autora: Jana Kundratová (rozená Bořucká)

Název práce: Úloha boru a světelných signálních drah v aktivitě buněčných transportérů boru během růstu rostlin

Typ práce: Disertační práce

Pracoviště: Katedra botaniky, Univerzita Palackého v Olomouci

Školitel: prof. RNDr. Martin Fellner, Ph.D.

Rok obhajoby: 2019

Abstrakt

Hlavním cílem mé disertační práce bylo pochopení mechanismu, jak světlo v interakci s borem a auxinem reguluje růst a vývoj rostlin. Vycházela jsem z hypotézy, že trojkombinace faktorů jako jsou světlo, koncentrace boru/auxinu a změny ve složení buněčné stěny mohou přinést nový pohled na reakci rostlin na abiotické podmínky.

V první části mé práce jsem studovala zapojení regulátorů buněčné stěny v transportu boru. Analyzovala jsem mutanty buněčné stěny s defektem v biosyntéze monosacharidů (*mur*), celulóza-deficientní mutanty z třídy radially swolen (*rsw*) a mutanta syntázy celulózy (*procruste, prc*). Mutanti *prc1-1 a rsw1-10* se projevovali malým vzrůstem, zejména v etiolizovaných kořenech a hypokotylech. Oba mutanti navíc ve tmě vykazovali schopnost stimulace růstu hypokotylu i v takových koncentracích, které byly pro kontrolní wild-type rostliny již toxické. Na základě těchto výsledků jsem se dále zaměřila na funkční propojení transportérů bóru *BOR1* and *NIP5;1* na molekulární úrovni. Data prokázala, že stimulace růstu u studovaných mutantů byla spojena s redukcí exprese transportérů bóru. Různá exprese obou transportérů u *prc1-1* and *rsw1-10* a kontrolních rostlin podporuje existenci mechanismu, kterým rostliny mohou tolerovat toxický efekt vyšších koncentrací bóru v růstu rostlin.

V druhé části mé disertační práce jsem studovala roli auxin binding proteinů (ABPs) v signální dráze světla během vývoje mezokotylů kukuřice (*Zea mays*). Dále jsem stanovovala expresi fytochromů PHYA a PHYB u loss-off-function mutantů *abp1* and *abp4*. Také jsem studovala vliv exogenního auxin NAA na expresi fytochromů a možné zprostředkování tohoto účinku signálními drahami ABP proteinů. Data z mého výzkumu potvrdila, že knock-out u ABP1 a ABP4 vede k redukci exprese *PHYB* ve tmě rostoucích mezokotylů. Tyto výsledky indikují, že ABP1 a ABP4 mohou zprostředkovávat inhibiční účinek auxinu na hladinu exprese *PHYB*.

Klíčová slova: *Arabidopsis thaliana*, auxin, auxin binding protein, bór, buněčná stěna, hypokotyl, světlo, mezokotyl, fytochrom, *Zea mays*

Jazyk: Anglický

Počet stran: 100

ABBREVIATIONS

ABCB	ATP-BINDING CASSETTE B
ABP	AUXIN BINDING PROTEIN
ARF	AUXIN RESPONSE FACTOR
Aux/IAA	AUXIN/INDOLE-3-ACETIC ACID
Aux-LAX	AUXIN-RESISTANT1/AUX-LIKE
BL	Blue light
CRY	Cryptochrome
COP1	CONSTITUTIVE PHOTOMORPHOGENIC 1
FR	Far red light
GARNET	Genomic *Arabidopsis* Resource Network
GTP	Guanosine triphosphate
HFR1	Long After Far-red light 1
HY5	LONG HYPOCOTYL 5
IAA	Indole-3-acetic acid
IAM	Indole-3-acetamide
IAOX	Indole-3-acetaldoxime
LAF1	Long After Far-red light 1
MIP	Major intrinsic protein
NIP	Nodulin-like intrinsic protein
IPA	Indole-3-pyruvic acid
PIF	Phytochrome interaction factors
PIN	PIN-FORMED proteins
Pfr	Far red light absorbing form
Pr	Red light absorbing form
R:FR	Red:Far-red
RL	Red light
ROS	Reactive oxygen species
SIP	Small basic intrinsic protein
TAM	Tryptamine pathway
TAIR	The *Arabidopsis* Information Resource
TILLING	Targeting Induced Local Lesions IN Genomes
TIP	Tonoplast intrinsic proteins

Trp	Tryptophan
TIR/AFB	TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN
WT	WILD TYPE
2,4-D	2,4-Dichlorophenoxyacetic acid

CONTENT

1. INTRODUCTION

Plants are the backbone of all life on Earth and their importance to humans and other organisms is staggering. Because all life on Earth depends on plants in some way, it is important to study the basic life processes of plants. Research on plants increases our knowledge about other life processes, and the results of such research can help us to understand how to approach problems in agriculture, health, and the environment (e.g., National Research Council (US), 1992). Recently, the scientific community has shown remarkably increased interest in both basic and applied research on plants. The modern laboratory equipment and consumables of a relatively affordable price as well as the power of novel methods to answer fundamental questions in plant biology has stimulated the interest of scientists all around the world. There are numerous reasons why scientists focus on plant research. Plants not only maintain the oxygen content of the air, supply food and energy to other organisms, but also provide variety of products for human use (e.g., Robbins, 1944).

During their lifetime, plants are affected by various environmental factors that threaten survival and negatively influence their growth, development and productivity. All plants need sunlight, water, minerals, oxygen, carbon dioxide and warmth to ensure their survival and successful reproduction. Especially light as a source of energy is crucial for developmental decision to germinate and flower as well as for metabolic activities (Casper, 2007). Because severe environmental stresses affecting plants often lead to enormous crop losses, which are recently higher due to increasing factors such as drought, high or low salinity, unsuitable temperature conditions, and heavy metal toxicity, researchers nowadays focus more on the understanding the interaction between plants and environmental factors. In order to overcome negative effects of abiotic stresses, plants have evolved sophisticated mechanisms of acclimation and adaptation to actual biotic and abiotic conditions including the fluctuations of temperature and available nutrients. Using the modern plant genetic engineering techniques, researchers are able to design stress-tolerant plants with a modified plant architecture and metabolism (Gururani *et al.*, 2015). Therefore, it is absolutely necessary to understand how plants respond to specific stresses at various levels.

Internal mediators as plant hormones play the crucial role in the interaction between plants and their surroundings. They occur in very low concentrations in the plant body where their levels are highly responsive to environmental changes (Santner *et al.*, 2009). Other factors include nutrition microelements as boron, nitrogen or sulfate. Nowadays it is well known that

abiotic stress induces changes in photosynthesis and phytohormone signaling and that these changes are often interlinked. This abiotic stress may change gene expression as well as plant signaling pathway. Understanding how plants can translate the signals from an unstable environment into physiological behavior is essential for reducing harmful effects caused by abiotic stresses such as toxicity or deficiency of various soil nutrients (e.g., Mukhopadhyay and Mondal, 2015; Mathur *et al.*, 2016; Bücker-Neto *et al.*, 2017). When we better understand the interactions between plants, hormone system and environmental factors, we can design plants which better react to drought, salinity, soil toxicity or nutrients imbalance (Zhu, 2016; Benkova *et al*., 2003). Thus, the rapidly evolving technologies can serve as a very useful tool for improving high-yielding, pathogen-resistant, stress-tolerant, and climate-smart crop (Varshney *et al.*, 2018). Although this field of plant biology research is very popular nowadays and our knowledge on the interactions between plants, phytohormones and fluctuating environment is rapidly increasing, there are still many questions to be addressed.

2. BACKGROUND

2.1. BORON

Boron is one of the essential micronutrients required for plant, animal and human health and it is involved in the evolution of life on Earth (Ahmed and Fujiwara, 2010). In human, it is essential for the healthy growth of bone, better and faster wound healing and well-keeping hormone balance (Pizzorno, 2015). Boron affects many enzymes, mineralization, metabolism of Ca, P and Mg and energy metabolism. It can also influence the activity of vitamin D and it is involved in treatment of bone structure disorder, reduction of cholesterol and triglyceride levels (Kabu *et al.*, 2015). In animals, boron is required for proper development of all taxa across the classification system (Fort *et al.*, 1998; Rowe and Eckhert, 1999). It affects metabolism, causes hyperinsulinemia and depression of inflammatory responses, and its deficiency promotes the deficiency of vitamin D (Armstrong and Spears, 2003). However, importance of boron was firstly recognized in plants (Warrington, 1923).

Plants require at least 14 mineral elements for their adequate nutrition. These macro- (N, P, K, Ca, Mg and S) and micronutrients (Cl, B, Fe, Mn, Cu, Zn, Ni and Mo) are generally obtained from the soil (Dhanapriya and Maheswari, 2015). One of the most important and recently rapidly studied elements is boron, which naturally occurs in the form of borates in the oceans, sedimentary rocks, coal, shale, geothermal steams and some soils (Woods, 1994). Recent studies have confirmed that boron affects not only yield but also quality of several crops by its deficiency or excess (White and Brown, 2010). Plants in general use 5% of boron in soils. The amount of boron useful for growth of plants varies between 0.5 to 2.0 mg/L^{-1} depending on species. The availability of boron in soil is limited in many regions in the world with a high rainfall or seasonal water availability (Miwa *et al.*, 2009; Ozturk *et al.,* 2010). In regions with high-boron groundwater, boron concentration in topsoil reaches to a toxic level for plants and reduces crop yields. South Australia, Egypt, Iraq, Jordan, Libya, Morocco, Syria, Turkey, California, and Chile are countries with severe boron toxicity problems in agricultural areas (Camacho-Cristóbal *et al.*, 2008; Ozturk *et al.,* 2010).

In general, there is a small concentration range between deficiency and toxicity. The optimum boron level for one species can be either toxic or insufficient for other species (Ozturk *et al.,* 2010; Fang *et al.*, 2016). Batar *et al.* (2009) established three categories of species regarding their tolerance to boron (reviewed in Ozturk, 2010): tolerant (2–5 mg/l; tomato, cotton, asparagus, cauliflower), semi-tolerant (1–2 mg/l; pepper, pear, lettuce, maize) or

sensitive (up to 1 mg/l; lemon, grapefruit, avocado) to boron. Symptomes of toxicity as well as deficiency depend on the species and the age of plant (Brown and Hu, 1997). Boron deficiency inhibits root elongation, affects leaf growth and development and prevents pollen tube organization that may exhibit as necrotic leaves, dwarf phenotypes, deformations in growth (Brown and Hu, 1997; Wang *et al.,* 2015). Boron deficiency can also severely damage the organization of vascular tissue as it was observed in coffee (*Coffea arabica*) or in pumelo and sweet orange leaves. Deficient symptoms in leaves, including chlorosis, necrosis or malformations was observed in many trees as well (Wang *et al*., 2015). Deficiency of boron may reduce also transport function in petioles and photosynthesis in leaves, which leads to the formation of brown rings in petioles of cotton (*Gossypium hirsutum,* L.) seedlings (Li *et al*., 2017).

2.1.1. BORON RESEARCH OVERVIEW

Already in the 1920's, Katherine Warington examined the importance of boron for the development of various plants and found out that a continual low concentration boron supply is necessary for their healthy growth (Warington 1923; reviewed in Josten and Kutschera, 1999). Haas and Klotz (1931) observed some anatomical and physiological changes in citrus species caused by boron deficiency. Eaton (1940) described symptoms of boron deficiency and toxicity on sunflowers (*Helianthus*) and beans (*Vicia faba*). Plants displayed whitish white young leaves during low boron concentrations, while boron toxicity showed up first on the mature leaves by death of tissues. It has been established that boron is one of the essential elements for the plants due to its involvement in cell wall synthesis and structure, metabolism and transport of sugars, stimulation of nuclei acids metabolism, pollen germination, hormone action, ascorbate/glutathione cycle, photosynthesis and several enzymatic activities and membrane transport (Lovatt & Dugger, 1984; Loomis and Durst, 1992; Blevins and Lukaszewski, 1994; Power and Woods, 1997; Abdulnour, 2000; Liu, 2000; Lou *et al.*, 2001; Reid, 2014; Fang *et al.*, 2016).

Because it was shown that 90% of boron content is localized in the cell wall (Loomis and Durst, 1992; Blevins and Lukaszewski, 1994), later research started to focus on elucidating boron function in the cell wall in model plants. Reiter *et al.* (1993) described *Arabidopsis* mutant *mur1* completely deficient in L-fucose, component of pectin and hemicellulose polysaccharides. These mutants show dwarf phenotype and their cell wall are more fragile than in WT plants. The *mur1* mutation affects all fucose containing polysaccharides, including pectin

components, rhamnogalacturonan RG-I and RG-II and xyloglucan, the main cross-linking glucan in dicots (Ryden *et al.*, 2003). Cakmak and Römheld (1997) further confirmed a crucial role of boron in cell wall expansion. The authors based their research on ability of boron to form complexes with the compounds having cis-diol configurations of two rhamnogalacturonan II molecules, and the borate cross-linked RG-II was shown to be essential for normal plant growth. Fleischer *et al.* (1999) and Ryden *et al.* (2003) showed that boron deficiency alter cell wall porosity and tensile strength. It has been suggested that boron may be necessary for cell-to-wall adhesion and for the organization of the architectural integrity of the cell (Lord and Mollet, 2002; Bassil *et al.*, 2004). Additionally, it can be also expected that boron toxicity alters the cell wall structure, actin organization and thus rapid change in mechanical strength of cell wall occurs (Fang *et al.*, 2016).

2.1.2. BORON TRANSPORTERS

To maintain the cell wall structure and optimal plant growth, sufficient boron must be transported to the growing regions of plants using various transport mechanisms. At the molecular level, facilitated diffusion is mediated by uniporters or channels. Passive transporters facilitate the infusion of 10 of 14 mineral nutrients across the plasma membrane of root cells. Among the elements, boron is the only one that is taken up by plants not as an ion but uncharged molecule by root system (Marschner *et al.*, 1995; Mengel and Kirkby, 2001; Hänsch and Mendel, 2009; Tewari *et al.*, 2010). The high permeability of lipid bilayer for boric acid lead scientists to an idea that the passive diffusion is the only mechanism for the boron transport (Marschner *et al.,* 1995). Dannel *et al.* (2000) suggested a putative boron transporter mechanism when describing boron uptake in sunflower. Using positional cloning, Takano (2002) identified first protein required for boron transport against its concentration gradient under low boron supply.

Up-to-now, three mechanisms are known for membrane transport of boric acid. The first one is the passive diffusion across lipid bilayer (Dannel *et al.*, 2000; Dordas and Brown, 2000; Frommer and von Wirén, 2002; Takano, 2002; Kuchel *et al.,* 2006). Most mineral nutrients have to be transported across the root from soil to the xylem. They need to move across plasma membrane at least twice because of the Casparian strips. These initial observations led researchers to further explore the need of membrane proteins to satisfy a plant's demand of boron, especially under its limitation.

The second mechanism is the active transport by BOR transporters responsible for the boron export in plant cells and represented by BOR1 and NIP5;1 as the efficient transport of boron across the plasma membrane under boron limitation (Miwa and Fujiwara, 2010; Reid, 2014; Fig. 1). Both types of boron transporters contribute to boron uptake by roots (Takano *et al.*, 2006; Durbak *et al.*, 2014; Hanaoka *et al.*, 2014), xylem loading and boron distribution (Takano *et al.*, 2001; Nakagawa *et al.*, 2007; Tanaka *et al.*, 2008) and boron utilization within the plant under boron-limited conditions (Miwa *et al.*, 2013). The boron pathway from the root surface to the shoot includes at least two transmembrane transport events. First, it is import of boron into epidermal, cortical, or endodermal cells (i.e. uptake) and second, the export from pericycle or xylem parenchyma cells into the stellar apoplast (i.e. efflux or xylem loading). In *Arabidopsis*, an efflux-type borate transporter BOR1 is involved in the xylem loading. BOR1 was identified based on analysis of *bor1-1* mutant as the first boron transporter (Noguchi *et al.*, 2000; Takano *et al.,* 2001). The mutant *bor1-1* shows visible defects as reduced expansion of rosette leaves, reduced fertility and loss of apical dominance (Noguchi *et al.*, 1997). BOR1 is required for the transport of boron from roots to shoots under conditions of low boron supply and is capable of conferring high boron tolerance to yeast by pumping boric acid out of the cell. However, in plants under high boron conditions, BOR1 is degraded via endocytosis (Takano *et al.,* 2005), and overexpression of BOR1 does not improve plant growth in the presence of toxic levels of boron (Miwa *et al.,* 2006, 2007). Takano (2002) suggested six more genes related to *BOR1*. One of the paralogs is *BOR4*, which accumulates in the presence of a high boron supply and it correlates with plant tolerance of boron BOR4 transporter is the efflux one. It has been localized on the distal part of root epidermal cells (Miwa *et al.,* 2007). During plant growth in excessive supplies of boron, the BOR4 efflux overexpression supports growth in these conditions.

The third type of membrane transport of boric acid includes the facilitated transport by nodulin–like intrinsic protein (NIP) channel, which is a member of major intrinsic protein family. The MIP family in plants can be subdivided into five evolutionarily distinct subfamilies, including nodulin-26-like intrinsic proteins (NIPs), plasma membrane intrinsic proteins (PIPs), small basic intrinsic proteins (SIPs), tonoplast intrinsic proteins (TIPs) and uncharacterized X intrinsic proteins (XIPs) (Ishibashi *et al.*, 2011). Especially NIPs are very important for the transport of boric acid in plants. Takano *et al.* (2005) demonstrated NIP5;1 as a protein functioning as boric acid channel for boron uptake. The authors also found NIP5;1 to be crucial for plant growth under boron limitation in *Arabidopsis*. *NIP6;1*, the gene most similar to *NIP5;1* has been established as another boric acid channel required for proper distribution of boric acid

in *Arabidopsis*, especially in young shoot tissues (Zimmermann *et al.*, 2004; Tanaka *et al.*, 2008). Both NIP5;1 and NIP6;1 are localized in the plasma membrane, but their boron transport functions are different: NIP5;1 is involved in the initial uptake process in root cells (Takano *et al.*, 2006), while NIP6;1 may function in xylem-phloem boron transfer into young growing tissues (Tanaka *et al.*, 2008). In contrast to NIP5;1 the transporter NIP6;1 is not permeable to water but it is permeable to boron (Tanaka *et al.*, 2008). The microarray data showed that the NIP5;1 is primarily expressed in root, whereas the NIP6;1 in stems (Wallace *et al.*, 2006).

All three above mentioned mechanisms are involved in regulation of boron transport in plants. However, these mechanisms remain still partly unclear and underinvestigated.

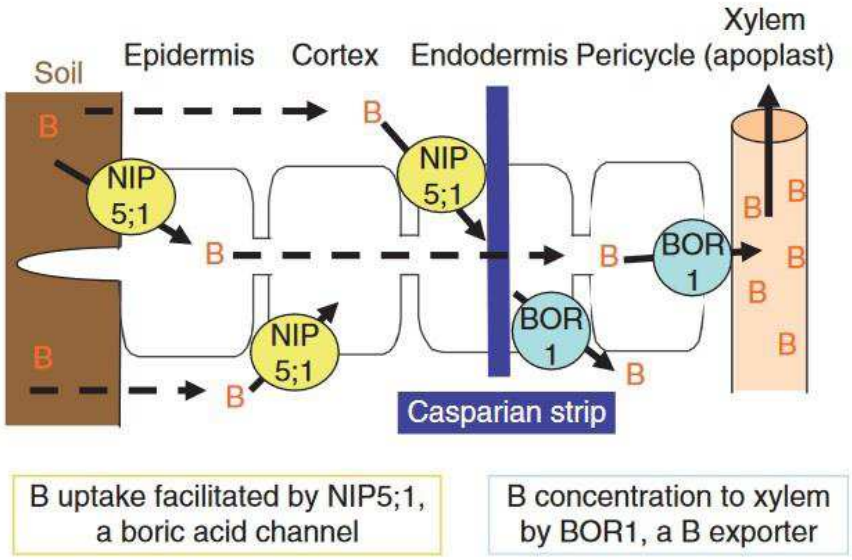

Fig. 1. Schematic model of boron transport in *Arabidopsis thaliana* roots under boron limitation (from Miwa and Fujiwara, 2010).

2.1.3. DEFICIENCY AND TOXICITY OF BORON IN PLANTS

Boron transporters as well as cell-wall related genes are most probably involved in mechanism how plants can respond to low or extensive supply of boron (Miwa and Fujiwara, 2010; Fig. 1). Boron deficiency probably originates from a combined effect of three processes: boron uptake, (re)translocation, and its utilization. Plants that grow in soil with low boron concentrations exhibit deficiency symptoms in early growth stages and are characterized by abnormal growth of the apical growing region. In later phases, plants show retarded growth, because boron deficiency affects physiological, biochemical and molecular processes and finally result in decreased yield (Tewari *et al.*, 2010). Root growth is also sensitive to boron deficiency,

primarily as a result of disturbing cell wall plasticity and additionally due to the inhibition of root meristem functioning (Herrera-Rodríguez *et al.*, 2010). In addition, boron deficiency and toxicity cause numerous other morphological changes including the browning of leaves, death of apical meristems, abnormal enlargement of root tips, dense appearance of lateral roots near to the root apex, lack of root hairs, and their growth inhibition (Goldbach *et al.*, 2001). Boron deficiency causes abnormally formed cell walls that are often thick and fragile (Brown *et al.*, 2002). Generally, the cell wall thickening process requires two elements: polysaccharides (e.g., cellulose and xylans) and aromatic components (e.g., lignins; Goujon *et al.*, 2003). Expression of cell-wall-related genes can be affected by boron deficiency. It can decrease production of molecules that are related to the cell wall synthesis as well as extension. Camacho-Cristóbal *et al.* (2008) described changes in *Arabidopsis* genes responsible for cell wall modification in roots under boron deficiency. Moreover, Camacho-Cristóbal *et al.* (2011) suggested cross-talk between boron and Ca^{2+} in stabilization of cell wall during deficiency. In *Citrus* species that belong to the group of highly sensitive plants, boron deficiency suppressed the expression of cell-wall-modifying genes as well as lignin biosynthesis pathway genes in roots (Zhou *et al.*, 2015) and leaf veins (Yang *et al.*, 2013). Takano *et al.* (2006) reported that boron could induce expression of genes as *NIP5;1* in response to stress. These results suggest that boron deficiency affects the expression of cell-wall-related genes not only in herbaceous plants but also in trees (Lehto *et al.*, 2010). Boron-deficient trees usually exhibit visible symptoms as deformation of growing organs as root tip, bud, flower, and young leaf. This is somehow linked with boron role in cell wall and membrane synthesis. It can damage structural development of vascular tissues resulting in wood quality and cold tolerance. Boron deficiency can also affect different metabolic processes, decrease leaf photosynthesis, and increase lignin and phenol content in trees (Lehto *et. al*, 2010). These negative effects definitely influence the quality and quantity of wood, fruit and agricultural products.

Similar to deficiency, boron toxicity causes severe plant growth disorders in areas including California, Turkey, Spain or Chile. The first observation of boron toxicity on plant growth was reported by Shear *et al*. (1946). They showed that high ratio of Ca+K/Mg and Ca+Mg/K in plants is associated with boron toxicity effect. Interestingly, ratio K+Mg/Ca has only little influence on this phenomenon. Later, Leece (1978) confirmed these ratios and reported that boron may be involved in the nutrition balance of plants. Boron toxicity in the similar way as deficiency delays development, inhibits plant growth and decreases weight, number and size of fruits (Fitzpatrick and Reid, 2009; Herrera-Rodríguez *et al.*, 2010). Accumulation of boron causes development of necrotic regions in leaf tips, that causes chlorosis

starting at the leaf tips and margins of mature leaves (Fitzpatrick and Reid, 2009; Reid, 2014). Toxicity symptoms include reduced root growth, abnormal cell division in root meristem as well as formation of hypodermis. It negatively influences uptake of water and other nutrients (Ghanati, 2005). Aquea *et al.* (2012) reported cross-link between boron toxicity and abscisic acid signaling. Furthermore, boron repressed the expression of genes encoding water-stress response in association with root growth inhibition and could cause different morphological traits in tomato (Princi *et al.*, 2016). Genetic studies revealed that the most tolerant species is barley, especially cultivar Sahara (Fitzpatrick and Reid, 2009). It is also well known that boron toxicity increases oxidative stress (Cervilla *et al.*, 2007; Aftab *et al.*, 2010; Landi *et al.*, 2014). For example, Tassi *et al.* (2011) and Giansoldati *et al.* (2012) described that excess of boron from contaminated soils causes oxidative stress in sunflower (*Helianthus annuus* L.). Production and activity of antioxidant enzymes could be one of the strategies that protect plants against multiple stresses induced by boron deficiency and toxicity (Juan *et al.*, 2005; Han *et al.*, 2008). As in most environmental stresses, boron toxicity leads to the formation of reactive oxygen species (ROS) which cause oxidative damage of cellular membranes and finally the cell death (Cervilla *et al.*, 2007). In order to reduce cellular damage produced by ROS, plants have developed the cleaning systems composed of antioxidant enzymes, such as superoxide dismutase or peroxidase (Chen and Dickman, 2005), and non-enzymatic system like ascorbate, glutathione or alpha-tocopherol (Blokhina *et al.*, 2003) and osmoprotective solutes like proline (Cervilla *et al.*, 2012). It has been well reported that in boron deficient plants antioxidant enzymes catch ROS to protect plants against stress condition (Karabal *et al.*, 2003; Gunes *et al.*, 2006; Molassiotis *et al.*, 2006; Han *et al.*, 2008; Tewari *et al.*, 2010; Saud *et al.*, 2017).

2.2. AUXIN

Plants as sessile organisms have developed a sophisticated mechanism to alter growth, development, and metabolism to adapt to the ever-changing environmental conditions. This mechanism includes primarily communications among the cells. Multiple signaling pathways and cell-to-cell communication are involved in processes to transduce various developmental signals. Plant hormones as auxin, brassinosteroid, cytokinin, gibberellins, ethylene, jasmonic acid, strigolactone, abscisic acid and salicylic acid belong to the group of important signaling molecules that act on different processes in plants during their growth and development (Druege *et al.*, 2016; Verma *et al.*, 2016).

Auxin was first recognized as a plant hormone because of its role in plant tropism to gravity or light stimuli which was based on the observation on canary grass made by Charles Darwin a century ago (Darwin, 1880) and later on *Avena* stems by Went (1935). In mid of 1930's, auxin was determined chemically as indole-3acetic acid (IAA). The name auxin is derived from the Greek "auxein" meaning "to grow" and "to extend".

Auxin regulates the most important cellular processes in morphogenesis such as elongation, division and differentiation, and it is required for plant viability (Davies, 2004; Vaneste and Friml, 2009). Hager *et al.* (1971) proposed the first model of auxin-driven cell wall acidification that leads to altered growth (reviewed in Arsuffi and Braybrook, 2018). Auxin activates the expression of cell wall-related genes and stimulates the synthesis of proton pumps, which leads to apoplast acidification (Velasquez *et al.*, 2016; Majda and Robert, 2018). The proton pumps hydrolyze available nucleotide triphosphate to power proton pressing (all these processes are addicted in aerobic condition). The result is extension and elongation of cell wall (Majda and Robert, 2018). All plants tissues as meristem, young leaves, fruits and seeds are the major source of indole-3-acetic acid (IAA), the endogenous form of natural auxin (Taiz and Zeiger, 2006; Sanchez-Garcia *et al.*, 2018).

Auxin IAA is structurally related to the amino acid tryptophan (Trp) and it was confirmed that tryptophan is a precursor of IAA biosynthesis. Two major pathways for IAA have been proposed: the tryptophan (Trp)-dependent and Trp-independent pathways. In Trp-dependent IAA biosynthesis, four pathways have been postulated in plants: (i) the indole-3-acetamide (IAM) pathway; (ii) the indole-3-pyruvic acid (IPA) pathway; (iii) the tryptamine (TAM) pathway; and (iv) the indole-3-acetaldoxime (IAOX) pathway (Mano and Nemoto, 2012).

Auxin is distributed differently within plant tissues and it appears to be regulated largely by pH. The way of auxin transportation depends on its gradient. The first one, long-distance source-to-sink, originates in young shoots (Marchant *et al*., 2002). The second one, for shorter distances, is polar transport depending on influx and efflux carrier proteins. These carriers include AUXIN-RESISTANT1/AUX-LIKE (AUX/LAX) family proteins for influx and PIN-FORMED (PIN) and ATP-BINDING CASSETTE B (ABCB) for efflux (Sanchez-Garcia *et al.*, 2018). Upon perception in the nucleus, auxin can trigger several specific transcriptional responses. The main components of the auxin signaling pathway belong to three protein families: the F-box TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX PROTEIN (TIR1/AFB) auxin co-receptors, the Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) transcriptional repressors, and the AUXIN RESPONSE FACTOR (ARF) transcription factors (Friml, 2007; Wang and Estelle, 2014; Salehin *et al.*, 2015; Lavy and Estelle, 2016; Fig. 2). It has been hypothesized that some auxin responses are mediated also by a candidate auxin receptor called auxin binding protein 1 (ABP1) (Shishova and Lindberg, 2010; Tromas and Perrot-Rechenmann, 2010). The ABP1 was first isolated from maize plants based on its ability to bind auxin (Jones, 1994). The crystal structure of ABP1 demonstrated clearly that ABP1 has an auxin-binding pocket and, indeed, binds auxin (Woo *et al.,* 2002). Differently from maize, where at least five ABPs were identified (including ABP1), *Arabidopsis* has only one homologue of ABP1. The ABP1 regulates cell division and cell cycle and it was proposed that it regulates auxin-mediated gene expression and cell elongation in addition to the TIR1-meditaed ubiquitination pathway (Fellner *et al.*, 1996; David *et al.*, 2007; Tromas *et al.*, 2009). Interestingly, Gao *et al.* (2015) reported that in *Arabidopsis,* in contrast with maize, ABP1 is not required for auxin signaling and plant development.

Iglesias *et al.* (2018) described how light quality directs altered growth through changing auxin biosynthesis and transport. Due to the fact that they are sessile organisms, plants use light as a mediator to optimize their growth and development. This ongoing process, which is crucial for plant survival, is dependent on interaction of light pathways with the auxin. At the molecular level, auxin rapidly activates the transcription of three gene families: *Aux/IAA*, *SAUR,* and *GH3* genes. The Aux/IAA proteins are a key class of proteins that bind to and repress the activity of ARF proteins. *SAUR* may operate in an auxin signal transduction pathway that involves calcium and calmodulin. The *GH3* genes encode a group of enzymes that adenylate IAA, salicylic acid, or jasmonic acid. These mechanisms are light-dependent. The strongest connections between light and auxin signaling have been observed in plants exposed to low R:FR-ratio light, which triggers the shade avoidance syndrome mediated by phytochromes,

the most strongest plant detectors. Recent studies suggest two possible roles of phytochrome phosphorylation in the control of protein-protein interaction between phytochromes and signal transducers. The first one is the phytochrome-mediated light signaling in plants that is initiated by conformational changes in phytochrome molecules triggered by the incoming light. Phytochrome can then stabilize Aux/IAA proteins that regulate auxin transport, distribution and its level in plants (Kim *et al.*, 2005; Salisbury *et al.*, 2007). The second role is based on turnover of Aux/IAAs as an important factor modulating the degree between skotomorphogenic versus photomorphogenic development, especially when plants are exposed to low R:FR ratio light (Sorin *et al.*, 2005; Halliday *et al.*, 2009).

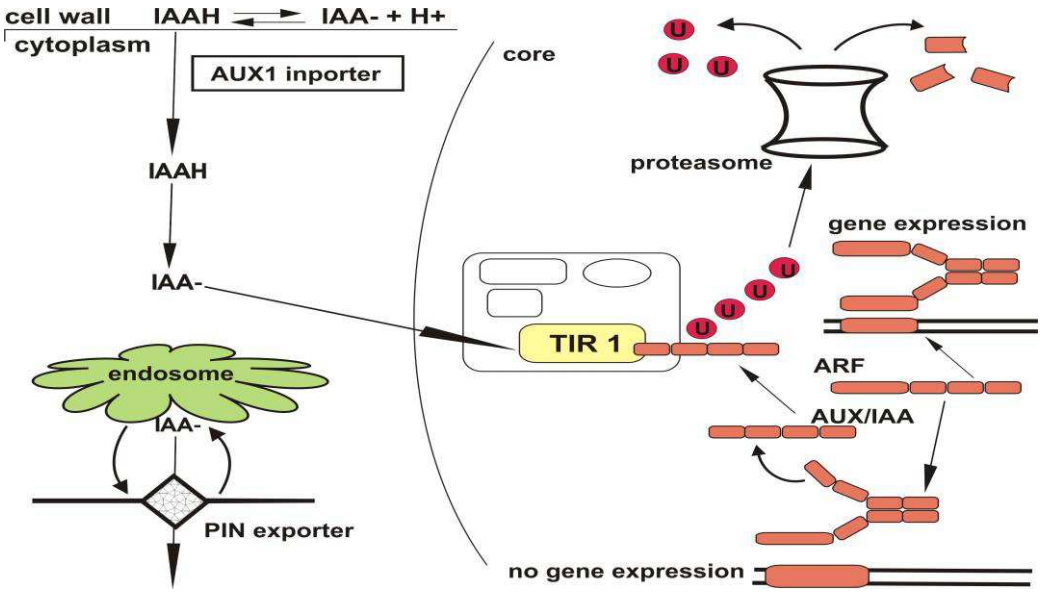

Fig. 2. Auxin transport and signaling pathway (edited from Friml, 2007).

2.3. LIGHT

Light plays a key role in plant growth and development not only as a source of energy but also an informational cue to control physiological responses during life cycle. Plants have developed unique photoreceptor system to mediate responses according to light spectrum. The combined absorption spectrum of the blue light receptors as well as UV-A/UV-B photoreceptors (cryptochromes, phototropins) to the red/far-red (R/FR) light response (phytochromes) allows to coordinate growth and development in plants including growth inhibition, differentiation of hypocotyl, maturation of cotyledons and activation of shoot apical meristem. Cryptochromes together with phytochromes regulate cell elongation and flowering, whereas phototropins mediate responses like phototropic curvature (Wang *et al.*, 2017). Phototropins are photoreceptors mediating blue light-induced phototropism (Briggs and Huala, 1999). PHOT1 and PHOT2 are two well-known phototropins in *Arabidopsis* plants (Briggs and Christie, 2002). Their role is mainly based on inhibition of hypocotyl growth during very first minutes of blue light irradiation. Another movement response mediated by phototropins is a stomatal opening (Zeiger *et al.*, 2008).

Plants have developed two different types of developmental programs depending on presence or absence of light. In dark conditions, plants set up skotomorphogenesis, which is characterized by long hypocotyl, closed cotyledons and etioplasts. Under light conditions, plants display a short hypocotyl, opened and green cotyledons, which are the main characters typical for photomorphogenesis. Hypocotyls play a crucial role as very photosensitive organ in reaching light before seeds reserves are exhausted (Wu *et al.*, 2010). Hypocotyl growth wasalso used as a good tool for identifying a large number of mutants involved in hormone biosynthesis and signaling.

Phytochrome-mediated responses of photomorphogenesis include germination, de-etiolation, shade avoidance, circadian rhythm and flowering (Han *et al.*, 2008). The phytochrome photoreceptors are present in all plant organs including roots, young cells and meristems. They are synthesized as apoproteins, which bind with chromophore, and together they act as functional dimers. Apoproteins are coded in the nucleus, whereas apoprotein-chromophore connection is created in cytoplasm. Based on their stability, they are categorized as type I (phyA) and type II (phyB-phyE) phytochromes. The localization of phytochrome is completely different depending on light conditions. PhyA displays rapid lability in Pfr form and can signal during photoconversion between Pr and Pfr. It is predominant phytochrome in etiolated seedlings. Conversely, phyB is the most abundant phytochrome in light-grown plants

(Sharrock *et al.*, 2003; Kim *et al.*, 2005; Sawers *et al.*, 2005). Upon red light exposure, phytochromes allosterically change their conformation from inactive red light absorbing form (Pr) to active far-red light absorbing form (Pfr) form. The inactive Pr form resides in cytosol, where it regulates mRNA translation, while the active Pr form translocates into the nucleus (Klose *et al.*, 2015). Although, phytochromes are not able to bind to DNA directly and thus transduce the signal via phytochrome interaction factors (PIF). PIFs act as a key regulator of transition from skotomorphogenesis to photomorphogenesis. They act as repressors of photomorphogenesis in the dark. These transcription factors belong to the bHLH (basic helix-loop-helix transcription factors superfamily) group and are crucial for negative regulation of genes involved in hypocotyl elongation, opening of cotyledons or de-etiolation. Upon light exposure, active Pfr form causes phosphorylation of PIF leading to their degradation in 26S proteasome. Light signal is thus transduced (Lin *et al.*, 2002).

Plants need both phytochromes and cryptochromes to sense environmental cues, such as irradiance, day-night transition, photoperiod, and light quality for optimal growth and development. Cryptochromes (CRY) are flavoprotein photosensory receptors that regulate growth, development, and the circadian clock in plants. They were first identified in *Arabidopsis* by Koornneef *et al.* (1980) by isolating of *hy4* mutant in the Landsberg erecta background. This mutant is defective in blue light control of elongation and lacks a protein with the characteristic of a blue light receptor (Ahmad and Cashmore, 1993). The *Arabidopsis* genome encodes three cryptochrome genes, *cryptochrome1* (*CRY1), cryptochrome2 (CRY2)*, and *cryptochrome3* (*CRY3)*. Genes *CRY1* and *CRY2* act primarily in the nucleus, whereas *CRY3* probably functions in chloroplasts and mitochondria (Facela *et al.*, 2017). Plant cryptochromes undergo photoexcitation to become physiologically active homodimers, which interact with CRY signaling partners such as cryptochrome signaling bHLHs and suppressor of PhyA1 (SPA1) to control BL-gene expression and developmental responses (Liu and Sharrock, 2017). CRY2 undergoes phosphorylation in etiolated seedlings exposed to blue light, whereas CRY1 is stable in light conditions. The last one, CRY3, probably functions as an additional blue light photoreceptor in *Arabidopsis* with low homology to other cryptochromes (Klar *et al.*, 2007).

2.4. LIGHT, AUXIN AND BORON CROSS-TALK

Different wavelengths of light are associated with various functions in plant development, changes in hormone levels and signaling and transport of signaling molecules and nutrition. Phytochrome and cryptochrome activities cause simultaneous elongation of the hypocotyl, reduction of primary root growth, and inhibition of lateral root formation (Canamero *et al.,* 2006; Salisbury *et al.,* 2007).

These effects are mediated at least in part by controlling auxin transport. The *phyA phyB* mutants show reduced shoot–root auxin transport (Salisbury *et al.,* 2007), and over time, auxin accumulates to higher levels in the *phyA phyB* mutant shoot, compared with those of the wild type (Nagashima *et al.,* 2008). Thus, light modulation of these genes provides a means to manipulate local auxin levels and cell expansion in the hypocotyl. Tao *et al*. (2008) described how phytochromes influence auxin levels by phyB action, when phyB triggers signaling pathway leading to increasing of IAA level. Active phyB (Pfr) reduce IAA level by coordinated activation of *SUR2* gene. Reduced level of Pfr induce by low R:FR ratio triggers the reciprocal control leading to increase in IAA levels (Tao *et al.*, 2008; Halliday *et al.*, 2009).

Additionally, cryptochromes are also involved in auxin signaling. It has been shown by Folta *et al.* (2001) that blue light acting through *CRY1* alters the expression of some *ARF* genes. Light influences also the direction of polar auxin transport by controlling the plasma membrane abundance of PIN proteins (Laxmi *et al.*, 2008; Sassi *et al.*, 2012; Wan *et al.*, 2012; Zhang *et al.*, 2013, 2014). In darkness, the expression of *PIN1* is largely reduced, thus reducing auxin delivery to the root system (Sassi *et al.*, 2012). Recent study by Gelderen *et al*. (2018) shows how HY5 regulates formation of lateral roots by decreasing the plasma membrane abundance of PIN-FORMED 3 (PIN3) and LIKE-AUX1 3 (LAX3) auxin transporters.

As light influences almost every step of plant growth and development, some possible cross-talk between nutrition balance and the light signals was also described. Tanada (1995) as one of the first presented boron as a key trigger in several processes initiated by light, gravity and some plant hormones. The author suggested possible mechanism of passage of ions through pores of the cell membrane to regulate leaves movement. In 2009, Kocábek *et al.* confirmed the role of cryptochromes and phytochromes in boron-induced hypocotyl elongation in *Arabidopsis*. Authors reported that boron stimulates hypocotyl elongation at concentration up to 3 mM, but has inhibitory effect on hypocotyl elongation at concentration of 5 mM. Interestingly, blue as well as red light did not alter the sensitivity to boron. Gonzáles-Fontes *et al.* (2015) used ethylene-insensitive *Arabidopsis* mutants to show that ethylene is involved

in responses of the primary root to boron deficiency. Under boron deficiency, the auxin level in the elongation zone would increase value of growth inhibition inducing local responses that inhibit the cell elongation. The authors suggest the cross-talk between these two phytohormones (auxin and ethylene) and mineral nutrition. Reddy and Finlayson (2014) described how phyB promotes branching in *Arabidopsis* by auxin suppression. The reduced branching of phyB-deficient plants was associated with enhanced auxin signaling in both seedling shoots and mature stem segments, as has been proven by expression of various auxin-responsive genes.

Light (especially blue light), can alter the hypocotyl responsiveness to auxins 2,4-D or IAA via the functional transporter BOR1. Stočes *et al.* (2012) suggested the mechanism how blue light and BOR1 transporter can influence the auxin transport in and out of the cell. Their model showed that higher supply of boron (over 2 mM) reduces the activity of AUX1/LAX auxin influx carrier. Their data also indicate that blue light could increase accumulation of boron and thus contribute to reduction of 2,4-D auxin influx. Another experiment made by Abreu *et al.* (2014) confirmed involvement of cytokinin and light under boron deficiency during early plant growth and development by inhibiting key genes in cytokinin signaling pathway.

3. AIMS OF STUDY

The main goal of my PhD research was to understand the mechanism how light, in interaction with boron and auxin, regulates the development and growth of plants. We hypothesized that the triple combination of factors such as light, boron/auxin concentration and differences in cell wall composition could bring new insight into plant response to abiotic conditions.

Main aims were:

- to investigate the response of light-induced growth of hypocotyl in different *Arabidopsis* cell wall mutants under different boron concentrations;

- to investigate the cross-talk between boron-induced stimulation of hypocotyl growth with boron-controlled expression of *BOR1* and *NIP5;1* genes in *Arabidopsis thaliana*;

- to study the interaction of auxin and light signaling using the expression of phytochrome genes PHYA and PHYB in loss-of-function mutants in *ABP1* and *ABP4* genes in *Zea mays*.

To investigate the above mentioned problems, two independent projects were carried out in the Group of Molecular Physiology (Head: Prof. RNDr. Martin Fellner, Ph.D.). The main outputs of these projects are listed in the Supplement 1 and 2, respectively. The Supplement 1 (Kundratova *et al*., submitted) is the manuscript titled "Cell wall mutants in *Arabidopsis thaliana* show increase responsiveness to boron-induced hypocotyl growth" has been submitted to *Acta Biochimica Polonica* (IF_{2017} 1.239). The Supplement 2 (Bořucká and Fellner, 2012) is the scientific paper titled "Auxin binding protein ABP1 and ABP4 are involved in the light- and auxin-induced down-regulation of phytochrome gene *PhyB* in maize (*Zea mays*, L.) mesocotyl" published in the international refereed journal *Plant Growth Regulation* (IF_{2012} 1.670; IF_{2017} 2.081; 5x cited in Web of Science). The main results of this project were also presented at international scientific conference in Istanbul, Turkey (European Biotechnology Congress 2011) and published as an abstract in the *Current Opinion in Biotechnology* ((IF_{2011} 7.711, IF_{2017} 8.380)) (Supplement 3).

4. MATERIAL AND METHODS

The detailed methodology is given in Supplements 1 and 2. Here, I list the general information on material and main methods used in both research projects.

Cell wall mutants in *Arabidopsis thaliana* show increase responsiveness to boron-induced hypocotyl growth (Supplement 1)

Experiments of this study were conducted with several *Arabidopsis thaliana* cell wall mutants *rsw1-10* (NASC stock code; N6554), *rsw2-1* (N6555), *rsw3-1* (N6556), *prc1-1* (N297), *mur1-1* (N6243), *mur2-1* (N8565), *mur3-1* (N8566), *mur4-1* (N8568), *mur5-1* (N8572), *mur6-1* (N8573), *mur7-1* (N8574), *mur8-1* (N8575), *mur9-1* (N8576), *mur10-1* (N8577) and their backgrounds Wassilewskija (Ws; N915) and Columbia (Col-0; N1092). All the seeds were stratified for 5 days in distilled water at the temperature of 4°C in dark condition and after that surface-sterilized with commercial surfactants. Seeds germinated on basal Murashige-Skoog basal medium at temperature of 23°C in darkness. After beginning of germination, seeds were transferred on the new basal medium supplemented or not with appropriate concentration of H_3BO_3. Seeds in Petri dishes were placed into growth chambers and incubated for 10 days in the dark or under blue or red light. After this, length of roots and hypocotyls were measured. For gene expression, total RNA was extracted from hypocotyls using TRI Reagent™ (Thermo Fisher Scientific) followed by chloroform-isoamyl alcohol extraction. Traces of genomic DNA were removed by DNAse I treatment and then by RNA purification on filtered columns. RNA quality and quantity were measured by NanoDrop™ 8000 Spectrophotometer. The cDNA product was directly used for quantitative real-time PCR (qRT-PCR). Statistical analysis of all experiments was performed in R 3.3.2. (R Development Core Team, 2018).

Auxin binding protein ABP1 and ABP4 are involved in the light- and auxin-induced down-regulation of phytochrome gene *PhyB* in maize (*Zea mays*, L.) mesocotyl (Supplement 2)

In experiments concerning *Zea mays* L. research I used 307 and 3394 hybrids and loss-of-function single mutants *abp1, abp4* and double mutant *abp1abp4* and their corresponding WTs. Both genes *ABP1* and *ABP4* are the most expressed genes between ABP family in maize. Bensen *et al*. (1995) used a reverse genetic strategy to identify loss-of function alleles of *ABP1* gene. The *abp* mutants contained the Robertson's mutator transposable elements in both genes (Bennetzen, 1996). In addition, the fytochromobilin-deficient mutant *elm1* (elongated mesocotyl 1) was used in experiments. The mutant *elm1* was initially identified in the W22 background (Sawers *et al.,* 2002) and was also introgressed into the B73 background (inbred maize line) by backcrossing five times (Dubois *et al*., 2010). Firstly, maize kernels were sterilized by process including 70% ethanol, SAVO solution and Tween20 surfactant, completed by washing several times in distilled sterile water. Secondly, seeds were grown on Murashige-Skoog basal medium with or without appropriate concentration of synthetic auxin NAA (5.10^{-5}–10^{-6} $mol.l^{-1}$). Thirdly, for genes expressions we used RNeasy Plant Mini RNA kit (Qiagen Inc., CA, USA) according to manufacturer's interactions, followed by DNAse I treatment and reverse transcription reaction. The PCR products were size fractioned by electrophoresis in 1% (w/v) agarose gel with ethidium bromide. Detected bands were evaluated by ImageJ software. For more details of each individual step see the Supplement 2.

5. MAIN RESULTS AND DISCUSSION

The detailed Results and Discussion sections are given in Supplements 1 and 2. This chapter is only a brief overview of the main results and corresponding discussion.

Cell wall mutants in *Arabidopsis thaliana* show increase responsiveness to boron-induced hypocotyl growth (Supplement 1)

Boron as an essential micronutrient for the growth and development plays crucial role in cell wall formation. In this paper, we investigated the possible induction of various *Arabidopsis* cell wall mutants by elevated boron on impact of their growth. We focused mainly on mutants*rsw1* and *prc1* due the fact that both of them are mutated in genes coding for CESA1 and CESA6 cellulose synthase catalytic subunit family, respectively. Mutant *prc1* shows decreased cell elongation, especially in roots and dark-grown hypocotyls. Mutations in *RSW1* cause similar cell wall defects in all cell types, including those in hypocotyls and roots.

We observed that of 2–3 mM of H_3BO_3 had strong stimulatory effect on the etiolated hypocotyl growth of Col-0. In comparison with WT, etiolated *rsw2-1*, *rsw3-1* and *mur4-1* reached the highest stimulatory effect in concentration range between 3-5 mM. Interestingly, hypocotyls of *prc1-1* were stimulated by boron at all concentration levels with maximum of 5–10 mM that are usually inhibitory for WT plants. In other ecotype, Wassilewskija, the growth of etiolated hypocotyl was significantly promoted by 3 mM H_3BO_3. On the other side, dwarf hypocotyl of *rsw1-10* reached the maximum stimulatory growth effect at 20 mM that is highly toxic for growth of WT plants. Thus, our physiological experiments showed that defects in *RSW1* and *PRC1* genes cause tolerance to high boron concentrations (10 mM and higher) that are toxic for WT plants.

Also some other recent studies show that plant response to boron deficiency as well as toxicity could be associated with changes in the transcript level of wide range of genes involved in several physiological processes (Camacho-Cristobal *et al*., 2008; Schnurbursch *et al*., 2010). To elucidate the mechanism of boron action on molecular level with respect to its role in cell wall composition, we studied effect of elevated boron concentrations on expression of genes for *BOR1* and *NIP5;1* boron transporters. In previous experiments, Kocábek *et al*. (2009) showed that mutation in *rsw1-10* affects light-induced expression of *BOR1*. In this paper, we

found out that high boron concentration stimulated expression of *BOR1* and *NIP5;1* genes in etiolated Col hypocotyls that was associated with hypocotyl growth. High boron concentration (10 mM) induced also weak reduction of *BOR1* but significant reduction of *NIP5;1* expression level. We observed different manner in high-boron-tolerant phenotype of *rsw1-10* mutant. The concentration of 10 mM H_3BO_3 was associated with reduction of *BOR1* but with any changes on *NIP5;1* expression.

Auxin binding proteins ABP1 and ABP4 are involved in the light- and auxin-induced down-regulation of phytochrome gene *PhyB* in maize (*Zea mays*, L.) mesocotyl (Supplement 2)

Fellner *et al.* (1996) previously reported that auxin interacts with light in regulation of growth and development of maize by action of ABP4 in this cross-talk. To go deeper, in this paper we studied whether the knockout of ABP1 and /or ABP4 affects expression of PHYA and PHYB in maize mesocotyls.

Firstly, we studied effect of single and double mutations in *ABP1* and *ABP4* on expression level of *PHYA* and *PHYB* on basal medium in etiolated and WL-grown corn mesocotyls. We revealed that mutation in *ABP* genes had no effect on expression of *PHYA* gene in the dark as well as under WL. Interestingly, single mutation in genes *ABP1* and *ABP4* caused reduction of *PHYB* expression, whereas the *PHYB* expression in double mutant was comparable to that observed in WT. Under light conditions, WL strongly reduced expression level of *PHYB* in WT, but it did not have any effect in single or double mutants. It suggests positive influence of both *ABP* genes in *PHYB* expression in the dark. Under light, ABPs seem to play different roles in *PHYB* expression.

Secondly, based on fact that ABP1 in maize binds not only native auxin IAA but also synthetic NAA (Dahlke *et al*., 2009), we studied the effect of exogenous auxin NAA on expression of phytochrome genes under light conditions. We found out that mutation in *ABP* genes caused higher tolerance to the inhibitory effect of NAA in dark-grown plants. Further, we revealed that *PHYB* transcripts were significantly reduced by NAA in etiolated WT mesocotyls but not in mutant plants. In contrast with *PHYB*, level of *PHYA* was not affected by mutation in *ABP* genes in dark as well as WL conditions. It seems that ABP1 and ABP4 do not play a role in auxin-mediated inhibition of *PHYA* expression.

In this paper, we showed for the first time the evidence that knock-out of ABP1 and ABP4 affects expression of *PHYB* in etiolated maize mesocotyls.

6. CONCLUSIONS

Light is one of the key factors regulating plant growth and development. Not only the intensity of light but also spectral quality has a great effect on many aspects of plant life such as photosynthetic performance, differentiation and flowering. Scientists study plant responses to actual biotic and abiotic conditions including the fluctuations of temperature and available nutrients. To elucidate the process, we studied light effects in cross-talk with auxin and boron in the two most studied model plants, *Arabidopsis thaliana* and *Zea mays* L.

To obtain more information about the interaction of auxin and light signaling during maize seedlings development, we investigated the gene expression of *PHYB* and *PHYA* in loss-of-function mutants in *ABP1* and *ABP4* genes in maize. For the first time, we found out that knock-out of *ABP1* or *ABP4* results in reduced expression of *PHYB* gene in dark-grown mesocotyl. White light reduced *PHYB* expression in WT but not in the ABPs knockout seedlings. The data indicate that *ABP1* and *ABP4* are positively involved in *PHYB* expression in etiolated mesocotyl. Our results further suggest that *ABP1*and *ABP4* are not likely involved in the expression of *PHYA* gene and neither in auxin-induced suppression of *PHYA* transcript accumulation. In this paper, we support the existence of cross-talk between auxin and light signaling and indicate for the first time that *ABP1*, *ABP4* and *PHYB* genes could share common signaling pathway(s).

Boron as an essential microelement is crucial for structure of the cell wall. We studied the response of *Arabidopsis thaliana* hypocotyl growth to the changes in boron concentrations with respect to light quality and cell wall composition. We analyzed mutants with defects in genes involved in synthesis of plant cell wall components for their responsiveness to elevated boron. We revealed that especially the mutations *rsw1-10* and *prc1-1* result in ability of etiolated hypocotyl to elongate at the boron concentrations highly toxic for wild type plants. Gene expression analysis confirmed that the stimulation of hypocotyl growth in *rsw1-10* is associated with reduction of *BOR1* expression in mutant hypocotyl in the dark. Similarly, the *prc1-1* hypocotyls grown on 10 mM boric acid showed inhibition of *BOR1* and *NIP5;1* expression. This differentially expressed boron transporters in *rsw1-10* or *prc1-1* versus wild type plants support the existence of mechanism by which wild type plants can tolerate toxic effects of high boron concentrations on plant growth.

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8. LIST OF SUPPLEMENTS

Supplement 1

Kundratova, J., Kocabek, T., Fellner, M. (submitted) Cell wall mutants in *Arabidopsis thaliana* show increase responsiveness to boron-induced hypocotyl growth. *Acta Biochimica Polonica*.

(IF_{2017} 1.239)

Supplement 2

Borucka, J., Fellner, M. (2012) Auxin binding protein ABP1 and ABP4 are involved in the light- and auxin-induced down-regulation of phytochrome gene *PhyB* in maize (*Zea mays*, L.) mesocotyl. *Plant Growth Regulation*, 68, 503–509.

(IF_{2012} 1.670; IF_{2017} 2.081; 5x cited in Web of Science)

Supplement 3

Borucka, J., Fellner, M. (2011) Auxin receptors ABPs and phytochromes interact in maize (*Zea mays* L.) growth. *Current Opinion in Biotechnology*, 22S, S77.

(IF_{2011} 7.711; IF_{2017} 8.380)

SUPPLEMENT 1

Kundratova, J., Kocabek, T., Fellner, M. (submitted) Cell wall mutants in *Arabidopsis thaliana* show increase responsiveness to boron-induced hypocotyl growth. *Acta Biochimica Polonica*.

(IF_{2017} 1.239)

Author's contribution: 60 %.

Cell wall mutants in *Arabidopsis thaliana* show increase responsiveness to boron-induced hypocotyl growth

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Running title: *Arabidopsis* cell wall mutants tolerate higher boron concentrations

Key words: *Arabidopsis*; boron toxicity; boron transporter; cell wall mutants; hypocotyl length; light-responsive growth.

Abbreviations: BL, blue light; BM, basal medium; CESA, cellulose synthase catalytic subunit; EMS, ethyl methanesulfonate; EXPB, expansin B; MS, Murashige-Skoog medium; mur, murus; prc, procuste; RGII, rhamnogalacturonan II; RL, red light; rsw, radial swelling; WT, wild-type.

Funding: The work was supported by grant no. KJB600510503 from the Grant Agency of the Academy of Sciences of the Czech Republic, and by grant no. MSM6198959215 from Ministry of Education, Youth and Sports.

Abstract

Boron is an essential microelement in higher plants present as a structural component of the cell walls. The response of plants to the changes in boron concentrations can be altered by diverse factors, such as light, temperature, or genotypic varieties. In this paper we describe effects of elevated boron concentrations on *Arabidopsis thaliana* hypocotyl growth in *in vitro* conditions with respect to light quality and cell wall composition. We analyzed mutants with defects in genes involved in synthesis of plant cell wall components for their responsiveness to elevated boron, *i.e.* cell wall mutants with defects in monosaccharide biosynthesis (*mur*), cellulose-deficient mutants from radially swollen class (*rsw*), and cellulose synthase mutant *prc*. Our results indicated that changes in the cell wall composition affect growth capacity and/or sensitivity of seedling to boron supplement. We revealed that mutations *rsw1-10, rsw2-1, rsw3-1, prc1-1,* and *mur4-1* result in ability of hypocotyl to elongate at the boron concentrations highly toxic for wild type plants. Gene expression analysis confirmed that the stimulation of hypocotyl growth in *rsw1-10* is associated with reduction of *BOR1* expression in mutant hypocotyl. Similarly, the *prc1-1* hypocotyls grown on 10mM boric acid showed inhibition of *NIP5;1* expression. Differential expression of *BOR1* transporter and/or *NIP5;1* channel protein genes in *rsw1-10* or *prc1-1* and wild type plants support the existence of mechanism by which wild type plants can tolerate toxic effects of high boron concentrations on plant growth. We hypothesize that changes in expression of cell wall-related genes can mediate boron- and light-responsive growth.

Introduction

Boron is a micronutrient essential for the growth and development of all vascular plants. Boron is a crucial component of cell wall formation, membrane integrity and calcium uptake, and may aid in the translocation of sugars (Hu & Brown, 1997; Tariq & Mott, 2010; Uluisik *et al.*, 2018). Additionally, it affects several functions in plants such as flowering, pollen germination, fruiting, cell division, water relationships and the movement of hormones (Blevins & Lukaszewski, 1998; Brown *et al.,* 2002; Bolaños *et al.,* 2004). Boron-deficient plants exhibit a wide variety of symptoms, including the inhibition of root cell elongation and leaf cell expansion (Dell & Huang, 1997, Funakawa & Miwa, 2015). There is a narrow concentration range between boron deficiency and toxicity (Princi *et al.,* 2016). Symptoms of boron toxicity include reduced growth of shoots and roots, reduced fertility and chlorosis followed by necrosis of older leaves, starting at the leaf tip and margins (Bennett 1993; Reid *et al.*, 2004; Fang *et al.*, 2016).

Most boron in plants is fixed in the cell wall where it plays a structural role. Boron maintains the integrity of cell walls by cross-linking rhamnogalacturonan-II (RG-II), a domain of plant cell wall pectins, with other RG-II domains using borate diester bonds (Takano *et al.,* 2002; Koshiba *et al.,* 2009; Funakawa & Miwa, 2015). The RG-II complex is essential for normal plant growth and development; mutant plants with altered RG-II structure display the dwarf phenotype (O'Neill *et al.,* 2001) due to the swelling of the cell walls and the formation of small and irregularly shaped cells (Hu & Brown 1997; Matoh 1997; Ishii *et al.,* 2001; Koshiba *et al.*, 2009; Wu *et al.*, 2017).

The main component of the plant cell wall is the cellulose. Secondary-wall cellulose chains in the xylem of *Arabidopsis* are synthesized by products of the cellulose synthase genes *CESA1*, *CESA3* and *CESA6* (Beeckman *et al.,* 2002; Persson *et al.,* 2007; Desprez *et al.,* 2007). The roles of CESA1 and CESA6 in the synthesis of cellulose were demonstrated by the identification of *Arabidopsis* mutants *rsw1* to *rsw3* (*radial swelling3*) and *prc1* (*procuste1*),

respectively. Defect in *RSW1* was found to impair cellulose synthase catalytic subunit CESA1 (Arioli *et al.,* 1998; Baskin *et al.,* 1995; Fagard *et al.,* 2000). Mutations *rsw2-1* and *kor1-1* affect allelic genes *RSW2* and *KORRIGAN* encoding membrane-bound endo-β-1,4-glucanase (e.g., Lane *et al.,* 2001). Both mutants are also cellulose deficient and show increased pectin content. Mutation *rsw3-1* identified in *RSW3* gene encoding a putative glucosidase II (Burn *et al.,* 2002) shows temperature sensitive radially swollen roots and deficiency in cellulose. Mutations at *PRC1* locus show decreased cell elongation in roots and dark-grown hypocotyls, and it was reported that *prc1-1* is a null mutation in CESA6 product (Desnos *et al.,* 1996; Fagard *et al.,* 2000; MacKinnon *et al.,* 2006).

Another group of cell wall mutants has altered monosaccharide composition. They represent 11 different loci, i.e. *mur1* to *mur 11* (murus), which fall into essentially three groups (Reiter *et al.,* 1993, 1997). Among them, mutant *mur1* shows complete absence of single monosaccharide fucose, whereas mutants *mur2* and *mur3* have only reduced level of fucose. Mutants *mur4* to *mur7* have reduction in arabinose, and *mur8* has reduced content of rhamnose. Finally, the *mur9* to *mur11* represent mutants with complex changes in monosaccharide composition (Reiter *et al.,* 1997). Analysis of the extremely dwarfed mutant *mur1-1* in *Arabidopsis* revealed that the mutation results in synthesis of an altered RGII structure indicating that unaltered borate cross-linked RGII is required for normal *Arabidopsis* growth (O'Neil *et al.,* 2001; Voxeur *et al.*, 2017). On the other hand, many alterations in cell wall composition can be tolerated without affecting normal plant development as shown in *mur* mutants (Reiter *et al.*, 1997, Fagard *et al.,* 2000b).

It is well known that many cellular processes depend on cell wall reconstruction involving the role of expansins. Expansins are extracellular proteins that directly modify the mechanical properties of plant cell walls, leading to turgor-driven cell extension (Cosgrove 2000; Li *et al.,* 2003; Marowa *et al.,* 2016). Expansins are usually divided into four subfamilies: expansin A, expansin B, expansin-like A, and expansin-like B (see Marowa *et al.,* 2016 for a review).

Although many recent studies indicated that expansins enhance plant's tolerance to abiotic stress and expansin genes are often transcriptionally upregulated by abiotic stress conditions (Han *et al.,* 2012; Tenhaken, 2016), this needs further examination because some other studies opposed this notion (e.g. Kwon *et al.,* 2008).

Boron enters plant roots as uncharged boric acid (Hu & Brown, 1997). At high concentrations, boron is transported by passive diffusion (Dordas & Brown, 2000), whereas under low boron conditions plants need an active transport mechanism (Tanaka & Fujiwara, 2008 see for review). Most studies describing active transport of boron focus on BOR (BOR1; BOR4) membrane proteins or NIP (NIP5;1, NIP6;1) channels. BOR1, a membrane protein is essential for efficient boron translocation from roots to shoots under boron limitation (Takano *et al.,* 2002, 2005). In *Arabidopsis* mutant *bor1* that normally suffers from boron insufficiency, dwarf growth and female sterility (Noguchi *et al.*, 2003. When the mutant plants are treated by borate their growth can be rescued and the extent of RG-II cross-linking in cell walls is comparable to that of wild-type plants (O'Neill *et al.,* 2001). Thus, reduced cross-linking of RG-II is likely responsible for dwarf stature of the *bor1* mutant. After boron application the BOR1 protein was observed in the cytoplasm and then transferred to the vacuole for degradation. These results suggest that endocytosis and degradation of BOR1 are regulated by boron availability to avoid accumulation of toxic levels of boron in shoots under high-boron supply (Takano *et al.,* 2005). Miwa *et al.* (2007) reported that BOR4, which is a paralog of BOR1, is not degraded even under high levels of boron supply in *Arabidopsis*. Therefore, it leads to the increase of tolerance of high boron level.

Other membrane proteins involved in boron uptake NIP5;1 and NIP6;1 belong to the NOD26-like intrinsic protein family (Takano *et al.,* 2006; Tanaka *et al.,* 2008; reviewed by Takano *et al.,* 2008 and by Robert & Friml 2009). NIP5;1 was found to be crucial for plant growth under boron limitation in *Arabidopsis* (Takano *et al.,* 2006). NIP6;1 facilitates the permeation of

boric acid across the membrane in shoots. Additionally, it is involved in xylem–phloem transfer of boric acid at the nodal regions (Tanaka *et al.,* 2008).

Although some recent studies focused on the connection between boron, cell wall and light signaling pathway (e.g. Kocábek *et al.,* 2009, Stočes *et al.*, 2012), there are still some underexplored areas in this field. The main aims of this study were (i) to analyze the response of light-induced growth of hypocotyls in different *Arabidopsis* cell wall mutants. Since we found very interesting data from our studies with *rsw1-10* and *prc1-1*, we decided (ii) to find a possible involvement of strong mutant phenotypes associated with CESA1 (*rsw1-10*) and CESA6 (*prc1-1*) with BOR1 and NIP5;1 proteins and (iii) to test the possible role of cell wall regulators in boron-induced growth by studying *EXP-β*.

Material and Methods

Plant material

Experiments were conducted with *Arabidopsis thaliana* (L.) Heynh. ecotypes Columbia (Col-0; NASC stock code N1092), and Wassilewskija (Ws; N915). The following cell wall mutants were used in the experiments: *rsw1-10* (generated by T-DNA insertion in Ws, Baskin *et al.,* 1995; Arioli *et al.,* 1998; Fagard *et al.,* 2000), *rsw2-1* (N6555; EMS, Col-0, Baskin *et al.,* 1992), *rsw3-1* (N6556; EMS, Col-0, Baskin *et al.,* 1992), *prc1-1* (N297; T-DNA, Col-0, Desnos *et al.,* 1996, Fagard *et al.,* 2000), *mur1-1* (N6243), *mur2-1* (N8565), *mur3-1* (N8566), *mur4-1* (N8568), *mur5-1* (N8572), *mur6-1* (N8573), *mur7-1* (N8574), *mur8-1* (N8575), *mur9-1* (N8576), *mur10-1* (N8577) (all *mur* mutants were generated by EMS, Col-0; Reiter *et al.,* 1997). The seeds of *rsw1-10* mutant were kindly provided by Dr. Herman Höfte, INRA, Versailles, France. The seeds of *rsw2-1, rsw3-1*, *prc1-1,* and *mur* mutants were ordered via TAIR and provided by NASC (The Nottingham *Arabidopsis* Stock Center - http://nasc.nott.ac.uk).

Culture conditions and measurement of hypocotyl growth

For sterile (*in vitro*) cultures, seeds were stratified for 3 to 5 days in the dark in distilled water at temperature 4°C. Afterwards, the seeds were surface-sterilized with commercial bleach containing 1.5% (v/v) sodium hypochlorite and one drop of Tween 20 for 15 min, and rinsed five times with sterile distilled water. Seeds germinated on 0.7% (w/v) agar basal medium (BM) in Petri dishes (90 mm in diameter; 50 seeds per dish). The BM contained Murashige and Skoog salts (Murashige and Skoog 1962), 1% (w/v) sucrose and 1 mM MES (2-(N-morpholino)-ethanesulfonic acid) (pH adjusted to 6.1 by KOH before autoclaving). Seeds in the Petri dishes were placed in a temperature-controlled growth chamber (Microclima 1000, Snijders Scientific B.V., The Netherlands) and incubated in the dark at a temperature of 23°C. The BM contained 0.1 mM boric acid (H_3BO_3), which is considered basic sufficient for plant growth as it corresponds to approximately 10 $mg.kg^{-1}$ boron typically found in many soils. After beginning of germination, seeds were transferred on new BM (0.1 mM boric acid) and BM supplemented with the following boric acid concentrations: 2, 3, 5, and 10 mM. In case of *Arabidopsis rsw1-10* mutant and its background Ws, we additionally tested the hypocotyl growth at 20 mM boric acid concentration due to the stimulatory effect observed at 10 mM concentration in *rsw1-10* mutant. Petri dishes with germinating seeds were vertically aligned and then placed in blue light (BL), red light (RL) or kept in the dark. For plant growth under BL or RL, Philips tubes TLD-36W/18-Blue and TLD-36W/15-Red, respectively, were used as a light source. Maximum irradiance of BL was at 440 nm with total photon fluence rate 19 $\mu mol.m^{-2}.s^{-1}$. Maximum irradiance of RL was at 660 nm with total photon fluence rate 10 $\mu mol.m^{-2}.s^{-1}$. The PFD of the lights was measured with a portable spectroradiometer (model LI-1800; Li-Cor, Lincoln, NE, USA) calibrated by the Department of Biophysics at Palacky University in Olomouc at the start of the experiments.

Ten days after the transfer, the seedlings grown under BL, RL or in darkness were spread on a foil with a millimeter screen and length of hypocotyl and root was measured to the nearest millimeter. The measurements were performed on at least 15 plants per experiment.

Each experiment was repeated at least three times and the presented data are mean values ± SE. Statistical significance of the treatment differences was assessed using Student's t-test. All data collected were analyzed using Microsoft Excel software.

Measurement of cell dimensions

In *rsw1-10* mutant and its wild-type Ws, we measured the size and number of hypocotyl cells in epidermal layers by light microscope Olympus BX50. Hypocotyls were excised from 10-day-old seedlings grown on BM and on the BM supplemented with 10 mM H_3BO_3. Hypocotyl sections were laid on a slide, stained with methyl-blue dye (0.01% water solution) in order to facilitate cell observations, and then covered by cover slide. Intact seedlings on culture media were observed using a SZ61 Olympus binocular and pictures were taken by digital compact camera SP-350.

Analysis of gene expression

For gene expression experiments, total RNA was extracted from hypocotyls of 10-day-old plants using TRI Reagent™ (Thermo Fisher Scientific) followed by chloroform-isoamylalcohol extraction. Traces of genomic DNA were removed by DNaseI (Thermo Fisher Scientific) treatment using mix of DNAseI and buffer for 40 min at 25°C on the thermal box. Then, RNA purification on filtered columns was performed by RNA Clean & Concentrator TM-5 kit (Zymo Research, Irvine, CA, USA) according to manufacture instructions. RNA quantity and quality was measured by NanoDrop™ 8000 Spectrophotometer. cDNA was synthesized from 1 µg of total RNA in mixture with appropriate volume of oligo dT(18) and water that was incubated in thermocycler programmed for 72°C for 5 min following by 12°C for 2 min. The reaction continued by adding M-MuLV Reaction Mix (New England BioLabs Inc., MA, USA) consisting of 0.6 µl M-MuLV enzyme , 2 µl M-MuLV enzyme buffer, 2 µl dNTPs and 2,4 µl water and incubating at 42°C for 90 min. cDNA synthesis was stopped by adding 180 µl stop buffer (Tris-HCl 10mM, EDTA-Na 1mM, pH=8). The cDNA product was directly used for quantitative real-time PCR

reaction (qRT-PCR). PCR amplification was performed in mixture containing 12.5 µl of SensiMix (Bioline, London, UK), 0.5 µl of each specific primer (Table 1), 6.5 µl of water and 5 µ of diluted cDNA. qRT-PCR was performed in MyiQ (Bio-Rad laboratories, Madrid, Spain) detection system. Program was set up for holding at 95°C for 30 sec, followed by 40 cycles consisting of 95°C for 15 sec, 60°C for 30 sec and 72°C for sec, ended by 95°C for 1 min and 73°C for 30 sec. The primer sequences are given in Table 1.

All RT-qPCR experiments were performed in darkness in presence of 0.1, 3, and 10 mM boric acid. Statistical analyses of gene expression were performed in R 3.3.2 (R Development Core Team, 2018). All Ct values were normalized against those for the *EF1-α* and *TON1* genes (Dekkers *et al.,* 2012). The differences in the cycle numbers of the samples during the linear amplification phase, along with the ΔΔCt method, were used to determine fold changes in gene expression. All results are expressed in term of ‘‘fold change’’. Relative expression was evaluated using geometrical means calculated from two reference genes in each independent experiment (Humplík *et al.,* 2015). The quoted values represent the mean relative expressions observed in three independent experiments. Overlapping 95% confidence intervals (hereafter CIs) suggest that compared treatments are not significantly different at the alpha = 0.05 level (Whitlock & Schluter, 2015).

Results

Hypocotyl growth responses to boron in cell wall mutants developed in dark and light

As boron is essential part of plant cell wall structure, we investigated possible induction of the dual growth responses in various *Arabidopsis* mutants with defects in cell wall synthesis by elevated boron.

In Col-0, H_3BO_3 at concentrations of 2 to 3 mM promoted growth of etiolated hypocotyls by approximately 20% more compared to the hypocotyls grown on BM at 0.1 mM H_3BO_3 (Fig. 1A). At concentration of 5 mM, boric acid had no essential effect on hypocotyl growth, and at the

highest concentration tested (10 mM), boron noticeably reduced hypocotyl growth by approximately 25% (Fig. 1A). Under RL, significant stimulation (about 20%) of hypocotyl growth in Col-0 was observed at concentrations of 2 to 3 mM. From concentration of 5 mM boric acid reduced hypocotyl elongation, while at concentration of 10 mM, the growth was inhibited by 54% compared to the hypocotyl grown on BM (Fig. 1A). In comparison to darkness and RL, hypocotyls grown under BL on BM were shorter by about 50% (Fig. 1A). Under BL, the maximum stimulation (reaching about 30%) was observed at concentration of 2 to 3 mM. At concentration of 5 mM, no stimulation or inhibition of hypocotyl growth by H_3BO_3 compared to the growth at 0.1 mM H_3BO_3 was observed. At concentration of 10 mM, H_3BO_3 inhibited hypocotyl elongation in BL-grown seedlings more intensively (by 43%) than in etiolated plants (Fig. 1A).

In comparison with Col-0, etiolated *rsw2-1* mutant seedlings developed slightly shorter hypocotyl on BM. The hypocotyl growth was strongly stimulated by boron at concentration of 5 mM (about 45%) (Fig.1B). Additionally, hypocotyl growth in *rsw2-1* mutant was still slightly stimulated by boric acid at concentration of 10 mM (by 15%) (Fig. 1B). Etiolated *rsw3-1* mutant seedlings on BM developed hypocotyl of about the same length as in the case of Col-0. The hypocotyl growth of etiolated seedlings was stimulated by boron at concentration of 3 to 5 mM (by about 27%) (Fig.1C). In both BL and RL, no significant stimulatory or inhibitory effect of boron on hypocotyl growth in the concentrations up to 5 mM was observed. At concentration of 10 mM, hypocotyl of *rsw3-1* mutant was inhibited by boric acid by 15% in darkness, 29% under RL, and 49% under BL (Fig. 1C).

On the BM in darkness, mutation *prc1-1* resulted in dwarf hypocotyl (reaching only 26% of the Col-0 length under the same conditions), and in BL and RL mutant hypocotyl was about 50% of the length of the hypocotyl in Col-0 seedlings (Fig. 1D). Elongation of etiolated hypocotyls in *prc1-1* was stimulated by boron at all concentration levels, with maximum (over 60%) at 5 to 10 mM (Fig. 1D). Mutant hypocotyl under BL and RL was not stimulated by boron at

any concentration level. At the concentration of 10 mM, the inhibition of the mutant hypocotyl by boron was approximately 60% in BL and 77% in RL (Fig. 1D).

The tested mutants with altered monosaccharide composition, i.e. *mur1* to *mur10*, can be classified into four groups based on the growth responses to elevated boron (Table S1). In the first group (*mur5-1* and *mur6-1*), hypocotyls exhibited slight but significant promotion of growth by boric acid in all etiolated, BL- and RL-grown seedlings, usually at the concentration range from 3 to 5 mM (Fig. S1D,E). The second group (*mur1-1*, *mur3-1*, and *mur4-1*) was characterized by lack of boron-induced stimulation of hypocotyl growth in BL. Hypocotyl growth of these mutants was significantly stimulated by H_3BO_3 in seedlings developed in the darkness and in RL at the concentrations from 3 to 5 mM. Interestingly, RL-grown hypocotyls were insensitive to the inhibitory effect of boron at the concentration of 10 mM (Fig 1E and Fig. S1A,C). In this group of mutants, hypocotyl growth in *mur 4-1* showed the highest responsiveness to boron. Elongation of etiolated hypocotyls in *mur4-1* was stimulated by boron at the concentration levels with maximum (around 100%) at 3-5 mM. The growth stimulation was still observed at concentration 10 mM and reached about 40% (Fig. 1E). Growth of mutant hypocotyl under RL was stimulated at concentration 3-5 mM by approximately 36 to 45%. At concentration 10 mM, no inhibitory effect of boron was observed (Fig. 1E). In the third group of the *mur* mutants (*mur7-1*, *mur8-1* and *mur10-1*), hypocotyls were susceptible to the significant stimulatory effect of boron only in plants developed in the dark, with the maximum stimulation at the concentration from 3 to 5 mM (Fig. S1F, G, I). The fourth group (*mur2-1* and *mur9-1*) was characterized by the absence of significant B-induced hypocotyl growth. Additionally, the boric acid essentially inhibited growth of hypocotyls at the concentration of 10 mM in all light conditions tested (Fig. S1B, H). Four of the *mur* mutants tested (*mur1-1*, *mur3-1*, *mur4-1*, and *mur8-1*) exhibited dwarf phenotype. These mutants were at least 30% shorter than their wild type Col-0 on the basal medium under different light conditions. At the concentration of 10 mM H_3BO_3, vast majority of *mur* mutants showed significant decrease of the hypocotyl length in all

light conditions tested but there were several exceptions. The highest boric acid concentration tested was not significantly toxic for *mur1-1* in RL (Fig. S1A), *mur2-1* in BL (Fig. S1B), *mur3-1* under all three light conditions (Fig. S1C), and *mur6-1* and *mur8-1* in the darkness (Fig. S1E, G).

In Ws ecotype, the growth of hypocotyl in etiolated seedlings was significantly promoted by boron at concentration from 1 to 5 mM boric acid, with the peak stimulation at 3 mM. Hypocotyls grown at concentration of 3 mM were by 20% longer than those developed on the BM. At concentrations of 10 to 20 mM boric acid, elongation of etiolated hypocotyl was inhibited by about 22 and 58%, respectively (Fig. 2A). Hypocotyl growth of the seedlings grown under RL showed nearly identical effect to that observed in darkness, however, the maximum of the hypocotyl growth stimulation was reached at concentration of 2 mM H_3BO_3. At concentrations of 10 to 20 mM boric acid, elongation of RL-grown hypocotyl was inhibited by about 41 and 67%, respectively (Fig. 2A). Differently, hypocotyl developed under BL showed only negligible growth promotion at the concentration levels up to 5 mM boric acid. At concentrations of 10 to 20 mM boric acid, elongation of BL-grown hypocotyl was inhibited by about 47 and 62%, respectively (Fig. 2B). Mutation *rsw1-10* led to dwarf hypocotyl regardless of the light conditions and reached only 18–22% of the Ws seedlings length on the BM (Fig. 2B). In darkness, hypocotyl growth was promoted by boric acid at the concentrations of 2 to 10 mM. In comparison with Ws, the maximum stimulation was shifted to the concentration of 10 mM H_3BO_3. At this concentration, the hypocotyl lengths increased by 145% compared to the BM-grown hypocotyls. At the concentration of 20 mM, the etiolated hypocotyls of *rsw1-10* mutant were about the same length as those grown at the BM (Fig. 2B). Under BL as well as RL, the maximum of hypocotyl growth stimulation in *rsw1-10* was induced at the boron concentration of 10 mM, similarly as in the etiolated seedlings. At this concentration, the hypocotyl lengths increased by 90% (BL) and 45% (RL) compared to the BM-grown hypocotyls (Fig. 2B). At the concentration of 20 mM, the hypocotyls of *rsw1-10* mutant were just slightly inhibited in their growth by boron (Fig. 2B).

The measurements of cell dimensions in Ws and *rsw1-10* mutant clearly showed that the

effect of boron on the etiolated hypocotyls caused the changes in cell length. In Ws hypocotyl, H_3BO_3 at the concentration 10 mM reduced length of hypocotyl cells, whereas boric acid at the same concentration distinctly stimulated cell elongation in the mutant *rsw1-10* (Fig. 3A-D).

Effect of prc1-1 and rsw1-10 mutations on boron-induced expression of BOR1 and NIP5;1 genes in etiolated hypocotyls

Boric acid in concentration range between 3 to 10 mM distinctly restored elongation of etiolated hypocotyls in both *prc1-1* and *rsw1-10* mutants (Fig. 1D and Fig. 2B). Here, we investigated the effect of boron on the expression of genes coding the boron transporters, *i.e.*, BOR1 and NIP5;1, in hypocotyls of the two above mentioned mutants.

Expression of *BOR1* in etiolated hypocotyls of Col-0 seedlings was significantly down-regulated by H_3BO_3 at concentration of 3 mM (to 10% of the BM level), whereas at concentration of 10 mM it was 4.1 times up-regulated (Fig. 4A). On the BM, the expression of *BOR1* gene in etiolated mutant *prc1-1* hypocotyls reached less than half of that observed in Col-0. When grown on the medium with concentration of 3 mM boric acid, expression of *BOR1* in the mutant hypocotyl was reduced to half compared to that observed on the BM, and *BOR1* transcript level in *prc1-1* hypocotyl was not distinctly altered by H_3BO_3 at concentration of 10 mM (Fig. 4A).

Expression of *NIP5;1* in etiolated hypocotyls of Col-0 was not significantly influenced by concentration of 3 mM of boric acid. However, 10 mM H_3BO_3 4.7 times upregulated expression of *NIP5;1* gene (Fig. 4B). Mutation *prc1-1* caused strong release of *NIP5;1* expression (4 times more than in the WT) when seedlings grew on the BM. However, increasing of boron concentration in the medium resulted in the gradual reduction of the *NIP5;1* gene expression in the mutant hypocotyls (Fig. 4B).

Expression of *BOR1* in etiolated hypocotyls of WT Wassilewskija was inhibited by boric acid by 80% at the concentration of 3 mM and by 60% at the concentration of 10 mM (Fig. 4D). In mutant *rsw1-10*, expression of *BOR1* on the BM was slightly reduced in comparison with WT.

Expression of *BOR1* in the mutant *rsw1-10* decreased to about half at concentration of 3 mM and to about 60% at concentration of 10 mM (Fig. 4D).

Expression of *NIP5;1* in etiolated hypocotyls of Ws gradually decreased under the increasing concentration of boric acid (Fig. 4E). Mutation *rsw1-10* led to suppression of *NIP5;1* gene expression. Transcript level of *NIP5;1* in *rsw1-10* hypocotyls was about 80% lower than in the corresponding WT on BM. In contrast to WT, there was no change in the *NIP5;1* expression level in mutant genotype at any boric acid concentration (Fig. 4E).

Effect of prc1-1 mutations on boron-induced expression of EXPB

To test the possible involvement of cell wall regulators in light-induced growth, we studied expression of Expansin B (hereinafter referred to as "EXPB", see Marowa *et al.,* 2016 for a review) in *prc1* mutant (background Col) due to strong boron-induced growth response. The level of *EXPB* transcript in Col-0 etiolated hypocotyls was strongly induced by boric acid at concentration of 3 mM (Fig. 4C), whereas at concentration of 10 mM, the expression was roughly similar to that observed in the BM-grown hypocotyls. Mutation *prc1-1* resulted in essential reduction (about three times) of *EXPB* expression on the BM. Differently from WT, *EXPB* expression in *prc1-1* was slightly reduced by boron at concentration of 3 mM, and up-regulated about three times at concentration of 10 mM (Fig. 4C).

Discussion

In this study, we investigated the effect of elevated boron on growth of *Arabidopsis thaliana* hypocotyl to contribute to elucidating the mechanism of boron action on molecular level with respect to its role in cell wall composition.

Although many of recent reports about boron in plants focus on root development (Aquea *et al.*, 2012; Miwa *et al.*, 2013; Abreu *et al.*, 2014; Camacho-Cristóbal *et al.*, 2015; Wang *et al.*, 2017), we have paid our attention on hypocotyl elongation as the organ sensitive, in addition to

hormonal control, to various environmental factors, such as light or nutrition (Boron and Vissenberg, 2014). We hypothesized that the triple combination of factors such as light-mediated response, elevated boron concentration and differences in cell wall composition could bring new insight about plant response to high concentration of boron. We built our research on the results of Kocábek *et al.* (2009) analysing the response of *Arabidopsis* photomorphogenic mutants to elevated boron. Kocábek *et al.* (2009) reported that hypocotyl elongation in *Arabidopsis* was stimulated by boron at concentrations ranging from 1 to 3 mM H_3BO_3. The stimulation of the hypocotyl cells elongation by elevated boron was proportionally more obvious with increasing irradiance. The authors showed that BL and RL did not alter the sensitivity of *Arabidopsis* hypocotyls to boron, but instead, light altered capacity of boron-induced hypocotyl elongation. Additionally, they found out that boron at high concentrations (above 5 mM) has a clear toxicity effect on plant growth. Our efforts were also supported by recent studies showing that plant response to boron deficiency as well as boron excess could be associated with changes in the transcript level of wide range of genes involved in several physiological processes (Schnurbusch *et al.*, 2010; Camacho-Cristobal *et al.*, 2011).

Since boron is an important structural component of the cell walls, we used mutants defective in various cell wall compositions. In our experiments performed on the basic Murashige-Skoog culture medium (the basic MS medium contains 0.1 mM boric acid) hypocotyl elongation was significantly stimulated by boron with maximum rate reached at concentrations from 1 to 3 mM, especially in the dark. In contrast, the highest stimulation of hypocotyl elongation in *prc1* and *rs*w mutants was observed at 5 mM H_3BO_3 where a clear toxicity effect of boron on hypocotyl elongation is usually apparent for WT plants. The most striking effect was noticeable in all studied genotypes in dark. The boron concentration 10 mM H_3BO_3 that is highly toxic for WT plants were still stimulatory for the *rsw1-10* and *prc1* mutant hypocotyls. These data indicate that defects in *RSW1* and *PRC1* genes induce tolerance of hypocotyl growth to high (toxic) boron concentration and therefore further detailed analyzes were focused to these two

mutants.

The CESA6 mutant *procuste1* (*prc1*) has a dwarfed phenotype with reduced cell elongation and radial swelling of the dark-grown hypocotyl (Desnos *et al.,* 1996; Fagard *et al.,* 2000). In contrast, *rsw1-10* mutant shows in addition to swelling of dark-grown hypocotyls radial swelling of root tips (Williamson *et al.,* 2001). Although our experiments were based on the changes of the hypocotyl length, the influence of elevated boron has also been observed on the roots. In our experiment, the root mutant phenotype of *rsw1-10* was completely suppressed at boron concentrations above 10 mM in dark. Similar effect was observed also in other root cell expansion *Arabidopsis* mutants grown under stress conditions, such as low concentration of sucrose (Hauser *et al.*, 1995).

Based on our previous experiments showing that mutation *rsw1-10* affects light-induced expression of *BOR1* (Kocábek *et al.*, 2009) we performed quantification of *BOR1* as well as *NIP5;1* expression in dark grown *prc1-1* and *rsw1-10* mutants at elevated boron. We showed that high boron concentrations stimulated expressions of *BOR1* and *NIP5;1* genes in etiolated Col hypocotyls, which was associated with inhibition of hypocotyl growth. Differently, distinct hypocotyl elongation in *prc1* induced by high boron concentration was associated with weak reduction of *BOR1* and significant reduction of *NIP5;1* expression.

We also found out that *Arabidopsis thaliana* mutant *rsw1-10* shows high tolerance to boron toxicity. This high boron-tolerant phenotype was accompanied by improved growth performance under boron conditions (c=10 mM) that is overly different from Ws in hypocotyl growth under boron toxicity. Distinct hypocotyl elongation in *rsw1-10* mutant by high boron concentration was associated with reduction of BOR1 expression but without any changes on NIP5;1 expression levels.

Our results indicate that cellulose synthase CesA6 is probably functionally connected with boron-induced expression of *BOR1* and thus enhances boron transport mediated by BOR1. Mutation in *PRC1* gene lead to inhibition of both transporters expression and thus might be

related with the plant tolerance to stress of extensive boron level. As shown by others, high boron levels lead to reduction of BOR1 or NIP5;1 transporters and decrease subsequent boron uptake by the plant (Takano *et al.*, 2006; Leaungthitikanchana *et al.*, 2013). We therefore hypothesize that increased tolerance of *prc1* mutant seedlings is mediated by the reduction of *BOR1* and *NIP5;1* expression and thus by reduced transport of boron. In other words, both cellulose synthases CESA1 and CESA6 could be probably involved in mechanisms by which plants can tolerate toxic effects of high boron concentrations on plant growth. However, this hypothesis must be experimentally confirmed in subsequent research.

It is well known that many cellular processes depend on cell wall reconstruction involving the role of expansins (Carpita and McCann, 2000). There is clear evidence that EXPB1 plays a role in boron deficiency, as it was significantly down regulated after 6 and 24 hours under boron deficiency (Camacho-Cristóbal *et al.*, 2008). Here we investigated expression of β-expansin in etiolated hypocotyls of Col-0 and mutant *procuste1* under increasing concentrations of boron. Our study confirms the direct evidence for a structural role of boron in the cell wall assembly and function. Our data indicate that the level of *EXPB1* transcript was essentially affected by mutation in *PRC1* gene. Obtained results from molecular experiments confirmed correlation between expression level of expansin *EXPB1* in *prc1* mutant and corresponding WT and the boron-induced hypocotyl elongation of both genotypes in darkness. According to the physiological experiments, boron at the concentration of 3 mM stimulated the etiolated hypocotyl growth of Col, whereas concentration of 10 mM boric acid had strong inhibition effect. The same pattern was apparent with *EXPB1* expression level in WT Col. Interestingly, mutant *prc1* is tolerant to high concentration of boron (10 mM boric acid) in the darkness. Furthermore, its dwarf genotype suggested low expression level of *EXPB1* that was not affected by boron level at the concentration of 3 mM, but was stimulated by 10 mM boric acid. It totally corresponds to growth curvature and confirms the physiological role of expansin B in cell wall expansion and remodeling during the growth and the cross-talk between different regulators of the cell wall. The

role of *PRC1* gene is totally fixed in the cell wall remodeling during growth under different boron conditions.

Conclusions

Hitherto only some mechanisms promoting tolerance to toxic levels of boron were discovered. Our physiological experiments showed that defects in *RSW1 and PRC1* genes cause tolerance of hypocotyl growth to high boron concentration (10mM) that is toxic for WT plants. Further, we suggest that light reduces responsiveness to boron. Quantitative expression data indicate that boron transport mediated by BOR1 and NIP5;1 proteins in *Arabidopsis* hypocotyl is a complex process regulated by light, level of exogenous boron with involvement of the cellulose synthase CesA6, coded by *prc1* gene. We hypothesize that BOR1 transporter is cross-linked in some signaling pathway with cell wall coding genes. Interestingly, boron, light and CesA6 are significantly involved in regulation of expression of *EXPB,* another member of the cellulose synthase catalytic subunit family CesA1. The obtained data indicate that boron transport is an important player in the process of *Arabidopsis* hypocotyl growth. Anyway, more experiments with specific settings are needed to elucidate the role of boron transporters especially in world-known crop mutants in boron sensitive plants like citruses or corn. One of the interesting aspects should be the cross-talk between boron transporters and antioxidant enzymes, boron and sugar metabolism or using CRISPR/Cas9 genome editing system as a new technology tool for easy targeted mutagenesis of genes involved in the processes associated with the response of a plant cells to elevated boron.

Acknowledgements

We thank all members of Laboratory of Molecular Physiology, especially Mrs. Renáta Plotzová, for excellent technical assistance. The authors thank Dr. Herman Höfte (INRA, Versailles, France) for providing seeds of *rsw1-10* mutants, and Dr. Junpei Takano and Toru Fujiwara from

University of Tokyo for *bor1-1* seeds. We are grateful to prof. Agustin Gonzales Fontes (Universidad Pablo Olavide, Sevilla) and all members of his laboratory for their kind hospitality and help with experiments. The authors declare no conflict of interest.

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Figure legends

Figure 1. Effect of light on hypocotyl growth of the *Arabidopsis thaliana* cell wall mutants *rsw2-1* (B), *rsw3;1* (C), *prc1-1* (D), *mur4-1* (E), and their background, Col-0 (A). The results are mean values ± S.E. obtained from three independent experiments (n=20 per each experiment). "*" indicates statistically significant difference ($P<0.05$) from the plants grown on 0.1 mM H_3BO_3.

Figure 2. Effect of light on hypocotyl growth of the *Arabidopsis thaliana* cell wall mutant *rsw1-10* (B) and its background, Ws (A). The results are mean values ± S.E. obtained from three independent experiments (n=20 per each experiment). "*" indicates statistically significant difference ($P<0.05$) from the plants grown on 0.1 mM H_3BO_3.

Figure 3. Size of epidermal cells in hypocotyls of 10-day-old etiolated Ws and *rsw1-10* mutant seedlings**.** Ws seedlings grown on basal medium without H_3BO_3 (A) and on basal medium with 10 mM H_3BO_3 (B). Mutant *rsw1-10* seedlings grown on basal medium without H_3BO_3 (C) and on basal medium with 10 mM H_3BO_3 (D). Scale bars: 100 μm (A, B), 50 μm (C, D).

Figure 4. Relative expression of *BOR1* (A), *NIP5;1* (B) and *EXPB* (C) in etiolated hypocotyl of WT (Col) and the mutant *prc1-1* at the boron concentrations of 0.1, 3, and 10 mM. Relative expression of *BOR1* (D) and *NIP5;1* (E) in etiolated hypocotyl of WT (Ws) and the mutant *rsw1-10* at the boron concentrations of 0.1, 3, and 10 mM. The results are mean values ± S.E. obtained from three independent experiments by quantitative real-time PCR. "a" indicates statistically significant differences ($P<0.05$) from WT in appropriate concentrations. "b" indicates statistically significant differences ($P<0.05$) from WT in absence of boric acid.

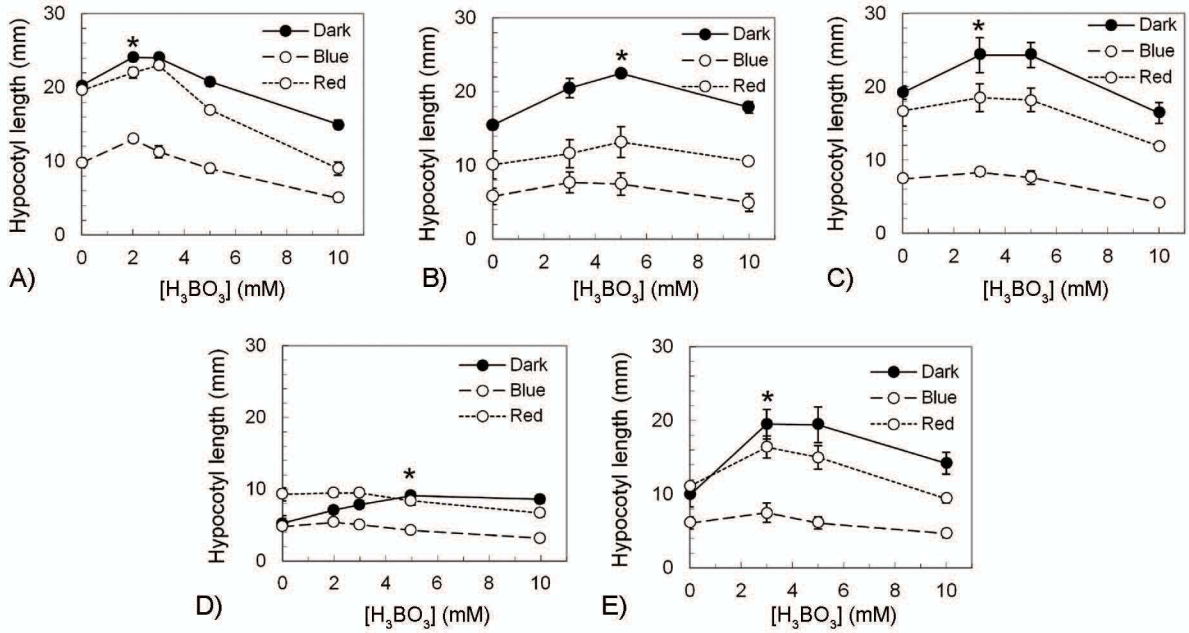
Table 1. Primers used for the qRT-PCR experiments in this study.

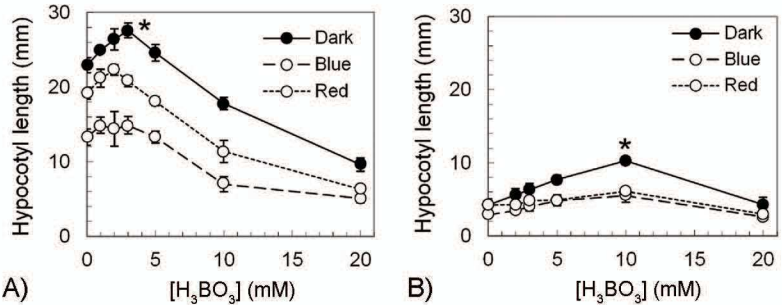
Gene	Primer	Sequence	length (bp)
AT5G60390	EF1-alpha F-	5‘-TTGACCAGATCAACGAGCCCAAGA-3‘	24
	EF1-alpha R-	5‘-TCAACCCTGTGGGAGCAAAGGTAA-3‘	24
AT3G55000	TON1 F-	5‘-TGTGAGGGATGGAACAAATG-3‘	20
	TON1 R-	5‘-AACGCAGTTGCAAATAAAGGA-3‘	21
AT2G47160	BOR1 F-	5‘-TCGCTTCTGCGATTCCTGTCATCT-3‘	24
	BOR1 R-	5‘-AAGCAGTGGCTGACCTCCGATAAT-3‘	24
AT4G10380	NIP5;1 F-	5‘-CACCGATTTTCCCTCTCCTGAT-3‘	22
	NIP5;1 R-	5‘-GCATGCAGCGTTACCGATTA-3‘	20
AT2G45110	EXPβ1 F-	5‘-CACCTCTCGCCTCAACTTTCT-3‘	21
	EXPβ1 R-	5‘-GATGGCTCACTCATTG-3‘	16

Supplementary Information

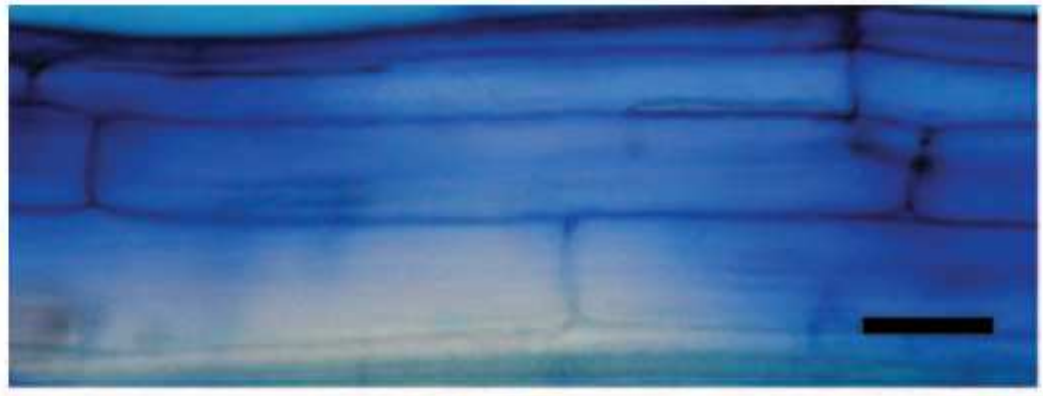
Figure S1. Effect of light on hypocotyl growth of the *Arabidopsis thaliana* cell wall mutants *mur1-1* (A), *mur2-1* (B), *mur3-1* (C), *mur5-1* (D), *mur6-1* (E), *mur7-1* (F), *mur8-1* (G), *mur9-1* (H), *mur10-1* (I). The results are mean values ± S.E. obtained from three independent experiments (n=20 per each experiment). * indicates statistically significant difference ($P<0.05$).

Table S1. Summary of hypocotyl growth responses to the effect of boron in *mur* mutants tested under different light conditions. BL, blue light; RL, red light; + indicates presence of boron-induced hypocotyl elongation; - indicates absence of boron-induced hypocotyl growth.

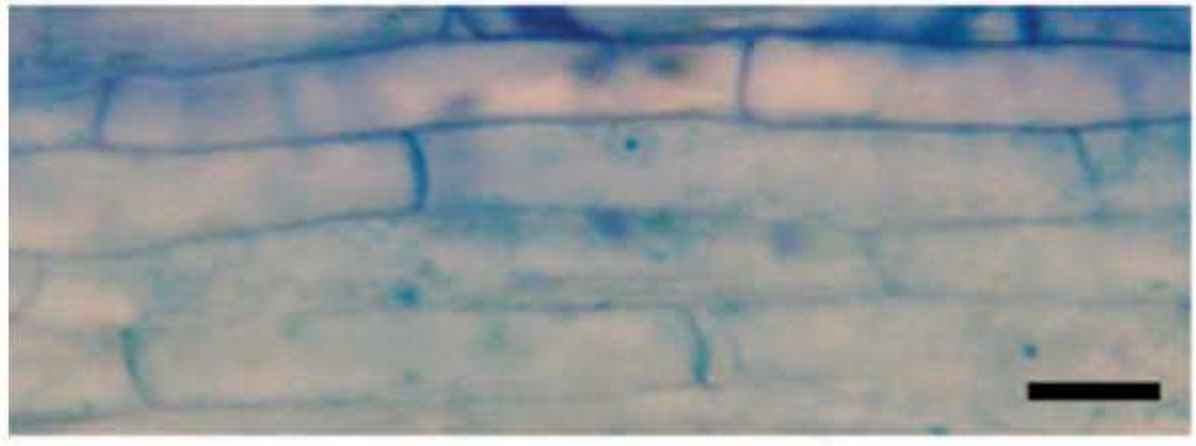

A)
B)
C)
D)
E)
Hypocotyl length (mm)
[H3BO3] (mM)
Dark
Blue
Red
*


Hypocotyl length (mm)
30
20
10
0
0
5
10
15
20
[H3BO3] (mM)
Dark
Blue
Red
*
A)
B)

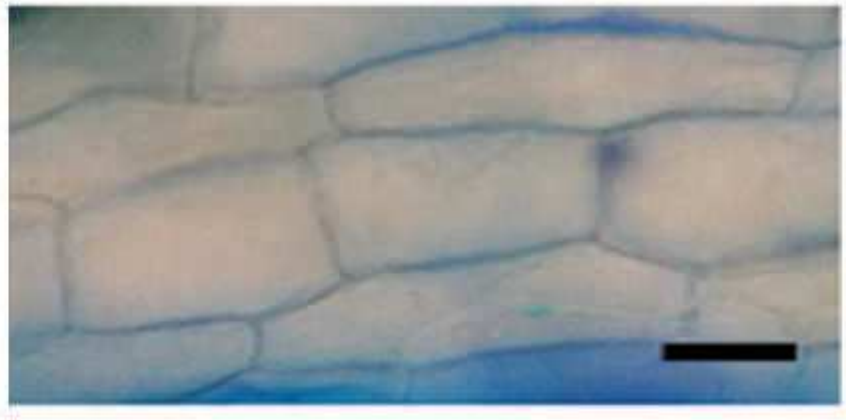
A)



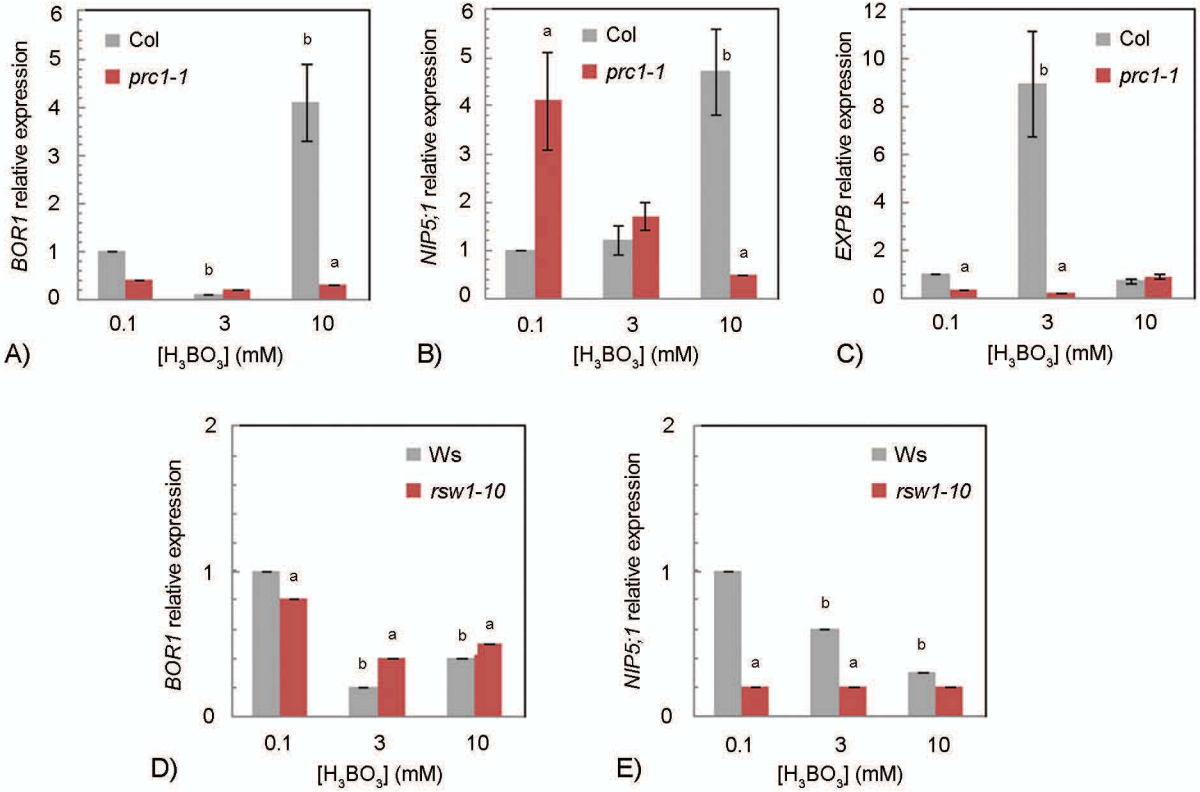
B)



C)



D)


A)
BOR1 relative expression
Col
prc1-1
0.1
3
10
[H3BO3] (mM)
B)
NIP5;1 relative expression
Col
prc1-1
0.1
3
10
[H3BO3] (mM)
C)
EXPB relative expression
Col
prc1-1
0.1
3
10
[H3BO3] (mM)
D)
BOR1 relative expression
Ws
rsw1-10
0.1
3
10
[H3BO3] (mM)
E)
NIP5;1 relative expression
Ws
rsw1-10
0.1
3
10
[H3BO3] (mM)

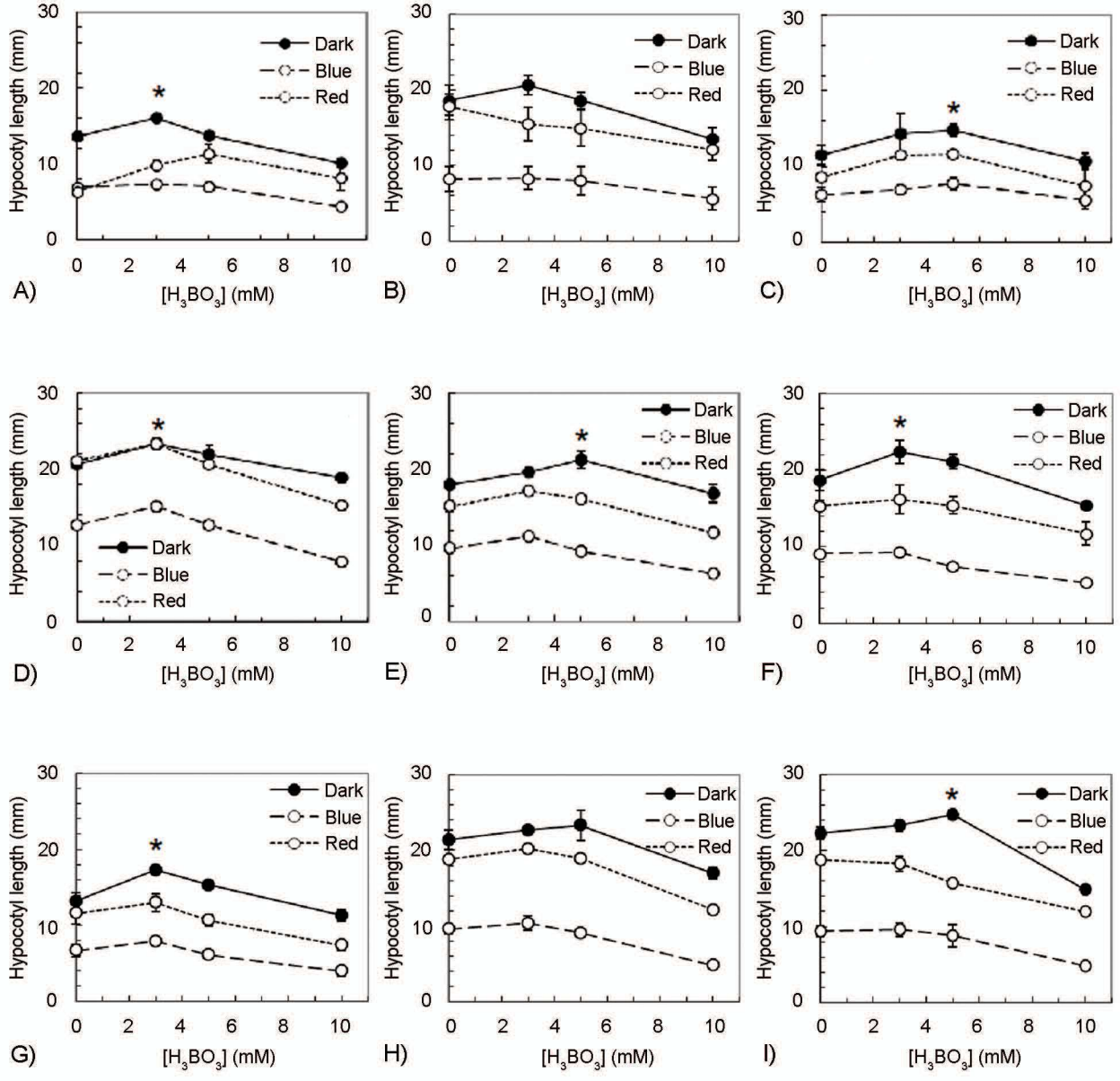

Hypocotyl length (mm)
Dark
Blue
Red
*
0
10
20
30
0
2
4
6
8
10
A)
B)
C)
D)
E)
F)
G)
H)
I)
[H3BO3] (mM)

Table S1. Summary of hypocotyl growth responses to the effect of boron in *mur* mutants tested under different light conditions. BL, blue light; RL, red light, **+** indicates presence of boron-induced hypocotyl elongation, **-** indicates absence of boron-induced hypocotyl growth.

Genotype	**Dark**	**BL**	**RL**
Col-0	+	+	+
mur5-1	+	+	+
mur6-1	+	+	+
mur1-1	+	-	+
mur3-1	+	-	+
mur4-1	+	-	+
mur7-1	+	-	-
mur8-1	+	-	-
mur10-1	+	-	-
mur9-1	-	-	-
mur2-1	-	-	-

SUPPLEMENT 2

Borucka, J., Fellner, M. (2012) Auxin binding protein ABP1 and ABP4 are involved in the light- and auxin-induced down-regulation of phytochrome gene *PhyB* in maize (*Zea mays*, L.) mesocotyl. *Plant Growth Regulation*, 68, 503–509.

(IF_{2012} 1.670; IF_{2017} 2.081; 5x cited in Web of Science)

Author's contribution: 80 %.

Plant Growth Regul (2012) 68:503–509
DOI 10.1007/s10725-012-9719-x

BRIEF COMMUNICATION

Auxin binding proteins ABP1 and ABP4 are involved in the light- and auxin-induced down-regulation of phytochrome gene *PHYB* in maize (*Zea mays* L.) mesocotyl

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Received: 4 February 2012 / Accepted: 17 May 2012 / Published online: 29 May 2012

Abstract Previous research in maize suggested a possible involvement of auxin-binding proteins (ABPs) in light signaling during maize seedling development. To obtain more information about the interaction of auxin and light signaling, we investigated the gene expression of phytochrome B (*PHYB*) and phytochrome A (*PHYA*) in loss-of-function mutants in *ABP1* and *ABP4* genes in maize (*Zea mays* L.). We studied how expression of the *PHYB* and *PHYA* genes in mesocotyl is regulated by white light (WL), and whether exogenous auxin NAA influences the expression of the phytochrome genes. We found that knockout of *ABP1* or *ABP4* results in essentially reduced expression of *PHYB* gene in dark-grown mesocotyl. WL reduced *PHYB* expression in WT but not in the *ABPs* knockout seedlings. The data indicate that ABP1 and ABP4 are positively involved in *PHYB* expression in etiolated mesocotyl. Our results also indicate that in etiolated mesocotyl, ABP1 and ABP4 mediate the inhibitory effect of exogenous auxin on level of *PHYB* transcript. In contrast, in our experimental conditions, WL does not reduce expression of *PHYA*. Our results further suggest that ABP1 and ABP4 are not likely involved in the expression of *PHYA* gene and neither in auxin-induced suppression of *PHYA* transcript accumulation. Our results support the existence of cross-talk between auxin and light signaling and indicate for the first time that *ABP1*, *ABP4* and *PHYB* genes could share common signaling pathway(s).

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Keywords Auxin binding protein · *ABP1* · *ABP4* · Gene expression · Mesocotyl · *PHYA* · *PHYB* · *Zea mays*

Abbreviations

ABP	Auxin binding protein
NAA	1-Naphthalene acetic acid
WL	White light
WT	Wild-type

Introduction

The primary step of auxin signaling is the binding of auxin to an auxin receptor. In addition to described function of auxin receptors from TRANSPORT INHIBITOR RESPONSE 1/AUXIN-BINDING F-BOX PROTEIN (TIR1/AFB) family, putative auxin receptor, the ABP1 (AUXIN-BINDING PROTEIN 1) has been identified (reviewed in Tromas and Perrot-Rechenmann 2010, and in Shishova and Lindberg 2010). ABP1 is a protein with high-affinity to auxin identified in maize coleoptiles over 30 years ago by its capacity to bind radiolabelled auxin (Hertel et al. 1972; Löbler and Klämbt 1985). Several studies demonstrated that ABP1 acts at the plasma membrane (Barbier-Brygoo et al. 1989; Leblanc et al. 1999). On the other hand, the predominant localization of ABP1 was found to be in the endoplasmic reticulum lumen (Jones and Herman 1993). It was demonstrated that ABP1 plays a role in cell expansion (Jones et al. 1998; Chen et al. 2001) and in cell division (Fellner et al. 1996; David et al. 2007). Effendi et al. (2011) provided evidence that ABP1 is involved in regulation of polar auxin transport thus affecting local auxin concentration and early auxin gene

regulation (Braun et al. 2008). Recently, the role of ABP1 was extensively reviewed (e.g. Tromas et al. 2010; Sauer and Kleine-Vehn 2011; Scherer 2011). Differently from *Arabidopsis*, where only one homolog of ABP1 is present, at least five ABPs, including ABP1, have been identified in maize so far (Schwob et al. 1993), but their roles in growth and development have yet to be elucidated.

Light as an external factor regulates plant growth in the complex interaction with internal factors including auxins. The mechanisms how auxins can be involved in light-induced growth inhibition is not yet fully understood. Various studies have shown a correlation between light responses and auxin levels or polar auxin transport (reviewed in Tian and Reed 2001; Liu et al. 2011), and a number of reports demonstrated the existence of signalling elements shared by light and auxin (reviewed in Halliday et al. 2009; Li et al. 2012) One of the hypotheses how light via phytochromes mediates the decrease in growth involves ABP1 (Walton and Ray 1981; Jones et al. 1989). This idea was supported by the observation that red light (RL) reduces the abundance of ABP1 (Jones and Venis 1989; Jones et al. 1991). Interestingly, the expression of another member of the ABP family, *ABP4* in maize mesocotyls was up-regulated in RL- and FR-grown seedlings (Fellner et al. 2006).

In *Arabidopsis*, the phytochrome family consists of five genes (*PHYA*, *PHYB*, *PHYC*, *PHYD* and *PHYE*) (Sharrock and Quail 1989; for review see Franklin and Quail 2000). In maize, the gene family consists of six phytochromes: *PHYA1*, *PHYA2*, *PHYB1*, *PHYB2*, *PHYC1* and *PHYC2* (Gaut and Doebley 1997; Sheehan et al. 2004; Sawers et al. 2005). Sheehan et al. (2004), the authors showed that all six phytochrome genes are transcribed in several seedling tissues, while the expression of *PHYA1*, *PHYB1*, and *PHYC1* predominate in all seedling tissues examined. The authors also showed that etiolated seedlings express higher levels of *PHYA* and *PHYB* than plants developing in light, whereas the expression of *PHYC* was not affected by light.

We previously reported that seedlings of modern maize hybrid 3394 with defect in *ABP4* gene expression show changes in its growth responses to auxin and light (Fellner et al. 2006). To obtain more information about the interaction of auxin and light signalling, we investigated the expression of phytochrome genes *PHYB* and *PHYA* in loss-of-function mutants in *ABP1* and *ABP4* genes in maize (*Zea mays* L.). We studied how the expression of the *PHYB* and *PHYA* in mesocotyls is regulated by white light (WL), and whether the artificial auxin NAA influences the expression of the phytochrome genes in darkness. Our results here support the existence of cross-talk between auxin and phytochrome signalling and indicate for the first time the involvement of ABP1 and ABP4 in phytochrome signalling pathways.

Materials and methods

The loss-of-function mutants in *ABP1* and *ABP4* genes in maize (*Zea mays* L.) were used for all experiments (Im et al. 2000). The *abp* mutants contained the Robertson's *Mutator* transposable elements (Bennetzen 1996) in *ABP1* and/or *ABP4* genes. Seeds of single mutants *abp1* (B2 allele) and *abp4* (B2/K1 allele), double mutants *abp1abp4* (B2/K1 allele) and a near isogenic line (here called WT) were a gift from Alan M. Jones (The University of North Carolina, Chapel Hill, NC). All *abp* mutants were tested for the lack of *ABP1* and/or *ABP4* gene expression, and they showed stable phenotypes. The fytochromobilin-deficient mutant *elm1* (*elongated mesocotyl 1*) was initially identified in the W22 background (Sawers et al. 2002) and was also introgressed into the B73 background (inbred maize line) by backcrossing five times (Dubois et al. 2010). Kernels of *elm1* and B73 were a gift from Thomas P. Brutnell from Boyce Thompson Institute for Plant Research, Ithaca, NY.

For experiments in vitro, seeds were surface sterilized (70 % ethanol for 3 min, soaked in 5 % sodium hypochlorite, and rinsed with sterile distilled water). The seeds germinated on a 0.7 % (w/v) agar medium in Magenta GA7 boxes (77 × 77 × 196 mm; Sigma-Aldrich, Prague, Czech Republic) (9 seeds per box). The basal medium (BM) contained Murashige and Skoog salts (MS medium; Sigma-Aldrich, Prague, Czech Republic) (Murashige and Skoog 1962), 1 % (w/v) sucrose and 1 mM MES (2-(N-morpholino)-ethanesulfonic acid; pH adjusted to 6.1 before autoclaving). In experiments with auxin, the BM was supplemented with 1-Naphthaleneacetic acid (NAA) in various concentrations. Seeds in the Magenta boxes were placed in a growth chamber (Percival PGC-10, IA, USA) and incubated in the dark or white light (WL; Philips PL-L-40 W/840/4P, USA; total photon fluence rate 150 μmol m^{-2} s^{-1}). For the development of etiolated seedlings, the boxes were wrapped in aluminium foil. In all light conditions, the seeds were incubated for 7 days at a temperature of 23 °C. The fluence rate was measured with a portable spectroradiometer (model LI-1800; Li-Cor; Lincoln, NE) calibrated by the Department of Biophysics at Palacky University in Olomouc. The mesocotyl length (from the scutellar to the coleoptilar node) was measured with a ruler to the nearest millimeter. Mesocotyl in 6–9 seedlings per treatment in each independent experiment was measured. Changes in mesocotyl growth (i.e. inhibition or stimulation) caused by auxin were expressed in percents based on the following formula: $X = 100 \times (A - B)/A$, where "X" is the change in growth (in %), "A" and "B" stand for growth (in mm) in the absence and presence, respectively, of auxin.

For gene expression experiments, total RNA was extracted from the mesocotyl of 7-day-old plants using an

RNeasy Plant Mini RNA kit (Qiagen Inc., USA, Valencia, CA) according to the manufacturer's instructions. After RNA extraction was performed, traces of genomic DNA from a batch of RNA was removed by a DNaseI treatment using RQ1 RNA-free DNase (Promega, USA) for 40 min at 37 °C in a water-bath. RNA was then mixed with phenol:chloroform:isoamylalcohol (25:24:1) vortexed and spun to allow the two phases to form. Afterwards, supernatant was transferred into a new tube and the volume completed with 20 μL of 1 M acetic acid and 550 μL of 96 % ethanol. The mixture was incubated overnight and then washed with ethanol. RNA quantity and quality was then measured by a spectrophotometer Smart Spec Plus 2000 (BioRad, Czech Republic).

A reverse transcription reaction was performed with 1 μg of total RNA by adding 4 μL of 5 × FS (First Strain) buffer, 1 μL of 10 mM dNTP, 1 μL of 0.1 M DTT, 1 μL of 50 μM oligo(dT)20 primer, 0.5 μL of RNaseOUT (InVitrogen, Carlsbad, CA, USA) and 1 μL of SuperScript III Reverse Transcriptase (InVitrogen Co., Carlsbad, CA, USA). The volume was filled up to 20 μL with RNase-free water. The reaction mixture was incubated in a thermocycler (MJ Mini Gradient Thermal Cycler, BioRad, Czech Republic) programmed for 50 °C for 60 min followed by 85 °C for 5 min and ended by cooling for 15 min. The cDNA product was directly used in PCR (Polymerase Chain Reaction). PCR amplification was performed in a mixture containing: 4 μL of 5X GoTaq polymerase buffer, 2 μL of 1 mM dNTP, 1 μL of 10 mM of each gene-specific primer, 1 μL of GoTaq polymerase (Promega, USA) and 1 μL of diluted cDNA. The *PHYA* (accession no. AT 1G09570) and *PHYB* (accession no. AT2G18790) gene specific primers (Table 1) were used and PCR reactions of 24 cycles were performed as described in Sheehan et al. (2004) and adjusted in initial experiments. Number of cycles for expression of *18S rRNA* was also carefully adjusted to detect possible differences in expression between genotypes and conditions. The template cDNAs were denatured at 95 °C for 15 min followed by cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Final elongation was performed at 72 °C for 10 min followed by cooling at 4 °C. The *18S rRNA* gene (accession no. AF168884) of maize was used as reference gene and amplified using the specific primers (Table 1). The template cDNAs were denatured at 94 °C for 3 min followed by cycles of 94 °C for 30 s, 55 °C for 45 s, and a 30 s extension at 72 °C for 22 cycles. The final extension was performed at 72 °C for 5 min followed by cooling at 15 °C. PCR products were size fractionated by electrophoresis in a 1 % (w/v) agarose gel stained with ethidium bromide. Detected bands were evaluated using the software package ImageJ to obtain semi-quantitative data on relative gene expression. The numbers on axis "y" in the expression graphs are number of pixels of band signals. When necessary, the statistical significance of the treatment differences was assessed using Student's *t* test.

Table 1 List of gene-specific primers used for semi-quantitative RT-PCR

Primer	Sequence
PHYA—F	5′-GAG AGA TCC ATG AAG CAA AAG GTT TAC-3′
PHYA—R	5′-GAA GGT TGA CAT GCC CAG CTT CCC TGA G-3′
PHYB—F	5′-GTT TTG GCT GAC TTC GCT AAG CAT G-3′
PHYB—R	5′-GGA CGA TGA GGA AGA AAC TCC GCT CTG-3′
18S rRNA—F	5′-ACG AAC AAC TGC GAA AGC-3′
18S rRNA—R	5′-CGG CAT CGT TTA TGG TTG-3′

Results and discussion

We previously reported that in the regulation of growth and development of young maize seedlings auxin interacts with light, and we hypothesized that *ABP4* plays an important role in this cross-talk (Fellner et al. 2006). To get insight into the interaction, we investigated whether the knockout of *ABP1* and/or *ABP4* affects expression of genes coding for phytochrome B (*PHYB*) and phytochrome A (*PHYA*) in maize mesocotyls developed in darkness or in white light (WL).

In maize seedlings with knockout *ABP1* or *ABP4* gene grown in darkness, the expression of *PHYB* was significantly lower than in mesocotyls of WT plants. Interestingly, when both genes were off in the double mutant *abp1/abp4*, expression of *PHYB* was similar to that observed in WT plants (Fig. 1). The results indicate that in darkness, ABP1 and ABP4 positively influence *PHYB* expression, and that ABP1 and ABP4 functionally depend on each other. Alternatively, it is possible that the knockout of *ABP1* and *ABP4* trigger alternative pathway(s) leading to the normal expression of *PHYB*. In our experimental conditions, WL strongly reduced level of *PHYB* transcript in WT plants, whereas it had not any essential effect on the level of *PHYB* transcript in single and double mutants (Fig. 1). Namely, the knockout of *ABP1* and/or *ABP4* gene led to the similar expression of *PHYB* in dark- and WL-developed mesocotyls. However, WL reduced the expression of *PHYB* in WT mesocotyl to the level similar to that in *abp* mutants. It is therefore possible that in the dark, ABPs do not stimulate *PHYB* expression directly but rather through positive regulation of some transcription factor,

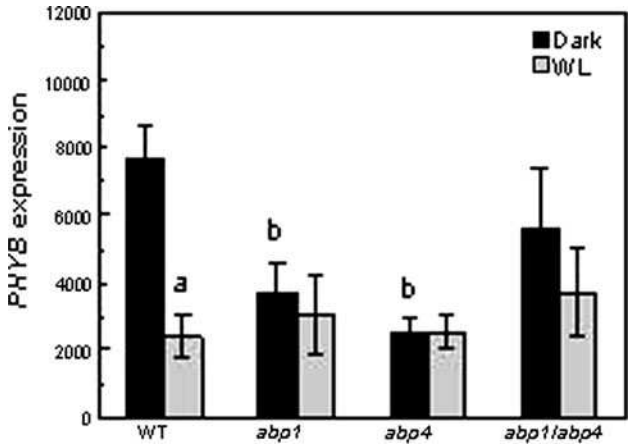

Fig. 1 Expression of *PHYB* gene in mesocotyls of etiolated or white light-grown WT and *abp* mutants. WT and mutant plants grew in conditions in vitro on the BM. *PHYB* expression analysis was performed by semi-quantitative RT-PCR, and *18S rRNA* was used as a reference gene. In each genotype and each condition, the data represent average from 10 identical independent experiments ±SE. In each experiment, in each condition and in each genotype, a mix of 20 mesocotyls was always used. **a** significantly different ($P < 0.05$) from the dark in each genotype; **b** significantly different ($P < 0.05$) from WT in the dark

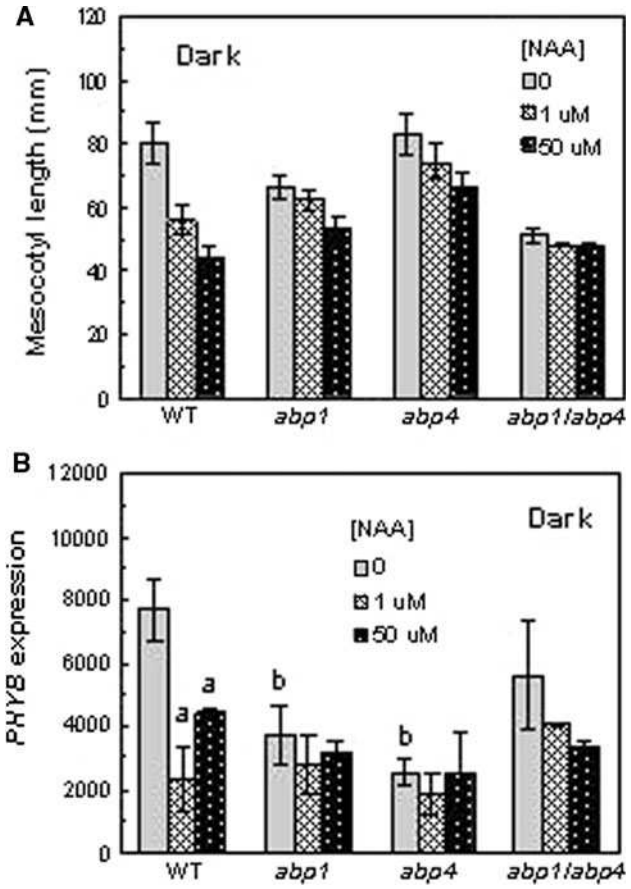

Fig. 2 Responsiveness of mesocotyls in etiolated WT and *abp* mutants to exogenous auxin in regards to elongation and *PHYB* gene expression. **a** Comparison of mesocotyl growth in etiolated single mutants *abp1*, *abp4*, double mutant *abp1abp4*, and corresponding WT, in response to NAA. Mesocotyl elongation was measured with a ruler to the nearest millimeter in 7-day-old seedlings grown in Magenta boxes in darkness, on the BM supplemented with 10^{-6} or 5×10^{-5} mol L^{-1} NAA. The results are the mean length ±SE obtained from 5 to 12 independent experiments. In each experiments, each genotype and in each condition, six to nine seedlings were measured. **b** Expression of *PHYB* in mesocotyls of WT and *abp* mutants developed in the dark as a function of exogenous auxin. WT and *abp* mutant plants grew in conditions in vitro on the BM supplemented with NAA at concentrations of 10^{-6} or 5×10^{-5} mol L^{-1}. *PHYB* expression analysis was performed by semi-quantitative RT-PCR, and *18S* rRNA as a reference gene was used. In each genotype and each condition, the data represent average from 10 identical independent experiments ±SE. In each experiment, in each condition and in each genotype, a mix of 20 mesocotyls was always used. **a** significantly different ($P < 0.05$) from corresponding control (absence of NAA); **b** significantly different ($P < 0.05$) from WT in the absence of NAA

which could be however blocked (destabilized) in WL conditions. The results on *PHYB* expression in WT developed under WL are not consistent with the results reported by Sheehan et al. (2004). The authors showed that dark- and WL-grown mesocotyls accumulates *PHYB* at similar levels. The discrepancy could reflect the differences of WL sources used by us and Sheehan et al. (2004), and/or that differently from the authors we used very young plants developed in conditions in vitro. On the other hand, the authors showed tissue-specific accumulation of *PHYB* is light dependent. Neither in the dark nor in WL, levels of *PHYB* transcript in WT and *abp* mutants correlate with mesocotyl length. However, this conclusion is obvious also from results of Sheehan et al. (2004).

It was reported that ABP1 in maize binds not only native auxin IAA but also artificial auxin NAA (Ray and Dohrmann 1977; Dahlke et al. 2009). Here we found that intact etiolated maize seedlings with knockout *ABP1* and/or *ABP4* are much less sensitive to the inhibitory effect of NAA than plants with the functional ABPs (Fig. 2a). We further revealed that the level of *PHYB* transcript was significantly reduced by NAA in etiolated WT mesocotyls but not in mesocotyls of the knockout plants (Fig. 2b). The data suggest that in etiolated mesocotyls, functional ABP1 and ABP4 are required for NAA-induced inhibition of mesocotyl elongation and NAA-induced inhibition of *PHYB* expression. As evident, the lack of NAA-induced inhibition of *PHYB* expression in the *abp* mutants correlates with the lack of the mutant sensitivity to exogenous auxin in elongation response. To support the existence of the cross-talk between *PHYB*- and *ABP*-mediated signalling pathway, we studied sensitivity to exogenous auxin of *elm1* (*elongated mesocotyl 1*) mutant deficient in fytochromobilin (Dubois et al. 2010). In agreement with our hypothesis, etiolated *elm1* mesocotyls showed distinctly less sensitivity to NAA than corresponding WT (Fig. 3).

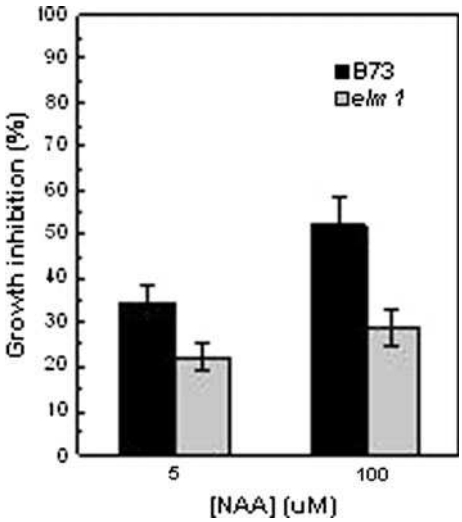

Fig. 3 Responsiveness of mesocotyls in intact etiolated *elm1* mutant to exogenous auxin. Growth responses of maize *elm1* mutant and corresponding WT (B73) cultured in the dark on the BM supplemented with NAA at concentrations of 5×10^{-6} or 10^{-4} mol L^{-1}. The values are the mean length ±SE from six independent experiments, with 6–9 plants measured in each experiment. Auxin-induced inhibition of mesocotyl growth (in %) was calculated as described in the "Materials and Methods"

In WL conditions, auxin had slight and very variable effects on *PHYB* expression in all genotypes tested. Although for each condition and each genotype, ten independent experiments were conducted, the obtained results were inconclusive (data not shown).

In comparison with experiments on *PHYB*, expression of *PHYA* was much variable. In contrast to *PHYB*, the level of *PHYA* transcript in etiolated maize mesocotyls was not affected by the loss of function in *ABP1* nor *ABP4* genes, suggesting that functional ABP1 or ABP4 do not play a role in regulation of *PHYA* expression. *PHYA* expression was not essentially influenced by WL in either genotype tested (Fig. 4a). It is unusual as PhyA belongs to light labile phytochromes (Sharrock and Quail 1989). Light-dependent decrease in *PHYA* transcript has been observed in several grass species (Cobert et al. 1989) and the same trend was observed in maize (Sheehan et al. 2004, and references therein). Here we found no reduction of the *PHYA* level by WL in maize mesocotyls. It could be explained by the fact that differently from Sheehan et al. (2004) our source of WL was not supplemented by incandescent lamps and has total photon irradiance 150 µmol m^{-2} s^{-1}. Interestingly, Franklin et al. (2007) reported that under high photon irradiances of RL (>100 µmol m^{-2} s^{-1}) degradation of a pool of nuclear-localized phyA was retarded. The authors demonstrated photoprotection of phyA at high photon irradiances of RL and provided evidence of significant functional activity for photoprotected phyA.

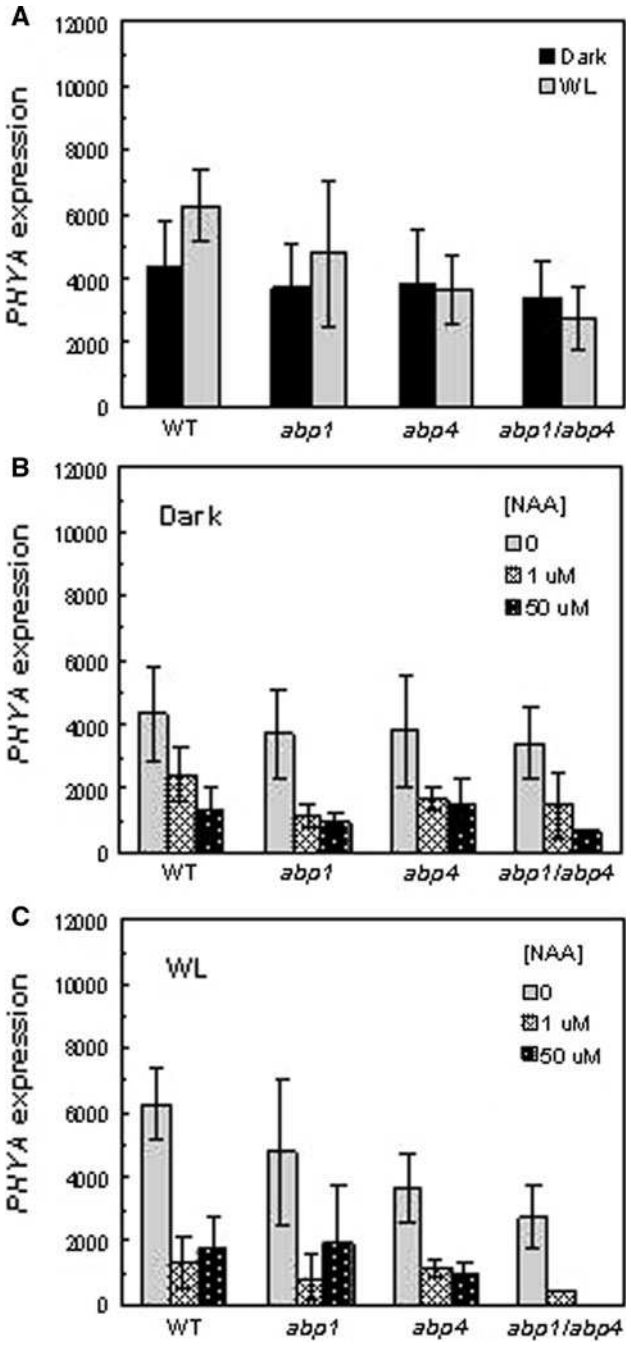

Fig. 4 Expression of *PHYA* gene in mesocotyls of etiolated or white light-grown WT and *abp* mutants as a function of exogenous auxin **a** Expression of *PHYA* in mesocotyls of WT and *abp* mutants developed in the dark or white light (WL). WT and mutant plants were grown in conditions in vitro on the BM. **b** Expression of *PHYA* in mesocotyls of WT and *abp* mutants developed in darkness or **c** in WL as a function of exogenous auxin. WT and mutant plants were grown in conditions in vitro on the BM supplemented with NAA at concentrations of 10^{-6} or 5×10^{-5} mol L^{-1}. *PHYA* expression analysis was performed by semi-quantitative RT-PCR, and *18S rRNA* was used as a reference gene. In each genotype and each condition, the data represent average from 10 identical independent experiments ±SE. In each experiment, in each condition and in each genotype, a mix of 20 mesocotyls was always used

Albeit the variability in *PHYA* expression, distinct trend of auxin-induced reduction of *PHYA* transcript was observed in etiolated as well as in WL-developed mesocotyls of all genotypes tested (Fig. 4b, c). Differently from *PHYB*, the inhibition of *PHYA* gene expression was however of similar trend in WT and the *abp* mutants. It therefore seems that ABP1 and ABP4 do not play a role in the auxin-mediated inhibition of *PHYA* expression. It supports the conclusion above that ABP1 and ABP4 are not involved in the expression of *PHYA*.

The possibility of the involvement of ABP in cross-talk between light and auxin signalling was brought out by Jones et al. (1991). The authors reported that RL reduces the abundance of the ABP1, which controls cell expansion in maize. In contrast, the expression of *ABP4* in maize mesocotyls was up-regulated in light grown seedlings (Fellner et al. 2006). Our results presented here show for the first time that knockout of *ABP1* and/or *ABP4* genes affects expression of *PHYB* in etiolated maize mesocotyls. We further report that etiolated mesocotyls of the *abp* mutants are insensitive to exogenous auxin NAA as regards to NAA-induced inhibitin of mesocotyl elongation and NAA-induced inhibition of *PHYB* expression. Similarly, reduction of sensitivity to exogenous auxin was observed in fytochromobilin-deficient mutant *elm1* (Fig. 3). Our results therefore indicate that in the dark, reduction of *PHYB* expression by exogenous auxin interferes with the signaling pathway involved in mesocotyl elongation. However, the molecular mechanism has to be elucidated.

Based on our results we hypothesize that in dark conditions, ABP1 and ABP4 activate a transcription factor, which trigger expression of *PHYB*, whereas in WL conditions, the TF could be blocked (destabilized) (Fig. 5). We further propose that after binding NAA, the ABPs lose their ability to activate the TF, which could come to the essentially reduced expression of *PHYB* gene. The fact that in the absence of exogenous auxin, ABP1 and/or ABP4 seems to play a positive role in *PHYB* expression (Fig. 1) may suggest a divergence of signaling pathways triggered by low (endogenous auxin) and high (endogenous plus exogenous auxin). Our results support the existence of cross-talk between auxin and light signaling and indicate for the first time that *ABP1*, *ABP4* and *PHYB* genes could share common signaling pathway(s).

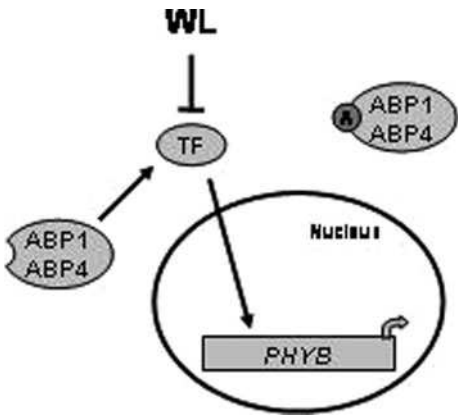

Fig. 5 Working model showing cross-talk between ABP and *PHYB* signaling pathways in etiolated or WL-grown maize mesocotyls in the absence or presence of exogenous auxin. The model shows that in dark conditions, ABP1 and ABP4 activate a transcription factor (*TF*), which trigger expression of *PHYB*, whereas in WL conditions, the TF could be blocked (destabilized). We further propose that after binding NAA (A), the ABPs lose their ability to activate the TF, which could come to the essentially reduced expression of *PHYB* gene. *Arrows* and *T-bars* represent positive and negative effects, respectively

Acknowledgments We thank Renáta Plotzová, Marie Karlická and Véronique Bergougnoux for excellent technical assistance. We specially thank Alan M. Jones (University of North Carolina) for providing seeds of the maize *abp* mutants. We thank Thomas P. Brutnell (Boyce Thompson Institute for Plant Research) for providing seeds of the maize *elm1* mutant. We also thank Adéla Fellnerová for her help in revising the English text. This work was supported by Ministry of Education of the Czech Republic (grant no. 1P05ME792 to MF) and by the EU (grant no. MRTN-CT-2006-035833 to MF).

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SUPPLEMENT 3

Borucka, J., Fellner, M. (2011) Auxin receptors ABPs and phytochromes interact in maize (*Zea mays* L.) growth. *Current Opinion in Biotechnology*, 22S, S77.
(IF_{2011} 7.711; IF_{2017} 8.380)
Author's contribution: 80 %.

OH• have been investigated. Hydroxyl radical was chosen, because of its high reactivity which makes it a very dangerous compound for the organism. Aim reaction mechanisms that OH• has been reacted with glycine and alanine, have been targeted modeling classical and quantum mechanical methods in detail and understanding the selectivities observed for different conformers of the studied amino acids. Method initially, conformational analysis has been done for glycine and alanine. Afterwards, the geometries of all conformations have been fully optimized for all the minimum energy conformers and frequency analysis has been carried out for characterizing stationary points at the B3LYP level of theory implementing the 6-31++G** basis set by using the Gaussian03 series of programs. Consequently, reaction mechanisms and energy surfaces have been obtained for glycine and alanine on the basis of two reactions have been mentioned (reactants, products, transition states). H abstraction by OH• reactions, that have been observed, are barrierless. But OH addition to Cα reactions are not barrierless. This study has been characterized like a prestudy that would been provided developing new experimental methods to minimize the damage of OH radical on metabolism.

doi:10.1016/j.copbio.2011.05.226

G40

Auxin receptors ABPs and phytochromes interact in maize (*Zea mays* L.) growth

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Previous research in old and modern maize hybrids indicated involvement of auxin binding proteins (ABPs) in light signaling during maize seedling development. Here, we investigated expression of phytochrome genes *PHYB* and *PHYA* in loss-of-function mutants in *ABP1* and *ABP4* genes in maize (*Zea mays* L.). Using a semi-quantitative RT-PCR we studied how expression of the *PHYB* and *PHYA* in mesocotyl is regulated by blue and white light, and whether auxin NAA influences the expression of the phytochrome genes in the dark. Experiments on *abp* mutant showed that blue light could decrease or block the expression of both phytochromes, and the results suggest that in the case of *PHYA*, *ABP1* and *ABP4* may be positively involved in phytochrome gene expression. We also revealed that auxin can reduce expression of *PHYB* genes, and the results suggest *ABP1* and/or *ABP4* mediate the effects of auxin. The results of our experiments support the existence of cross-talk between light and auxin signaling pathway, and provide some evidence that ABPs may function as elements of phytochrome signaling pathways involved in development of maize seedlings.

Acknowledgements: The work was supported by Ministry of Education of the Czech Republic (grant no. 1P05ME79) and by EU (grant no. MRTN-CT-2006-035833).

doi:10.1016/j.copbio.2011.05.227

G41

Genetic diversity of Romanian *Rhizobium leguminosarum biovar.trifolii* strains isolated from root nodules of clover grown in heavy metal polluted soil

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The isolation and characterization of *Rhizobium leguminosarum* strains from *Trifolium repens* and *T. pratense* root nodules, from plants grown in soil containing heavy metals (Zn, Cu and/or Pb). The new isolates were investigated for nodulation efficiencies and growth performance in the presence of heavy metal and antibiotics. The genetic diversity of these isolates, compared with reference strains was examined using PCR fingerprinting (PCR-RFLP analysis, rep-PCR, PCR-RFLP of *nifH* and *nodC* genes). The bacterial isolates originated from unpolluted soils were very sensitive both to antibiotics and heavy metals, except Pb. Contrary, the strains isolated from contaminated soil were resistant to these compounds. Moreover, some of these isolate were able to grown in different concentrations of NaCl. The analysis of nod genes using nodCF/nodCI primers allowed the detection of differences in amplicon profiles between isolates. The differences encountered could be related to heavy metal concentrations and were of practical interest in order to select some strains and use them for inoculums preparation, for plant treatment. Five strains were resistant to antibiotics and heavy metals and tolerant to some concentrations of NaCl, but their symbiotic and nodulation abilities were not affected, proving their usefulness as bioinoculants.

doi:10.1016/j.copbio.2011.05.228

G42

Unknown picophytoplankton in Transylvanian salt lakes: great biotechnological potential in extreme environments

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The picophytoplankton (PPP; <2 μm) plays an important role in most aquatic environments contributing significantly to the biomass and the primary production of the phytoplankton. The surface/volume ratio of the PPP cells is the largest among algae, which not only provide an advantage in the competition for limiting nutrients, but the picoplankton cells are able to utilize the light more efficiently than the nanoplankton and microplankton organisms. These features could be very important for biotechnological purposes. Although PPP has been studied extensively during