Maize *AUXIN-BINDING PROTEIN 1* and *AUXIN-BINDING PROTEIN 4* impact on leaf growth, elongation, and seedling responsiveness to auxin and light

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Abstract: In maize, at least five auxin-binding proteins (ABPs) have been identified, yet their functions remain unclear. The present study reports the use of maize *abp1*, *abp4*, and *abp1abp4* mutants to investigate the role of ABPs during maize growth and development. Single and double *abp* mutant plants grown in a greenhouse differ from the wild type (WT) in their leaf declination and leaf blade growth. The effect of the dark (D), blue light (BL), red light (RL), and exogenous auxin on the development of mutant seedlings was also studied. Relative to WT, etiolated mutant seedlings were shorter and showed a reduced responsiveness to exogenous auxin. In BL or RL, the responsiveness of maize seedlings to auxin was distinctly less than in D. The reducing effect of light on seedling responsiveness to auxin is mediated at least by phytochromes. The suppression of *ABP1* and (or) *ABP4* led to a distinct accumulation of free indole-3-acetic acid (IAA) in etiolated and light-grown seedling organs. We concluded that ABP1 and ABP4 participate in the growth of maize seedlings, mediate seedling responses to auxin, and interact with light signaling pathway(s). We also deduce a functional interaction between ABP1 and ABP4, which is that the relationship between them is light-, organ- and response-dependent.

Key words: auxin, auxin-binding protein, growth, light, maize.

Résumé : Les auteurs ont identifié cinq protéines liant l'auxines « auxin-binding proteins » (ABPs), chez le maïs, sans pouvoir établir clairement leurs fonctions. Ils ont utilisé trois mutants de maïs (*abp1*, *abp4* et *abp1abp4*) pour comprendre le rôle des ABPs dans la croissance et le développement du maïs. En serre, les mutants diffèrent du témoin par la croissance de la feuille et son angle d'insertion sur la tige. Ils ont également étudié la croissance des mutants cultivés in vitro, dans le noir, exposés à la lumière bleue (BL) ou rouge (RL), en présence d'auxine exogène. Les plantules étiolées des mutants s'avèrent plus courtes et moins sensibles à l'auxine que les témoins de type sauvage. Les BL et RL atténuent la sensibilité des plantules à l'auxine, cette réaction étant partiellement contrôlée par les phytochromes. La suppression de *ABP1* et (ou) *ABP4* se caractérise par une accumulation différente d'auxine dans les organes des plantules étiolées ou cultivées à la lumière. En conclusion, ABP1 et ABP4 jouent un rôle dans la croissance des plantules de maïs et leur réponse à l'auxine, et interagissent avec la(es) voie(s) de signalisation de la lumière. Nous suggérons une interaction entre ABP1 et ABP4, toutefois dépendante de la lumière, de l'organe et de la réaction étudiés.

Mots-clés : auxine, protéine liant l'auxine, croissance, lumière, maïs.

Introduction

Auxins play a central role in plant growth and development, as they are involved in broad spectrum processes, including embryogenesis, stem elongation, apical dominance, photo- and gravitropism, and lateral root formation (reviewed in Davies 2004 and Vanneste and Friml 2009). At the cellular level, auxin acts to regulate these processes through changes in cell division, cell expansion, and differentiation (reviewed in Teale et al. 2006 and Perrot-Rechenmann 2010). At the plant level, it has been demonstrated that endogenous, as well as externally supplied, indole-3-acetic acid (IAA) stimulates the growth of intact maize coleoptiles (Baskin et al.1986; Iino 1995; Haga and Iino 1998). Epstein et al. (1980) reported that maize kernels supply the coleoptile tip with auxin in a conjugate form, from which free IAA is released by specific enzymes and moved from the coleoptile tip to the elongation zone via polar transport (Goldsmith

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1977; Lomax et al. 1995). The underlying mechanisms of cell elongation still remain the subject of debate, although distinct interpretations of auxin-induced growth have been proposed (Hager et al. 1971; Rayle and Cleland 1972, 1992; Hoth et al. 1997; Claussen et al. 1998; Philippar et al. 1999; Bauer et al. 2000; Becker and Hedrich 2002). On a longer time scale (days), the elongation of various plant organs in intact plants was shown to be inhibited by exogenous auxin (Marten et al. 1991; Zimmermann et al. 1994; Boerjan et al. 1995; King et al. 1995; Keller and Van Volkenburgh 1996; Thomine et al. 1997; Ephritikhine et al. 1999; Fellner et al. 2003, 2006).

The primary step of auxin signaling is the binding of an auxin to an auxin receptor. In addition to the described function of auxin receptors from the TRANSPORT INHIBITOR RESPONSE 1/AUXIN-BINDING F-BOX PROTEIN (TIR1/AFB) family, putative auxin receptor, the AUXIN-BINDING PROTEIN 1 (ABP1) has been identified (reviewed in Tromas and Perrot-Rechenmann 2010, Shi and Yang 2011, Sauer and Kleine-Vehn 2011, and Scherer 2011). ABP1 is a protein with a high-affinity to auxin that was identified in maize coleoptiles over 30 years ago by its capacity to bind to 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), conversely the predominant localization of ABP1 was found to be in the endoplasmic reticulum lumen (Jones and Herman 1993). It was demonstrated that ABP1 mediates very early auxin responses including modifications of ion fluxes across the plasma membrane through the activation or deactivation of ion channels (K^+ cations) or transporters (H^+) (Rayle and Cleland 1980; Cleland et al. 1991; Ephritikhine et al. 1987; Barbier-Brygoo et al. 1989, 1991; Leblanc et al. 1999; Napier et al. 2002). ABP1 specifically binds auxin and the level of ABPs roughly correlates with the extent of cell responses to auxin (Ray et al. 1977; Shimomura et al. 1988; Jones et al. 1989). In addition, constitutive over-expression of *ABP1* in maize or tobacco cells resulted in larger cells; this effect was auxin dependent. These facts are consistent with ABP1 having an auxin receptor function (Jones et al. 1998; Chen et al. 2001). The results of Chen et al. (2001) suggest that in tobacco leaf cells, ABP1 acts with a relatively low level of auxin to mediate cell expansion, whereas high auxin levels stimulate cell division potentially via an unidentified receptor TIR1. However, the involvement of ABP1 in cell division has also been identified in tobacco (Fellner et al. 1996), and the characterization of the *Arabidopsis* loss-of-function mutant in *ABP1* indicates a dual role for the protein in cell expansion and cell division during early embryogenesis (Chen et al. 2001). Previously, it was shown that ABP1 acts on the control of the cell cycle (David et al. 2007). The authors demonstrated that cell cycle arrest provoked by ABP1 inactivation could not be bypassed by exogenous auxin, suggesting the role of ABP1 in an auxin-mediated control of the cell cycle. Until 2008, no evidence had been provided that the ABP1 was involved in auxin-regulated gene expression. However, recently Braun et al. (2008) demonstrated that ABP1 coordinates cell division and cell expansion during postembryonic shoot development in *Arabidopsis* and tobacco. The down-regulation of ABP1 activity in *Arabidopsis* results in a decrease of the expression of a large spectrum of *Aux/IAA* genes during shoot development (Braun et al. 2008), and ABP1 is required for the auxin-induced expression of a subset of *Aux/IAA* genes in roots (Tromas et al. 2009). The authors present a model in which ABP1 is the major regulator of auxin action on the cell cycle and regulates auxin-mediated gene expression and cell elongation along with the TIR1-mediated ubiquitination pathway. Recently, evidences were provided that ABP1 is involved in the regulation of polar auxin transport (PAT), thus affecting local auxin concentration, cell expansion, and early auxin gene regulation (Braun et al. 2008; Robert et al. 2010; Xu et al. 2010; Effendi et al. 2011). In addition to ABP1, at least four other closely related ABPs have been identified in maize so far (Hesse et al. 1989; Schwob et al. 1993), but their roles in growth and development have yet to be elucidated. Moreover, as far as we know the existence of maize mutant knockout in genes other than *ABP1* or *ABP4* (Im et al. 2000) have not been reported.

Light is an external factor that essentially regulates plant growth in a complex interaction with internal factors including auxins (reviewed in Tian and Reed 2001 and Halliday et al. 2009). 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 accumulation or PAT (reviewed in Tian and Reed 2001; Liu et al. 2011). In fact, light reduces the intensity of PAT in the etiolated coleoptile segments (Huisinga 1964, 1967; Naqvi 1975; Fellner et al. 2003) and reduces the content of free IAA in etiolated maize seedlings (Briggs 1963; Bandurski et al. 1977; Iino 1982*a*). Similarly, light was shown to decrease auxin transport in maize mesocotyls, revealing the important role of light and PAT in the mesocotyl photomorphogenesis (Van Overbeek 1936; Vanderhoef and Briggs 1978; Iino 1982*b*; Jones 1990; Barker-Bridgers et al. 1998). Recent research efforts led to the revelation that light pathways can modify auxin distribution by controlling the abundance of P-glycoproteins and PIN-FORMED proteins (Sidler et al. 1998; Friml et al. 2002; Nagashima et al. 2008; Zazimalova et al. 2010). A number of reports demonstrated the existence of signaling elements shared by light and auxin during elongation. Several links between light and auxins have been defined using primary auxin-response genes as genetic and molecular tools (Abel et al. 1995; Abel and Theologis 1996; Sitbon and Perrot-Rechenmann 1997; Tepperman et al. 2001; Devlin et al. 2003). Other hypotheses on the mechanism of light-regulated growth have included ABP1 (Walton and Ray 1981; Napier et al. 1988; Jones et al. 1989; Jones and Venis 1989). This idea was supported by the observation that a reduced abundance of ABP1 and a reduced level of free IAA in red light (RL)-grown seedlings were associated with a decrease in the mesocotyl growth (Jones et al. 1991). It was also reported that the expression of the *ABP1* gene in leaf tissues of 2-week-old green maize seedlings and in mature green leaves is much less than in etiolated seedlings (Im et al. 2000). By contrast, the expression of another member of the ABP family, *ABP4*, in maize mesocotyls was up-regulated in RL- and far-red light (FR)-grown seedlings (Fellner et al. 2006).

We previously reported that in comparison to old maize hybrids, cells of modern maize varieties developing upright leaves were insensitive to auxin- and light-induced hyperpolarization of the plasma membrane. The expression analysis

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revealed an up-regulation of the *ABP4* gene by auxin and light in old hybrids but not in the modern ones (Fellner et al. 2003, 2006). Although Im et al. (2000) reported no phenotypic changes in loss-of-function mutants in *ABP1* and *ABP4* genes in maize, they found that a knockout of the *ABP4* gene resulted in a strong elevation (four to seven times) of the ABP1 protein in etiolated seedlings. In contrast to their observations, we previously found and reported distinct differences between the maize *abp* mutants and the wild type (WT) in leaf declination (Fellner et al. 2006). To uncover more information about the role of ABP1 and ABP4 in the growth and development of maize seedlings and their cross-talk with light signaling pathways, we investigated in more detail the phenotypes and growth responses in the loss-of-function mutants in *ABP1* and *ABP4* genes.

Material and methods

Plant material, growth conditions, and measurement of seedling growth

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 mutant *abp1abp4* (*B2/K1* allele), and a near isogenic line (inbred line A619, here called WT) were a gift from Alan M. Jones (The University of North Carolina, Chapel Hill, N.C.). All mutants were tested for the lack of *ABP1* and (or) *ABP4* gene expression (see Supplemental Fig. S1)[1]; they all showed stable phenotypes. The fytochromobilin-deficient mutant *elm1* (*elongated mesocotyl 1*) was initially identified in the W22 background (Sawers et al. 2002), and it was also introgressed into the B73 background (inbred maize line) by backcrossing five times (Dubois et al. 2010). Kernels of *elm1* and B73 were kindly provided by Thomas P. Brutnell (Boyce Thompson Institute for Plant Research, Ithaca, N.Y.).

For experiments in Magenta plant culture boxes, seeds were first washed with 70% ethanol for 3 min, rinsed with distilled sterile water, and then soaked in Savo original solution (~5% sodium hypochlorite; Bochemie, s.r.o, Czech Republic) that was supplemented with a drop of Tween20 (Calbiochem, USA). Finally, the solution was shaken on a stirrer for 30 min and then rinsed extensively with sterile distilled water. These seeds were germinated on a 0.7% (*w*/*v*) agar medium in Magenta GA-7 boxes (77 mm × 77 mm × 196 mm; Sigma-Aldrich, Prague, Czech Republic) (six to nine 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 mmol·L^{-1} MES (2-(*N*-morpholino)-ethanesulfonic acid); pH was adjusted to 6.1 before autoclaving. In experiments with auxin, the BM was supplemented with 1-naphthalene acetic acid (NAA) in various concentrations. Seeds in the Magenta boxes were placed in a growth chamber (Microclima MC1000E, Snijders Scientific, the Netherlands) and incubated at a temperature of 23 °C. For the development of etiolated seedlings, the boxes were wrapped in aluminum foil. Maize seeds were also incubated under continuous blue light (BL) with a maximum irradiance at 460 nm or in continuous red light (RL) with a maximum irradiance at 660 nm and both at 23 °C. BL and RL were provided by blue (Philips TLD-36W/18-Blue, Phillips, USA) and red (Philips TLD-36W/15-Red, Phillips, USA) fluorescent tubes. The total photon fluence rates of BL and RL were 10 μmol·m^{-2}·s^{-1}. The fluence rate was measured with a portable spectroradiometer (model LI-1800; LI-COR; Lincoln, Nebr.) calibrated by the Department of Biophysics at Palacky University in Olomouc. In all conditions, 5-day-old seedlings were measured. For experiments in the greenhouse, plants were grown in soil (Potgrond H, Klasmann Deilmann GmBH, Germany) in small pots (190 mm × 190 mm; one seed per pot; 1 cm deep) and regularly watered. In summer, the plants grew in natural light conditions with temperatures of 15 °C and higher. In winter, the plants grew under high-pressure sodium lamps using PlantaStar E40/ES 400 W (Osram GmbH, Germany) to maintain a 16-h photoperiod. The temperature was adjusted from 15 to 27 °C.

The size of various organs was measured with a ruler on 5-day-old intact seedlings developed in Magenta boxes on BM. The mesocotyl length was measured from the scutellar to the coleoptilar node, and the coleoptile length was measured from the coleoptilar node to the tip of the coleoptile. The primary root length was measured from the scutelar node to the root tip. For genotype, six to nine seedlings per treatment that germinated on the same day were measured in each experiment. When necessary, changes in growth (i.e., inhibition or stimulation) caused by an effector (light, exogenous auxin) in the individual genotype were expressed in percents based on the following formula: $X = 100 \times (A - B) / A$, where X is the change in growth (in %) and A and B stand for growth (in mm) in the absence and presence, respectively, of the effector.

For the study of the leaf characteristics, plants were grown in soil in a greenhouse as described above and watered regularly. Leaf angle (declination from vertical) was measured with a protractor held at the leaf base, as described by Fellner et al. (2003), in 5–10 intact plants of each genotype from 2 to 4 weeks after seed germination. Blade length was measured with a ruler to the nearest millimetre. Leaf blades were scanned with a scanner and the width and blade surface were determined using ImageJ software (Abramoff et al. 2004).

Mesocotyl cross-section light microscopy and cell diameter

Cross-sections (~0.5 mm) of etiolated maize mesocotyls were performed using a razor blade. The sections were stained ~5 min in methylene blue dye, subsequently washed with distilled water, and then placed on a microscopy slide into a drop of distilled water and overlapped with a cover glass. Samples were examined using a light microscope model OLYMPUS BX-60 and microphotographs were taken using a CCD camera OLYMPUS DP71 (Olympus Czech Group, Prague, Czech Republic). The diameter of cortical cells was measured on microphotographs using ImageJ soft-

[1]Supplementary data are available with the article through the journal Web site (http://nrcresearchpress.com/doi/suppl/10.1139/b2012-071).

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ware (Abramoff et al. 2004). In each genotype tested, the diameter of the cells in five cross-sections was measured, and in each microphotograph, the diameter of ~70 randomly selected cells was measured.

Extraction and quantification of endogenous auxin

For analysis of endogenous free IAA in coleoptiles, mesocotyls, and primary roots, 5-day-old maize seedlings grown in Magenta boxes in dark (D), RL, or BL conditions as described above were used. Organs were each separately excised from the seedling, placed individually into prechilled aluminum foil envelopes, immediately frozen in liquid nitrogen, and then stored at –80 °C. Samples of ~10 mg of fresh mass were extracted and purified by solid-phase extraction on C8 column as described by Pěnčík et al. (2009). The eluates were evaporated until dry in vacuo and analyzed for free IAA content by UPLC (Acquity UPLC System, Waters) coupled to triple quadrupole mass detection (Quatro micro, Waters).

Analysis of *ABP* transcripts

The presence or absence of *ABP1* and (or) *ABP4* transcripts in the loss-of-function mutants in *ABP1* and *ABP4* genes in maize was confirmed by reverse transcription PCR (RT–PCR) as described in Fellner et al. (2006) with a slight modification. PCR of 25 cycles for *ABP1* and of 28 cycles for *ABP4* were performed by denaturing the template cDNAs at 94 °C for 3 min followed by cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, and 72 °C for 5 min. An *ABP1* fragment (maize *ABP1* gene accession No. L08425) was amplified using oligonucleotides 5′-CCGCAAAGCAGCTATGGGATT-3′ from exon 2 and 5′-CGAAGGGGAATTTCAGTACCGCG-3′ from exon 5. An *ABP4* fragment (maize *ABP4* gene accession No. L08426) was amplified using oligonucleotides 5′-CAGCAGCGCAACTACGGGAGG-3′ from exon 2 and 5′-AGTAGGGGAATTTCAGCTTTGCA-3′ from exon 5. The primer pairs were previously described in Fellner et al. (2006). PCR products (*ABP1* as well as *ABP4*, each 403 bp in size) were size fractionated by electrophoresis in a 1% (*w*/*v*) agarose gel stained with ethidium bromide.

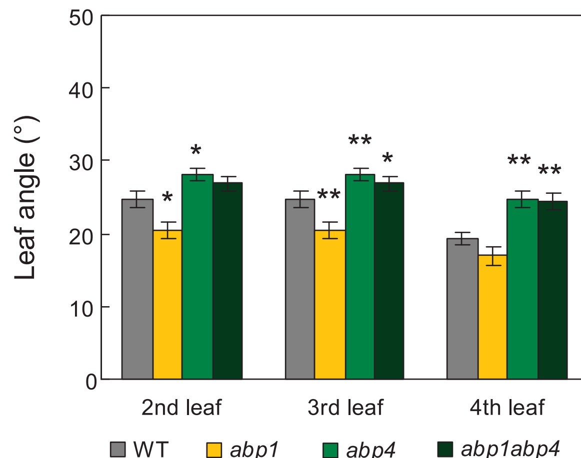
Statistical analysis

When needed, the statistical significance of the treatment differences was assessed using Student's *t* test.

Results

Contrary to Im et al. (2000) who reported no phenotypic differences between *abp* mutants and the corresponding WT, we previously observed distinct differences between the genotypes in leaf angle development when plants were grown in growth chambers (Fellner et al. 2006). Similarly, we observed a number of other phenotypes in the *abp* mutants developed in the greenhouse in WL conditions and in the Magenta boxes on the BM when grown in D, BL, or RL conditions. Im and co-authors observed that the level of the ABP1 protein in the *abp4* mutant was four to seven times higher than in WT, indicating that the elimination of the *ABP4* gene activates *ABP1* expression post-transcriptionally because the *ABP1* transcript levels did not differ among WT and *abp4* mutants (Im et al. 2000). Based on this, and on our previous

Fig. 1. Leaf angle of single maize mutants *abp1* and *abp4*, double mutant *abp1abp4*, and corresponding wild type (WT) grown in the greenhouse. The leaf angle, measured as a declination from vertical in 25-day-old plants (2nd leaf), 32-day-old plants (3rd leaf), and 35-day-old plants (4th leaf), was determined with a protractor to the nearest degree. For each genotype, 4 to 10 seedlings were measured in every experiment. The values show the leaf angle (mean ± SE) obtained in 12 independent experiments. * and ** indicate significant differences (*t*-test; $P \leq 0.05$ and $P \leq 0.01$, respectively) from the corresponding WT leaf.

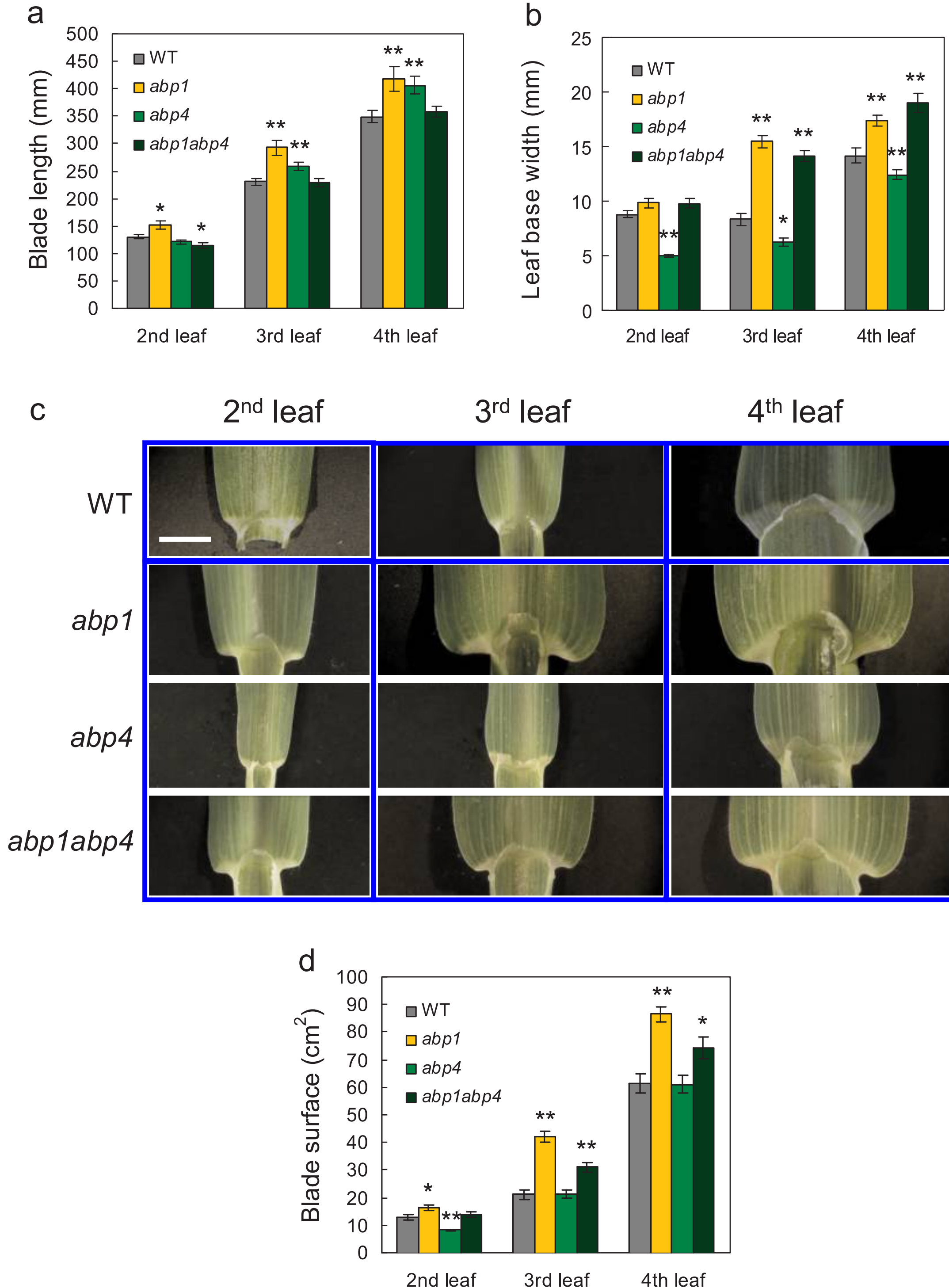


observations of differential leaf angles in WT and *abp* mutants, we expected some phenotype patterns in single or double *abp* mutants. First, we expect that the single mutants *abp1* and *abp4* will show opposite phenotypes. The opposite phenotypes were observed for example in leaf angle and width or length of the etiolated coleoptile (see below). Second, if the increased ABP1 protein is partly responsible for the *abp4* single mutant phenotype, we expect that the *abp1abp4* double mutant will show a less extreme phenotype than *abp4*, possibly similar to WT. This was found for example in leaf blade length, diameter of etiolated mesocotyl, mesocotyl length in RL, and the sensitivity of the etiolated primary root to NAA (see below). Third, we also expect a phenotype more extreme than single mutants or WT in the case that the two genes are acting together, rather than in opposition. This would be for example, in the case of the length of the etiolated mesocotyl and the primary responsiveness of the etiolated coleoptile and mesocotyl to NAA, or the level of endogenous IAA in the etiolated coleoptile and primary root (see below).

Loss-of-function mutants in *ABP1* and *ABP4* genes show differential leaf growth

In this study, we investigated leaf development in 4-week-old plants that were grown in a greenhouse. In WT, declination of the 2nd and 3rd leaf reached ~25° (Fig. 1). The mutation in *ABP1* led to the development of a significantly smaller leaf declination (~20°), whereas the defect in the *ABP4* gene resulted in the development of leaves with a larger angle (~28°). Interestingly, the loss of function in both genes resulted in leaves with a declination similar to that observed in the *abp4* mutant. A comparable situation was observed in the fully developed 4th leaf (Fig. 1). Leaf declination in the double mutant and WT was not significantly different for the 2nd leaf.

Fig. 2. Length of leaf blade, leaf width, and leaf surface of single maize mutants *abp1* and *abp4*, double mutant *abp1abp4*, and corresponding wild type (WT) grown in the greenhouse. (*a*) The blade length and (*b*) leaf width in 25-day-old plants (2nd leaf), 32-day-old plants (3rd leaf), and 35-day-old plants (4th leaf) were measured with a ruler to the nearest millimetre. (*c*) Examples of the widths of the bases in the 2nd, 3rd, and 4th leaf in 1-month-old WT and *abp* mutant plants are shown. Scale bar = 5 mm. (*d*) The blade surface in 2nd, 3rd, and 4th leaf plants was measured by scanning the leaf blades and afterwards determined using ImageJ software. For each genotype, 4 to 10 seedlings were measured in every experiment. The values show the measured parameters (mean ± SE) obtained in 12 independent experiments. * and ** indicate significant differences (*t*-test; $P \leq 0.05$ and $P \leq 0.01$, respectively) from the corresponding WT leaf.



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Mutations in *ABP1* or *ABP4* affected the overall features of the leaves. In the single mutant *abp1*, leaf blades of the 2nd, 3rd, and 4th leaf were longer than in WT plants. In *abp4* mutants, the 2nd leaves were the same size as in WT, and blades of the 3rd and 4th leaves were significantly longer than those in WT (by ~17%) (Fig. 2*a*). The 2nd leaf of the double mutant *abp1abp4* was shorter than in WT plants, and the 3rd and 4th leaves reached lengths similar to those observed in

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WT plants (Fig. 2*a*). Leaf width was also affected by the mutations (Figs. 2*b*, 2*c*). The base of the leaf in *abp1* and the double mutant was wider than in WT plants, especially in the 3rd and 4th leaf. The narrowest leaf base was found in the *abp4* mutant (Fig. 2*b*). The examples of the base width in the 2nd, 3rd, and 4th leaves in WT and the mutants are shown on Fig. 2*c*. We also estimated the blade surface in the 2nd, 3rd, and 4th leaves using ImageJ software. Figure 2*d* shows that the greatest blade surface was measured in plants where *ABP1* or both *ABP1* and *ABP4* were knocked out. The single mutant *abp4* showed a blade surface similar to that observed in WT plants with the exception of the 2nd leaf, where the mutant blade surface was distinctly smaller than that measured in the WT plants (Fig. 2*d*).

Defects in *ABP1* and (or) *ABP4* cause changes in the development of young maize seedlings in darkness or light

The effect of light on plant growth is extremely evident in seedlings in the early stages of development. Based on the differential phenotypes of *abp* mutants and WT in the greenhouse, we determined the phenotypes of WT and mutant seedlings developed in Magenta boxes on the BM 5 days after kernel germination (Fig. 3). The single mutant *abp1* grown in D developed a coleoptile similar to that of the WT seedlings, whereas in *abp4* and the double mutant the coleoptile was about 20%–30% shorter than the WT plant (Fig. 3*a*). Different from the etiolated plants, mutations in *ABP1* and *ABP4* only slightly affected elongation of coleoptiles in seedlings developed under BL and had no effect on the coleoptile elongation in plants grown in RL (Fig. 3*a*).

Whereas the etiolated seedlings of single mutants *abp1* and *abp4* did not show distinct differences in the mesocotyl length compared with WT, the double mutant developed mesocotyls significantly shorter than that observed in WT (Fig. 3*b*). Whatever the genotype, mesocotyls grown in BL were much shorter in comparison with the etiolated organs, and the lengths of BL-grown mesocotyls were comparable to WT and mutant seedlings. RL also strongly inhibited the mesocotyl elongation in all genotypes. However, the knockout of the *ABP4* gene led to the development of a significantly longer mesocotyl than in WT, *abp1*, and the double mutant (Fig. 3*b*).

Defects in *ABP1* and (or) *ABP4* also had an impact on the length of the primary root (Fig. 3*c*). In etiolated seedlings, loss of function in *ABP1* or *ABP4* resulted in a significant reduction in the primary root growth. Notably, the double mutation caused development of a primary root almost half the length of the one observed in WT. The growth of the primary root in WT was reduced in BL by ~35%, whereas in single mutants BL did not have an inhibitory effect on root elongation. Compared with WT, the *abp1* root in BL was even longer. The length of the primary roots in BL-grown *abp4* seedlings was similar to those developed in WT plants, whereas the lack of both genes led to the development of significantly shorter primary roots than observed in WT plants. Primary roots of WT seedlings developed under RL were about 30% shorter than in etiolated plants. RL did not inhibit the root growth in single or double mutants but roots in *abp1* had a tendency to be longer (no significant difference was observed) than the primary roots in WT. In *abp4* and the

Fig. 3. Comparison of seedling growth in single maize mutants *abp1* and *abp4*, double mutant *abp1abp4*, and corresponding wild type (WT) in response to darkness and light. Elongation of the (*a*) coleoptile, (*b*) mesocotyl, and (*c*) primary root was measured with a ruler to the nearest millimetre in 5-day-old seedlings grown in Magenta boxes on the BM in dark (D), blue light (BL), or red light (RL) conditions. The results are the mean length ± SE obtained from 5 to 12 independent experiments. In each genotype, six to nine seedlings were measured in every experiment. * and ** indicate significant differences (*t*-test; $P \leq 0.05$ and $P \leq 0.01$, respectively) from WT in D; letters a, b indicate significant differences (*t*-test; $P \leq 0.01$ and $P \leq 0.05$, respectively) from dark-grown seedlings of the same genotype; c indicates a significant difference (*t*-test; $P \leq 0.05$) from WT in BL; d indicates a significant difference (*t*-test; $P \leq 0.05$) from WT in RL.

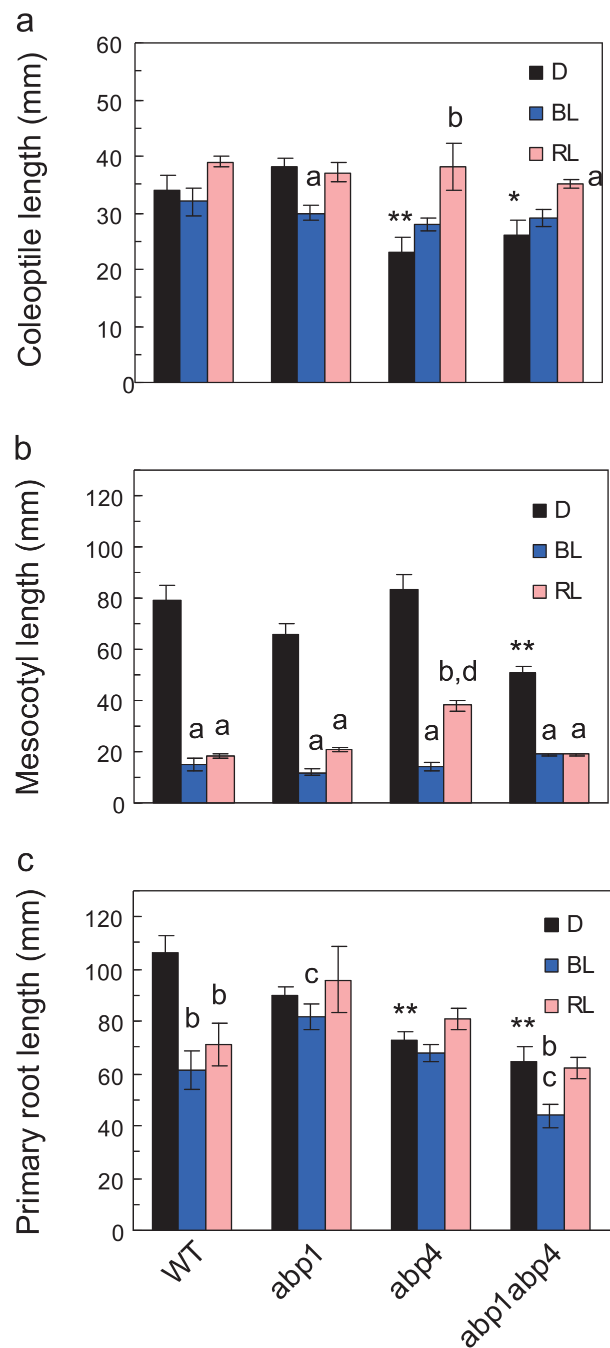
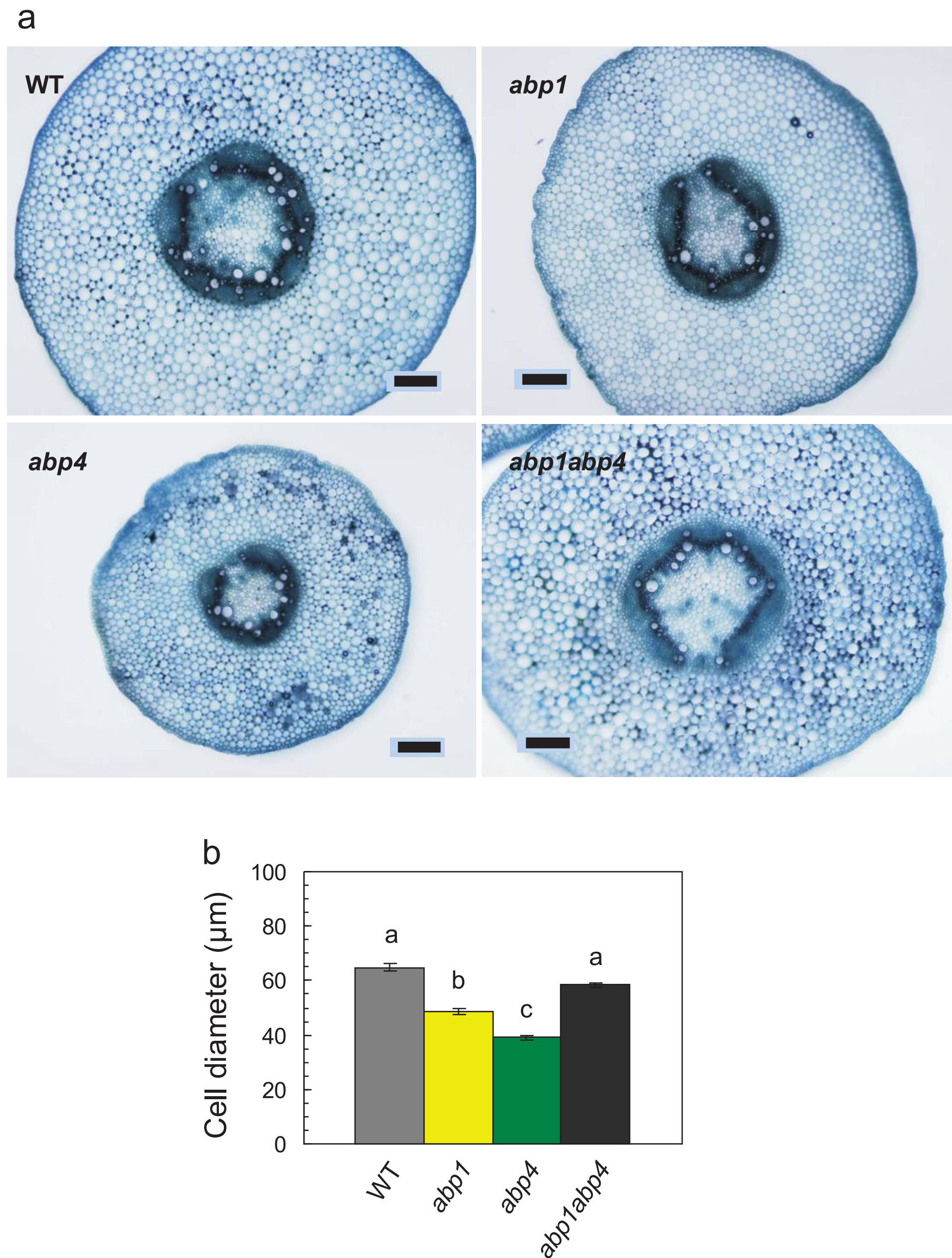


Fig. 4. Comparison of mesocotyl diameter in single maize mutants *abp1* and *abp4*, double mutant *abp1abp4*, and corresponding wild type (WT) seedlings grown in Magenta boxes in darkness. (*a*) Mesocotyl cross-section microphotograps (Scale bar = 200 µm). (*b*) Diameter of cortical cells in etiolated mesocotyls of the WT and *abp* mutants. The values represent the mean ± SE obtained by measurement of cells in five cross-section microphotographs for each genotype tested. In each cross-section, the diameter of ~70 randomly selected cortical cells was measured. Values with different letters are significantly different (*t*-test; $P \leq 0.05$) from each other.



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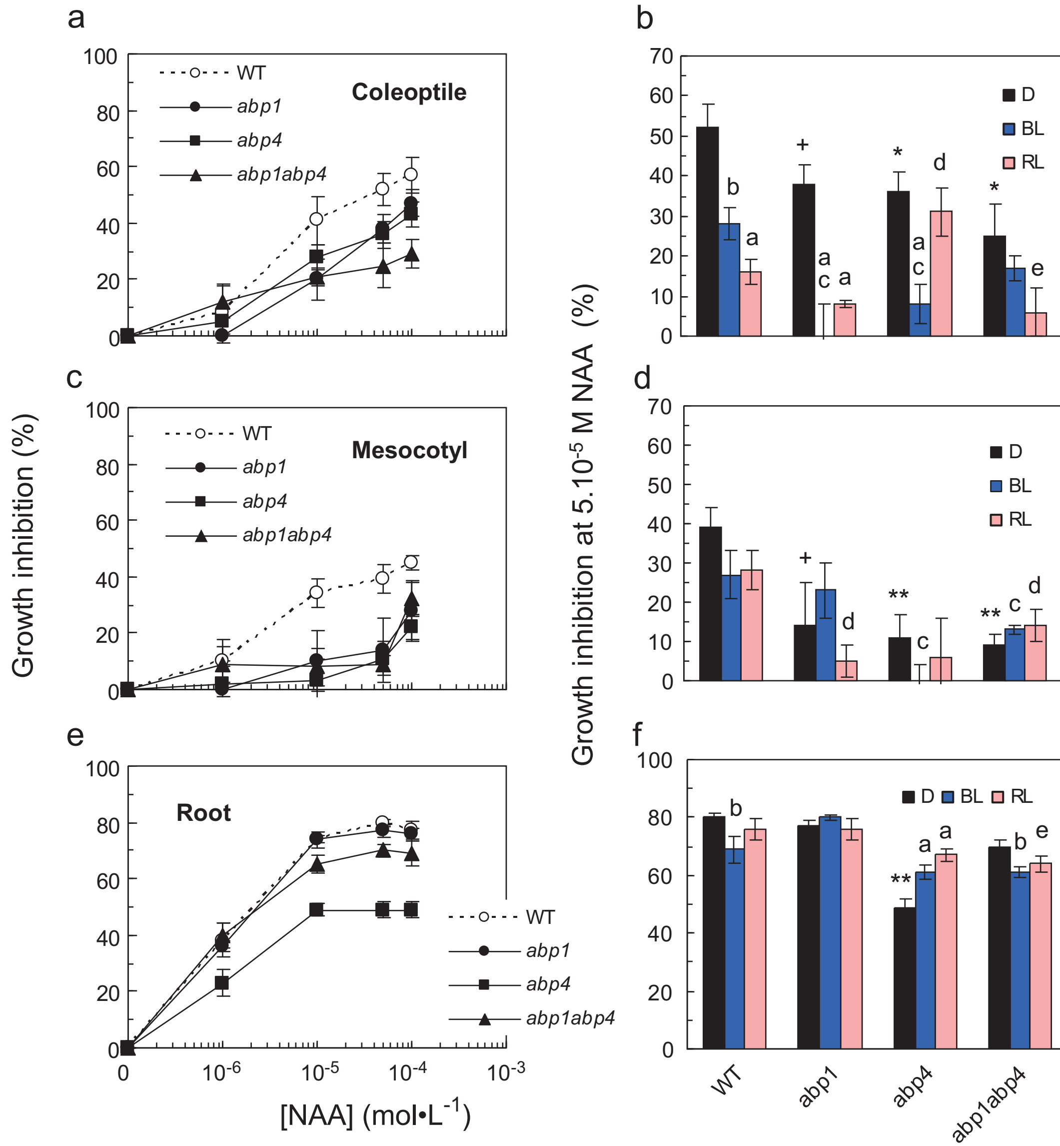
double mutant *abp1abp4*, primary roots were of a length similar to those observed in WT plants (Fig. 3*c*).

In etiolated mesocotyls, the mutation in *ABP1* or *ABP4* affected the cell size. Figure 4*a* shows the microphotography of the mesocotyl cross-sections stained with methylene blue dye. It is manifested that in single mutants, the cells in all rows are essentially smaller in radial direction than the cells of WT plants. Interestingly, cell expansion was not significantly affected in the double mutant. As shown in Fig. 4*b*, a reduced mesocotyl diameter in single mutants *abp1* and *abp4* corresponded with the reduced diameter of cortical cells. It is also evident that the loss of function in *ABP1* or *ABP4* does not affect cell division, as the number of cells was the same in WT and mutant plants (Fig. 4*a*).

Elimination of the *ABP1* and (or) *ABP4* gene alters seedling growth responses to NAA

We previously reported that exogenous auxin reduces the elongation of the etiolated maize coleoptile, mesocotyl, and primary roots in intact maize hybrid seedlings (Fellner et al. 2003, 2006). We also revealed that the etiolated seedlings of modern maize hybrids with reduced expression of the *ABP4* gene showed reduced auxin-induced responses (Fellner et al. 2006). To determine the role of ABP1 and ABP4 in seedling

Fig. 5. Elongation of the coleoptile, mesocotyl, and primary root in 5-day-old wild type (WT) and *abp* mutants in maize grown in vitro on the BM in the absence or presence of auxin 1-naphthalene acetic acid (NAA; 10^{-6} to 10^{-4} mol·L^{-1}), in dark (D), blue light (BL), or red light (RL) conditions. Dose-response curves of the (*a*) etiolated coleoptile growth, (*c*) etiolated mesocotyl growth, and (*e*) primary root growth. For each genotype and condition, the organ length in six to nine seedlings was measured in every experiment. The values represent the mean organ length ± SE obtained from 5 to 12 independent experiments. NAA-induced growth inhibition of the (*b*) coleoptile, (*d*) mesocotyl, and (*f*) primary root in seedlings developed in D, BL, or RL at an auxin concentration of 5×10^{-5} mol·L^{-1}. The values are the mean growth inhibition ± SE calculated from the mean values in 5 to 12 independent experiments. $^{+}$, *, and ** indicate significant differences (*t* - test; $P \leq 0.1$, $P \leq 0.05$ and $P \leq 0.01$, respectively) from WT in D; letters a, b indicate significant differences (*t*-test; $P \leq 0.01$ and $P \leq 0.05$, respectively) from dark-grown seedlings of the same genotype; c indicates a significant difference (*t*-test; $P \leq 0.01$ or 0.05) from WT in BL; d indicates a significant difference (*t*-test; $P \leq 0.05$) from WT in RL; e indicates a significant difference (*t*-test; $P \leq 0.1$) from *abp1abp4* in RL.



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responses to auxin, we studied the effect of the auxin NAA on the growth of *abp* mutants in D. The elongation of the coleoptile in etiolated seedlings of all genotypes was gradually inhibited by auxin in a concentration-dependent manner. However, as shown in Fig. 5*a*, the inhibition of the coleoptile growth was essentially greater in WT plants than in all *abp* mutants. Except for the lowest auxin concentration tested, the double mutant showed half of the reduction of the coleoptile growth by NAA compared with the inhibition observed in WT plants. For example, the coleoptile growth in WT at the concentration 5×10^{-5} mol·L^{-1} NAA was inhibited by ~50%, whereas in the double mutant the inhibition reached ~25% (Fig. 5*b*).

Earlier we revealed that the modern hybrids respond less than the old hybrids to light (Fellner et al. 2003, 2006). To determine the role of ABP1 and ABP4 in possible auxin-light cross-talk we further characterized the growth response of the *abp* mutants in the presence of auxin in BL or RL conditions. WT coleoptiles developed under BL or RL were essentially less inhibited by NAA than the coleopiles grown in D. For a clearer demonstration of the light effects on the coleoptile responsiveness to auxin, the data obtained for the growth

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of seedlings in BL and RL are shown in percentage of inhibition by NAA at the concentration 5×10^{-5} mol·L^{-1} (Figs. 5*b*, 5*d*, 5*f*), and similar results were obtained for the other auxin concentrations tested (data not shown). The figure shows that in BL, NAA inhibited the coleoptile growth in WT by ~28% and in RL by ~16%, i.e., two and three times less, respectively, than in D (50%). The figures further show that in BL, the coleoptile responsiveness to NAA of the single mutants *abp1* and *abp4* was significantly less than in WT, whereas BL-grown seedlings of the double mutant responded to NAA similar to WT plants. Under RL, *abp1* and the double mutant showed very low responsiveness to auxin similar to that in WT. Interestingly, the knockout of the *ABP4* gene resulted in a high sensitivity to the auxin of the coleoptile developed in RL (Fig. 5*b*), i.e., similar to that observed for coleoptiles in etiolated mutant plants.

Exogenous auxin inhibited the elongation of etiolated WT and *abp* mesocotyls in a concentration-dependent manner, and again, a stronger response to exogenous auxin was observed for the WT seedlings (Fig. 5*c*). Auxin-induced inhibition of the WT mesocotyl growth was only slightly reduced by BL or RL. Except for *abp1*, the percentage of growth inhibition by NAA in *abp4* and double mutant mesocotyls in BL was distinctly less than in WT. In addition, the RL-grown mesocotyls of all the mutants were markedly less responsive to auxin than in WT plants (Fig. 5*d*).

The strongest inhibition of growth by exogenous auxin was observed in the primary roots (Fig. 5*e*). Etiolated WT, *abp1*, and double mutants showed similar responsiveness to NAA (70%–80% at concentrations from 10^{-5} to 10^{-4} mol·L^{-1}), whereas etiolated roots in *abp4* mutants were distinctly less inhibited by auxin in all the concentrations tested. BL and RL slightly decreased (in WT and double mutant), increased (in *abp4*), or did not essentially influence (in *abp1*) the primary root responsiveness to NAA (Fig. 5*f*). A similar trend was observed at all higher auxin concentrations tested (data not shown).

Knockout of *ABP1* and (or) *ABP4* has a strong impact on the levels of endogenous auxin

Differential responsiveness of WT and *abp* mutants to exogenous auxin could reflect a differential level of endogenous auxin. This led us to examine the amounts of free endogenous IAA in all experimental genotypes. Free IAA was analyzed in 5-day-old coleoptiles, mesocotyls, and primary roots of seedlings grown in D, BL, or RL conditions.

In etiolated coleoptiles, mutation in the *ABP1* or *ABP4* gene did not result in any essential change in the accumulation of free IAA. However, a twofold augmentation of free IAA was observed in etiolated coleoptiles of the double mutant (Fig. 6*a*). In WT, *abp1*, and double mutants, the amount of IAA in the coleoptiles was markedly reduced in seedlings grown in BL compared with those grown in D. Of note was that the reduction was not significant in seedlings with the *ABP4* gene knocked out. In these cases the auxin level was similar to that observed in the etiolated coleoptiles. RL distinctly reduced the accumulation of free IAA in the coleoptile and the amount of auxin was similar in WT and *abp* mutants (Fig. 6*a*).

In etiolated seedlings, mesocotyls of WT and *abp* mutants accumulated a similar amount of free IAA and no matter the

Fig. 6. Level of free indole-3-acetic acid (IAA) in (*a*) coleoptiles, (*b*) mesocotyls, and (*c*) primary roots of 5-day-old seedlings of wild type (WT) and *abp* mutants developed in Magenta boxes on the BM in dark (D), blue light (BL), or red light (RL) conditions. Auxin analysis was performed using an immunoaffinity column with an immobilized polyspecific rabbit polyclonal antibody against IAA. The final analysis was performed by HPLC coupled to MS/MS detection with the use of a triple quadrupole mass spectrometer. The data represent mean ± SE obtained from three independent replicates. + and ** indicate significant differences (*t*-test; $P \leq 0.1$ and $P \leq 0.01$, respectively) from WT in D; letters a, b indicate significant differences (*t*-test; $P \leq 0.01$ and $P \leq 0.05$, respectively) from dark-grown seedlings of the same genotype; c, d indicate significant differences (*t*-test; $P \leq 0.01$ and $P \leq 0.05$) from WT in BL; e indicates a significant difference (*t*-test; $P \leq 0.05$) from WT in RL.

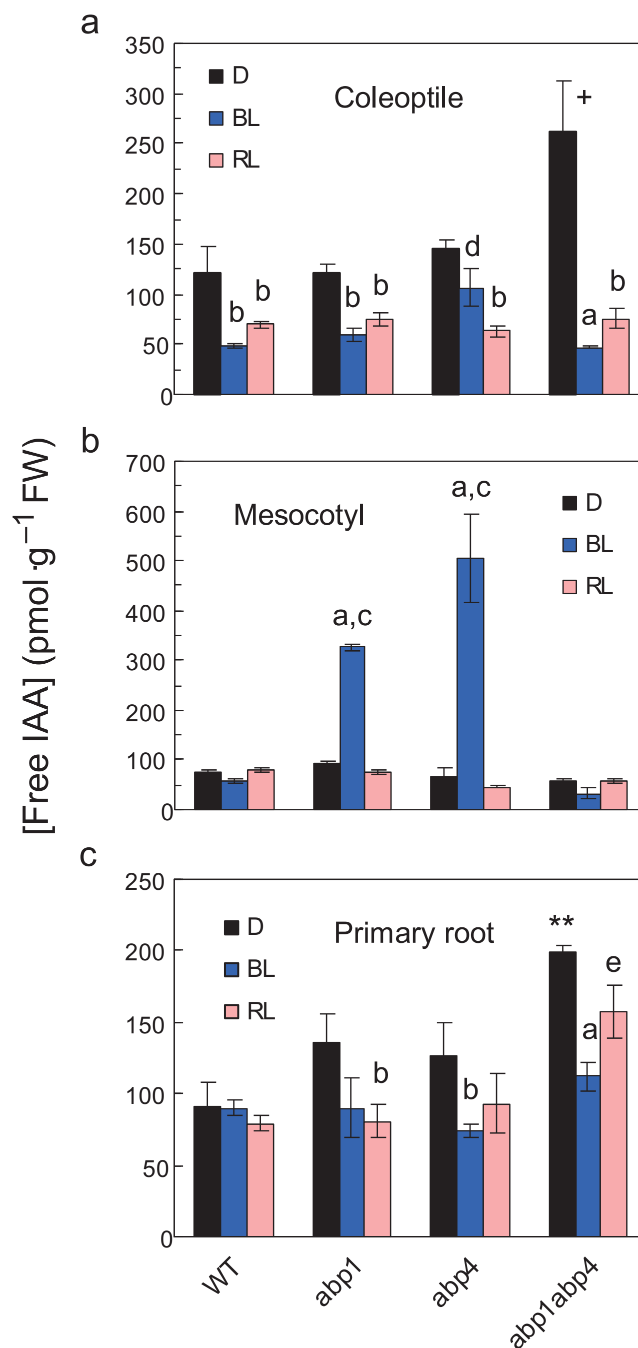
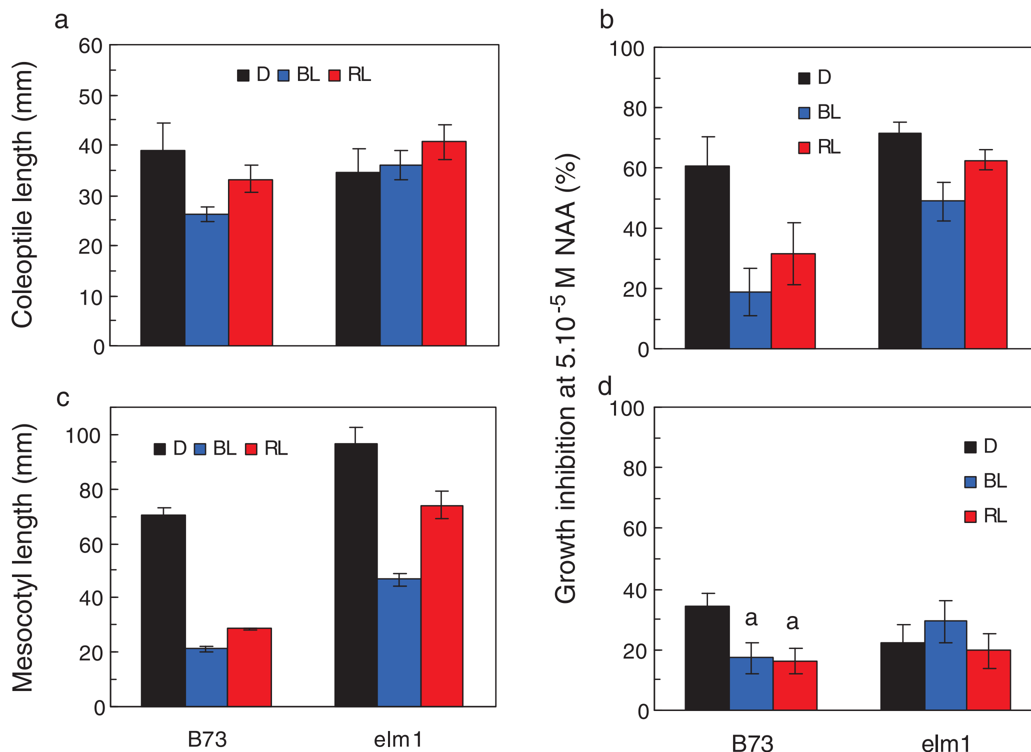


Fig. 7. Growth responses of the maize *elm1* mutant and corresponding wild type (WT; B73) cultured in dark (D), blue light (BL), and red light (RL) conditions on the BM, some of which were supplemented with 1-naphthalene acetic acid (NAA; 5×10^{-5} mol·L^{-1}). The effect of BL or RL on the elongation of etiolated (*a*) coleoptiles and (*c*) mesocotyls are shown. The values are the mean length ± SE from six independent experiments, with six to nine plants measured in every experiment in each of the light conditions. The growth inhibition of the (*b*) coleoptile and (*d*) mesocotyl in seedlings was developed in D, BL, or RL conditions on the BM, supplemented with NAA (5×10^{-5} mol·L^{-1}). The values are the mean growth inhibition ± SE calculated from the mean values in six independent experiments. The letter a represents a significant difference (*t*-test; $P \leq 0.05$) from B73 in D.



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genotype, RL had a negligible effect on the auxin content. Interestingly, the loss of function in the *ABP1* or *ABP4* gene led to a dramatic increase of IAA amounts in mesocotyls of seedlings developed under BL. Even though the single mutants exhibited changes in IAA amounts, the double mutant grown in BL accumulated a similar amount of IAA to the WT in their mesocotyls (Fig. 6*b*).

Knockout of the *ABP1* or *ABP4* gene led to a slight (not significant) increase of IAA content in the primary roots of etiolated seedlings. However, when both genes were switched off the IAA amount was two times higher than in WT roots (Fig. 6*c*). In the primary roots of WT seedlings grown under BL or RL, the amount of free IAA was found to be similar to that in etiolated roots. In comparison to WT roots, the mutation in both genes led to a minor (not significant) augmentation of IAA in BL-grown roots and to a distinct increase of auxin amounts in primary roots of seedlings developed in RL (Fig. 6*c*).

The phytochrome-deficient mutant *elm1* shows light-dependent super-sensitivity to exogenous auxin

We showed that in WT seedlings BL and RL reduced the inhibitory effect of exogenous auxin on the coleoptile elongation (Fig. 5*b*). We also revealed that coleoptiles of *abp4* mutants developed in RL were super-sensitive to the inhibitory effect of exogenous auxin (Fig. 5*b*). The data indicated that functional ABP4 could be an element or a target of the RL signaling pathway, leading to the loss or reduction of the sensitivity to exogenous auxin of the coleoptile. To test the hypothesis if phytochromes are involved in this response, we investigated the phytochrome-deficient mutant *elm1* in maize for its sensitivity to exogenous auxin. On the BM, etiolated seedlings of WT and *elm1* mutants developed coleoptiles of similar length, and BL- or RL-induced de-etiolation of coleoptiles was less evident in *elm1* than in WT plants (Fig. 7*a*). In D, NAA inhibited the coleoptile elongation in a concentration-dependent manner in both genotypes. The inhibition of the coleoptile growth is shown in Fig. 7*b* for the concentration 5×10^{-5} mol·L^{-1}. In BL and RL, auxin inhibited the coleoptile growth in B73 significantly less than in D. However, the responsiveness of the *elm1* coleoptile to auxin in BL and RL was comparable to that observed in the etiolated coleoptile (Fig. 7*b*). In the absence of NAA, BL and RL inhibited the mesocotyl growth in *elm1* essentially less than in B73 (Fig. 7*c*). When seedlings grew in the presence of NAA, the inhibition of mesocotyl elongation in B73 was much less in BL and RL compared with the etiolated plants. In contrast, auxin inhibited mesocotyl growth in *elm1* in the same manner as in D, BL, or RL conditions (Fig. 7*d*).

Discussion

In *Arabidopsis*, the sole *ABP* gene, *ABP1*, was identified (Palme et al. 1992), and its knockout resulted in an arrest of seedling development at the globular stage of the embryo

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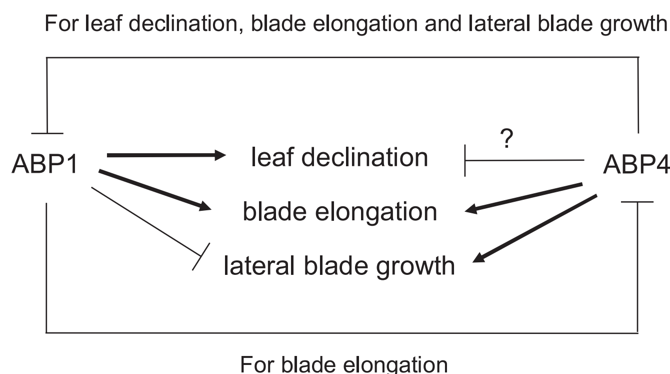
(Chen et al. 2001). Different from *Arabidopsis*, the maize ABP family contains at least five closely related members (Hesse et al. 1989; Schwob et al. 1993). It was reported that two available loss-of-function mutants in *ABP1* and *ABP4* genes do not show a distinct phenotype (Im et al. 2000), suggesting a functional redundancy in maize. We previously reported that the *abp1* and *abp4* mutants develop more erect and more horizontal leaves, respectively, than corresponding WT plants (Fellner et al. 2006). Our results therefore suggest that ABP1 and ABP4 play an important role in the development of maize leaf declination. The aim of this study was to characterize the phenotype of maize *abp* mutants with more detail to indicate other possible roles of ABPs in the growth and development of maize plants. In addition, based on our previous work (Fellner et al. 2003, 2006), we investigated whether defects in *ABP1* and *ABP4* genes may affect light- and auxin-mediated changes in the growth of maize seedlings.

ABPs control different aspects of the maize plant architecture

Our experiments on leaf declination in plants grown in the greenhouse support our previous data obtained in growth chamber conditions (Fellner et al. 2006), i.e., *abp1* mutants develop more erect, whereas *abp4* less erect, leaves than WT plants. Im et al. (2000) previously reported that knockout of *ABP4* results in a dramatic increase of ABP1 level. We did not analyze the accumulation of ABP1. However, in agreement with the results of Im and co-authors, our data suggest that functional *ABP4* may negatively regulate ABP1, which would positively mediate leaf angle development. Thus, the *abp4* phenotype reflects the overproduction of the ABP1 protein. The absence of ABP1 and ABP4 transcripts in the double mutant resulted in expectation of a leaf angle similar to *abp1* or even smaller. However, the opposite occurred with leaves in the double mutant showing a big declination similar to that in the *abp4* mutants, and thus suggesting that the simultaneous knockout of *ABP1* and *ABP4* triggers substitute pathway(s) for maintaining development of leaf declination. These pathways may include the involvement of other members of the ABP family (Schwob et al. 1993) or, of course, other signaling elements.

Phenotypic analyses of *abp* mutants further revealed that ABP1 and (or) ABP4 control the elongation and the lateral growth of the leaf blade and therefore the final blade surface. Based on our results, it seems that later in the plant development, ABP1 or ABP4 positively influences blade elongation, but simultaneously they reduce the activity of each other in this growth response. The WT phenotype of the double mutant knockout of *ABP1* and *ABP4* thus confirms the phenotype expectation above (see Results). The phenotypes of single mutants further indicate that the functional ABP1 keeps leaf blades narrower, while a positive role of ABP4 in the blades lateral growth cannot be excluded. The phenotype of the double mutant then indicate that when both genes are knocked out, an alternative and even stronger pathway may assure blade growth. Altogether, the model in Fig. 8 proposes that the functional ABP1 is positively involved in leaf declination and blade elongation but negatively regulates lateral blade growth. We further suggest that ABP4 reduces the activity of ABP1 in all these growth processes, and that for blade elongation ABP1 may suppress the activity of ABP4.

Fig. 8. A working model showing the effect of ABP1 and ABP4 on the development of the leaf angle and the growth of the leaf blade. We propose that ABP1 is positively involved in the leaf angle development and blade elongation, and it negatively contributes to lateral blade growth. ABP4 then seems to play a negative role in leaf declination, whereas it contributes positively to blade elongation and likely in lateral blade growth. Simultaneously, it seems that in all the growth responses tested, ABP4 (or *ABP4*) suppress the activity of ABP1, whereas for blade elongation, ABP1 (or *ABP1*) suppress the activity of ABP4. The arrows and T-bars represent positive and negative effects, respectively.



ABPs regulate development of maize seedlings differentially in darkness and light

Our experiments on adult mutants suggested that ABP1 and ABP4 are involved in maize leaf growth. We therefore investigated whether the ABPs control the development of seedlings. Our results indicate that ABP1 and (or) ABP4 are involved in the growth of maize seedlings, and that their effects and functional relationship are organ- and light-dependent (Table 1). First, our analysis of mutant seedlings suggests that ABP4, but not ABP1, is required for the growth of the etiolated coleoptile. The short coleoptile in the double mutant *abp1abp4* supports this idea. Since the loss of function of *ABP1* and (or) *ABP4* genes had no significant effect on the coleoptile growth in BL or RL, we conclude that for growth of the coleoptile in light conditions, functional ABP1 and ABP4 are not essential.

The development of a markedly shorter etiolated mesocotyl in double but not in single mutants indicates the requirement of both ABP1 and ABP4 during the elongation of the etiolated mesocotyl and suggests that ABP1 and ABP4 may substitute for each other. Our results further indicate that these genes are not essential for the BL-induced mesocotyl de-etiolation. In RL, the defect in *ABP1* does not influence the mesocotyl growth, whereas the mesocotyl of *ABP4* knockout plants was twice as long as in the WT. Considering that the *abp4* mutant contains high levels of the ABP1 protein (Im et al. 2000) even in RL, our data indicate that ABP1 has a positive effect on the mesocotyl elongation, whereas the direct effect of ABP4 remains obscure. Our results may support the hypothesis of Jones et al. (1991) that the RL-induced inhibition of the mesocotyl growth could be partially caused by the RL-induced reduction of ABP1 abundance. The normal mesocotyl elongation in the double mutant *abp1abp4* grown in BL or RL leads to the conclusion that in the light

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Table 1. Proposed functions of ABP1 and ABP4 during growth of maize seedlings cultured for 5 days in sterile conditions in the Magenta boxes in dark (D), blue light (BL), or red light (RL) conditions.

Measured response	Light condition	Effect of ABP1 and (or) ABP4
Coleoptile		
Elongation	D	ABP4(+)
	BL	x
	RL	x
IAA accumulation	D	ABP1(–), ABP4 (–)*
	BL	ABP1(+), ABP4 (?)
	RL	x
Mesocotyl		
Elongation	D	ABP1(+), ABP4 (+)*
	BL	x
	RL	ABP1(+), ABP4 (?)
IAA accumulation	D	x
	BL	ABP1(+), ABP4 (+)■
	RL	x
Cell size	D	ABP1(–), ABP4 (–)
Primary root		
Elongation	D	ABP4(+)
	BL	ABP4(+)■
	RL	ABP4(+)■
IAA accumulation	D	ABP1(–), ABP4 (–)*
	BL	x
	RL	ABP1(–), ABP4 (–)*

Note: x, indicates no involvement of ABP1 or ABP4; +, a positive effect; –, a negative effect; *, a substitution for each other; ■, where ABP1 (*ABP1*) affects negativey ABP4. Based on Im et al. (2000) post-transcriptional activation of ABP1 by *ABP4* is assumed in all the responses measured.

Table 2. Proposed involvement of ABP1 and ABP4 in NAA-induced inhibition of maize seedlings cultured for 5 days in sterile conditions in the Magenta boxes in dark (D), blue light (BL), or red light (RL) conditions.

Measured response	Light condition	Effect of ABP1 and (or) ABP4
Coleoptile		
Growth inhibition	D	ABP1(?), ABP4 (+)
	BL	ABP1(+), ABP4 (+)
	RL	ABP1(+)
Mesocotyl		
Growth inhibition	D	ABP1(?), ABP4 (+)
	BL	ABP4(+)
	RL	ABP1(+), ABP4 (+)
Primary root		
Growth inhibition	D	ABP4(+)
	BL	x
	RL	x

Note: x, indicates no involvement of ABP1 or ABP4; +, a positive effect; –, a negative effect. Based on Im et al. (2000) post-transcriptional activation of ABP1 by the *ABP4* is assumed in all the responses measured.

the elimination of both *ABP1* and *ABP4* triggers an alternative pathway regulating the mesocotyl growth.

The knockout of the *ABP4* gene causes the reduction of the primary root length in etiolated single mutants and even more in the double mutant, whereas the knockout of ABP1 only weakly affect the root growth. The data suggest that ABP4 is preferentially involved in the stimulation of the elongation of the etiolated primary root. Contrary to *Arabidopsis*, in maize seedlings BL or RL inhibits primary root elongation. Our results show that the knockout of *ABP1* enables roots to grow significantly more in BL (and RL, but insignificantly), whereas the knockout of *ABP4* results in normal (WT-like) root growth. It therefore seems that especially in BL, ABP1 itself has no effect on the root elongation but it suppresses the promoting effect of ABP4 on this growth response. The fact that in BL and RL the primary root of the double mutant grew more or less similar to WT just confirms this conclusion (expected phenotype, see Results).

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The knockout of *ABP1*, but especially the lack of the *ABP4* gene, causes a reduction of the radial expansion of cells of all types in the etiolated mesocotyl (Fig. 4). This indicates that ABP1 and ABP4 are negatively involved in the mesocotyl cell enlargement but also that both proteins may belong to a regulatory loop, affecting their mutual activity. Therefore, as expected (see Results), the double mutant *abp1abp4* shows WT phenotype.

Knockout of *ABP1* and (or) *ABP4* affects the accumulation of endogenous auxin in maize seedlings

Analysis of the endogenous auxin revealed that functional ABP1 and ABP4 are, depending on the organ and light conditions, involved in the accumulation of free endogenous IAA (Table 1). Based on our measurements, we suggest that in the etiolated coleoptile, functional ABP1 and ABP4 reduce the level of endogenous IAA and that they likely substitute for each other in the maintenance of the normal (WT-like) level of IAA. We further deduce that in BL conditions, the level of endogenous IAA in the coleoptile is positively controlled by ABP1, whereas the role of ABP4 remains unclear. Finally, the level of IAA in RL-developed coleoptile does not seem to be controlled by ABP1 nor ABP4. It further seems that in the mesocotyl, the level of IAA is positively regulated mainly via ABP1 (or possibly via ABP4) only in seedlings developed in BL conditions, while ABP1 and ABP4 suppress each other's activity (Fig. 6*b*). This is supported by the expected WT level of IAA in the mesocotyl of the double mutant developed in BL. Levels of IAA in the primary roots in D and RL conditions seem to be regulated similarly as observed in etiolated coleoptiles. It does not appear likely that ABPs regulate the IAA levels in roots developed in BL.

Growth responses of loss-of-function mutants indicate that ABP1 and ABP4 are involved in maize seedling responses to exogenous auxin

The concentration of and sensitivity to hormones is the controlling aspect of hormone action (Davies 1995). To better understand the role of ABP1 and ABP4 in the auxin- and light-regulated growth of maize seedlings, we analyzed, in addition to the amounts of endogenous free IAA, the sensitivity (responsiveness) of maize seedlings to auxin NAA in various organs of the seedlings reacting to light conditions. The study of intact seedling responses to exogenous auxin (often roots) became one of the methods for the selection of various mutants affected in auxin physiology (e.g., Maher and Bell 1990; de Souza and King 1991; reviewed in Fellner 1999). Therefore, the testing of plant responses to exogenous auxin is one of the appropriate methods for the investigation of auxin-regulated growth and development. It is not still fully understood why exogenous auxin, in contrast to endogenous auxin, inhibits elongation growth in various plant species. It is expected that the mechanism of this auxin effect is quite complex and may involve for example, cross-talk between various hormones (Ephritikhine et al. 1999). However, one of the proposed mechanisms is that the application of exogenous auxin results in a supraoptimal concentration of auxin in the tissues that results in the inhibition of elongation (Hasenstein and Evans 1988). In agreement with this, we clearly have shown that the etiolated WT coleoptile, with a high level of free IAA, is sensitive to the inhibitory effect of NAA much more than light-developed coleoptiles, which contain significantly less free IAA when compared with coleoptiles of etiolated seedlings (Fig. 5*a*). In the mesocotyl and primary root, the levels of endogenous IAA was not significantly altered by BL or RL. In agreement with that, the sensitivity of the organs did not change much in BL or RL (Figs. 5*b*, 5*c*).

Our experiments further showed that etiolated coleoptiles and mesocotyls of the single mutants partially lost their responsiveness to exogenous auxin, and the responsiveness to NAA in the double mutant was the lowest. The data indicate that the ABP1 and ABP4 act together in responsiveness to exogenous auxin. The results suggest that rather than ABP1, ABP4 mediates the growth inhibition of the etiolated maize coleoptile and mesocotyl induced by exogenous auxin (Table 2).

In comparison to the responsiveness in D, the responsiveness of the coleoptile and mesocotyl in light-developed WT seedlings to the inhibitory effect of exogenous auxin was reduced. Using the phytochrome mutant *elm1* in maize, we demonstrated that the effect of BL and RL on the coleoptile and mesocotyl responsiveness to exogenous auxin is mediated at least partially by phytochromes. Our results further suggest that under BL and especially in RL conditions, ABP1 and ABP4 contribute to the responses of coleoptiles to the inhibitory effect of exogenous auxin. Again, this is supported by the expected WT-like responsiveness to NAA of the double mutant. Our results further indicate that in BL the inhibition of the mesocotyl elongation by exogenous auxin is mediated by ABP4 without the essential participation of ABP1, whereas both ABP4 and ABP1 seem to be involved in the mesocotyl responses to exogenous auxin in RL conditions.

A loss of function in the *ABP4* gene, but not in *ABP1*, resulted in decreased sensitivity of the etiolated primary roots to exogenous auxin. The data suggest that auxin-induced inhibition of elongation in the etiolated primary roots is controlled via ABP4. The high responsiveness of the double mutant roots to exogenous auxin indicates that the simultaneous knockout of *ABP1* and *ABP4* results in a substitute pathway that enables the primary root to respond almost fully to exogenous auxin. BL and RL did not essentially affect WT root growth responses to the inhibitory effect of auxin. It indicates that the responsiveness of light-grown roots to exogenous auxin is controlled via ABP1- and ABP4-independent pathways.

In conclusion, we have shown that the loss-of-function mutants in *ABP1* and (or) *ABP4* genes in maize show striking phenotypes. Our results indicate that functional maize ABP1 and ABP4 are involved in the leaf development and growth, and in the elongation of etiolated seedlings. Furthermore, we have shown that ABP1 and (or) ABP4 mediate the responses of etiolated seedlings to exogenous auxin. Results of the analyses of endogenous auxin suggest that in darkness or light the growth capacity of WT and mutant seedlings does not simply correspond with the observed changes in levels of free IAA in coleoptiles, mesocotyls, and primary roots (Table 1). Differentially, the changes in the levels of endogenous IAA in etiolated and light-grown WT seedlings likely influences their responsiveness to exogenous auxin. This confirms that the auxin-regulated growth of maize seedlings

is a complex process and certainly involves the activity of the putative receptors ABPs and endogenous IAA. Here, we further showed that in BL or RL the responsiveness of maize seedlings to exogenous auxin is less than in D, and our data led to the conclusion that the light-induced reduction of the responsiveness to exogenous auxin is mediated at least by phytochromes. Based on our results, we propose that ABP1 and ABP4 cross the phytochrome pathway, and thus they can function as common elements of the light and auxin signaling pathways involved in the development of maize seedlings. However, because our data also showed that the functional relationship between ABP1 and ABP4 is organ- and response-dependent, additional research is required to elucidate the molecular mechanisms of the interaction between light and ABPs in the growth and development of maize plants.

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