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ARTICLE

AUXIN BINDING PROTEIN 4 is involved in the Ca^{2+}/auxin-regulated expression of *ZCAX3* gene in maize (*Zea mays*)

Dejana Jurišić-Knežev, Véronique Bergougnoux, David Milde, and Martin Fellner

Abstract: As a second messenger, calcium is involved in auxin signaling, and previous studies demonstrated that *Arabidopsis* CAX1 (Ca^{2+}/H^+ exchanger) is involved in the auxin transduction pathway. This study was performed to investigate the possible role of auxin-binding proteins ABP1 and ABP4 in Ca^{2+}/auxin-regulated growth in maize (*Zea mays* L.). We identified and cloned two AtCAX1 homologs in maize, *ZCAX2* and *ZCAX3*. Using maize loss-of-function *abp1* and *abp4* mutants, the role of ABPs in Ca^{2+}-dependent growth and in the Ca^{2+}/auxin-regulated expression of the *CAX* genes was investigated in etiolated maize seedlings. Exogenous Ca^{2+} enhanced mesocotyl but not coleoptile growth in WT, *abp1*, *abp4*, and *abp1*/*abp4* mutants, but the maximum stimulation was in *abp4*. As well, in the *abp4* mutant, maximum accumulation of Ca^{2+} was observed when seedlings were exposed to exogenous Ca^{2+}. In the mesocotyl of *abp4* and double mutants, the expression of *ZCAX3* was significantly reduced in the absence of exogenous Ca^{2+}, whereas exogenous Ca^{2+} significantly up-regulated its expression in both mutants. This effect of Ca^{2+} was not observed in the coleoptile. In the absence of NAA, knockout of *ABP4* led to significant drop of *ZCAX3* expression in the mesocotyl, and exogenous auxin significantly inhibited expression of the *ZCAX3* in WT, but not in *abp* mutants. This effect of auxin was not observed in the coleoptile. Our results indicate that in the absence of NAA, functional ABP4 is required for *ZCAX3* expression and that ABP4 mediates the inhibitory effect of NAA on *ZCAX3* expression. We provided evidence for a cross talk between *ABP4*, exogenous auxin, Ca^{2+}, and *ZCAX3* during growth of etiolated maize mesocotyl.

Key words: auxin-binding protein, ABP4, AtCAX1, calcium, CAX, growth, maize, mesocotyl.

Résumé : En tant que messager secondaire, le calcium joue un rôle dans la voie de signalisation de l'auxine. Des études ont démontré que CAX1 (échangeur Ca^{2+}/H^+) d'*Arabidopsis* est impliqué dans cette voie de signalisation. Cette étude a été menée de façon à déterminer le rôle des protéines liant l'auxine, ABP1 et ABP4, dans la croissance du maïs (*Zea mays* L.) régulée par Ca^{2+}/auxine. Nous avons identifié et cloné chez le maïs deux homologues de AtCAX1, *ZCAX2* et *ZCAX3*. En utilisant les mutants de maïs perte-de-fonction *abp1* et *abp4*, nous avons étudié le rôle des ABPs d'une part dans la croissance dépendante du Ca^{2+} et d'autre part dans l'expression des gènes *CAX* régulée à la fois par le Ca^{2+} et l'auxine. Le Ca^{2+} exogène stimule la croissance du mésocotyle mais pas du coléoptile chez le WT, les mutants *abp1*, *abp4* et *abp1*/*abp4*, même si l'effet optimum est observé pour le mutant *abp4*. De la même façon, chez le mutant *abp4*, l'accumulation maximale de Ca^{2+} est observée lorsque les plantules sont exposées au Ca^{2+} exogène. Dans le mésocotyle de *abp4* et du double mutant, l'expression de *ZCAX3* est significativement réduite en l'absence de Ca^{2+} exogène ; à l'opposé, l'application de Ca^{2+} induit significativement l'expression de *ZCAX3* chez ces deux mutants. Cet effet du Ca^{2+} n'est pas observé dans le coléoptile. En l'absence de NAA, l'absence du gène *ABP4* se caractérise par une diminution significative de l'expression de *ZCAX3* dans le mésocotyle, et la présence d'auxine exogène inhibe significativement l'expression de *ZCAX3* chez le WT mais pas chez les mutants *abp*. Cet effet de l'auxine n'est pas observé au niveau du coléoptile. Nos résultats indiquent qu'en l'absence de NAA, ABP4 est requis pour assurer l'expression de *ZCAX3* et que ABP4 sert d'intermédiaire dans l'effet inhibiteur de NAA sur l'expression de *ZCAX3*. Nous apportons ici l'évidence d'une interaction entre *ABP4*, auxine exogène, Ca^{2+} et *ZCAX3* pendant la croissance du mésocotyle étiolé de maïs.

Mots-clés : protéine liant l'auxine, ABP4, AtCAX1, calcium, CAX, croissance, maïs, mésocotyle.

Introduction

For growth and development, plants require metal ions and mineral nutrients whose intracellular concentration has to be maintained at an optimal level. Calcium (Ca) is one of the essential nutrients for plants as it acts also as a second messenger mediating a wide range of cellular responses (Sanders et al. 2002). Its concentration in the cytosol fluctuates during growth, development, and responses to environmental perturbations (Sanders et al. 1999; Curran et al. 2000). The vacuole serves as a primary pool of Ca^{2+} in plant cells (Ueoka-Nakanishi et al. 1999). During

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Abbreviations: ABP, auxin-binding protein; BM, basal medium; CaD, Ca^{2+} domain; CAX, cation exchanger; IAA, indole-3-acetic acid; NAA, 1-naphthalene acetic acid; PCR, polymerase chain reaction; PIN, PIN-FORMED; qRT–PCR, quantitative reverse transcription polymerase chain reaction; RT–PCR, reverse transcription polymerase chain reaction; WT, wild-type.

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biological responses, Ca^{2+} can be mobilized from and to the vacuole to act as an intracellular signaling molecule (Marty 1999). Thus, Ca^{2+} transported into the vacuole may regulate the cytosolic level of Ca^{2+} during and after signaling transduction events.

Ca^{2+}/H^+ exchangers belong to the group of proteins that export cations from the cytosol out of the cell or to storage compartments to maintain an optimal ionic concentration in the cytosol (Sanders et al. 2002) At first, *CAX1* and *CAX2* were cloned from *Arabidopsis thaliana* because of their ability to sequester Ca^{2+} into vacuoles of *Saccharomyces cerevisiae* mutants deficient in Ca^{2+} vacuolar transport (Hirschi et al. 1996). Shigaki and Hirschi (2000) identified the first monocot *CAX-like* gene in maize (*Zea mays* L.) named *ZmHCX1*. Despite a high homology to previously characterized CAXs (Ueoka-Nakanishi et al. 1999, 2000; Hirschi 2001), *ZmHCX1* could not overcome the Ca^{2+} growth defect in yeast mutants defective in Ca^{2+} vacuolar transport (Hirschi et al. 1996).

As a second messenger, Ca^{2+} is involved in different signaling pathways including auxin signaling. It has been shown that auxin causes an increase in the cytosolic-free Ca^{2+} concentrations (Felle et al. 1986; Shishova and Lindberg 1999). At the cellular level, auxin is involved in the regulation of cell elongation, cell division, and cell differentiation (Perrot-Rechenmann 2010). One of the primary steps of auxin signaling consists of the binding of the auxin to a receptor. In addition to the auxin receptor TRANSPORT INHIBITOR RESPONSE 1/AUXIN-BINDING F-BOX PROTEIN (TIR1/AFB family), another auxin receptor, AUXIN-BINDING PROTEIN 1 (ABP1) has been identified (for review, see Shishova and Lindberg 2010;Tromas et al. 2010; Scherer 2011). ABP1 was identified first in maize coleoptiles over 30 years ago (Hertel et al. 1972; Löbler and Klämbt 1985). Despite a predominant localization in the endoplasmic reticulum (Jones and Herman 1993; Napier et al. 2002), several studies demonstrated that ABP1 acts at the plasma membrane (Ephritikhine et al. 1987; Barbier-Brygoo et al. 1989, 1991; Leblanc et al. 1999). The constitutive over-expression of *ABP1* in maize or tobacco cells resulted in larger cells and this effect was auxin dependent, consistent with ABP1 having an auxin receptor function (Jones et al. 1998; Im et al. 2000; Chen et al. 2001). The role of ABP1 in cell division and expansion during early embryogenesis and postembryonic development was also reported in tobacco and *Arabidopsis* (Fellner et al. 1996; Braun et al. 2008). Moreover, cell cycle arrest provoked by ABP1 inactivation cannot be bypassed by exogenous auxin, suggesting that ABP1 mediates the control of the cell cycle by auxin (David et al. 2007). ABP1 is required for the auxin-induced expression of a subset of *Aux/IAA* genes (Tromas et al. 2009). Effendi et al. (2011) provided the evidence that ABP1 is involved in the regulation of the polar auxin transport by modulation of the localization of PIN transporters. Recently, Scherer (2011) proposed a model connecting ABP1-dependent auxin signaling with TIR1-dependent signaling pathway.

The *Arabidopsis cax1* mutant shows typical symptoms of auxin deficiency, indicating that AtCAX1 is involved not only in the mobilization of intracellular Ca^{2+} but also in the auxin transduction pathway (Cheng et al. 2003). Moreover, this *Arabidopsis* mutant shows alterations in growth responses to auxin and altered expression of auxin-regulated promoters, reinforcing the idea that CAXs are important components of the auxin signaling pathway. Very recently, it was demonstrated that *Arabidopsis* CAX1 and CAX3 influence auxin transport in guard cells via the regulation of the apoplastic pH, and that the *cax1* and *cax3* mutants are less responsive than WT to the IAA-induced inhibition of hypocotyl elongation (Cho et al. 2012). Shishova and Lindberg (2010) proposed that ABP1 interacts with the Ca^{2+}-permeable ion channel, which could serve as a docking protein, and as a part of the receptor complex. It can therefore be assumed that auxin action, mediated via ABP1, could change the cytosolic Ca^{2+} concentration via the regulation of Ca^{2+} transport by AtCAX1, which is sensitive to and regulated by cytosolic pH (Pittman et al. 2005). However, up to date, no information is available concerning the possible interaction between ABP1 and CAXs.

In maize, at least five ABPs have been identified (Hesse et al. 1989; Schwob et al. 1993), but their roles in growth and development is poorly understood. Recently, we showed that ABP1 and ABP4 participate in the growth of maize seedlings, mediate seedling responses to auxin, interact with light signaling pathway(s), and a functional interaction between ABP1 and ABP4 was proposed (Jurišić-Knežev et al. 2012; Bořucká and Fellner 2012). In this study, using maize loss-of-function mutants in *ABP1* and *ABP4* genes, we investigated the role of these ABPs, in Ca^{2+}-dependent growth, and in the Ca^{2+}/auxin-regulated expression of two identified maize *CAXs*, designated as *ZCAX3* and *ZCAX2*.

Material and methods

Plant material and growth conditions

The loss-of-function mutants in *ABP1* and *ABP4* genes in maize were used for the experiments (Im et al. 2000). The *abp* mutants contained 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 (inbred line A619, here called WT) were a gift from Alan M. Jones (The University of North Carolina, USA). All mutants were tested for the lack of *ABP1* and (or) *ABP4* gene expression and they showed stable phenotypes (Jurišić-Knežev et al. 2012). For experiments, seeds were first washed with 70% ethanol for 3 min, rinsed with distilled water, and then soaked in Savo original solution (BOCHEMIE, Czech Republic) (~5% sodium hypochlorite) supplemented with a drop of the detergent Tween 20 (Calbiochem, USA), shaken on a stirrer for 45 min and then extensively rinsed five times with sterile distilled water. Seeds germinated on 0.7% (*w*/*v*) agar medium in Magenta GA7 boxes (77 mm × 77 mm × 196 mm) (Sigma–Aldrich, Prague, Czech Republic) (six to nine seeds per box). The basal BM contained Murashige and Skoog salts (MS medium, Sigma–Aldrich) (Murashige and Skoog 1962), 1% (*w*/*v*) sucrose, and 1 mmol·L^{-1} MES (2-(*N*-morpholino)-ethanesulfonic acid) (pH adjusted to 6.1 before autoclaving). Medium without Ca^{2+} was prepared using components of BM excluding $CaCl_2$ (41.2 mmol·L^{-1} NH_4NO_3, 18.8 mmol·L^{-1} KNO_3, 1.5 mmol·L^{-1} $MgSO_4$, 1.25 mmol·L^{-1} KH_2PO_4, 100 μmol·L^{-1} H_3BO_3, 100 μmol·L^{-1} $MnSO_4$, 30 μmol·L^{-1} $ZnSO_4$, 5 μmol·L^{-1} KI, 1 μmol·L^{-1} Na_2MoO_4, 0.1 μmol·L^{-1} $CuSO_4$, 0.1 μmol·L^{-1} $CoCl_2$, 0.2 mmol·L^{-1} Na_2EDTA, 0.1 mmol·L^{-1} $FeSO_4$, 1% sucrose (*w*/*v*) and 1 mmol·L^{-1} MES). The pH was adjusted to 6.1 before autoclaving. In the experiments with auxin, BM was supplemented with 50 μmol·L^{-1} 1-NAA. In the experiments with Ca^{2+}, the prepared medium without Ca^{2+} was supplemented with $CaCl_2$ to a final concentration of 3 (concentration of the commercial MS medium) or 10 mmol·L^{-1}. For the development of etiolated seedlings, Magenta boxes were wrapped in aluminum foil and placed into the growth chamber (Percival PGC-10, Iowa, USA) at a temperature of 23 °C. In all conditions, coleoptile and mesocotyl of five-day-old seedlings were measured. For the experiments on the expression of *CAX* genes, the seeds germinated and seedlings were grown in darkness in medium depleted in Ca^{2+} or auxin. In the tested conditions, mediums were supplemented with 10 mmol·L^{-1} $CaCl_2$ and 50 μmol·L^{-1} NAA, respectively. Seedlings were harvested 5 days after germination.

Calcium content determination

Ca^{2+} content was determined in the coleoptile and mesocotyl of five-day-old plants grown in medium without Ca^{2+} and in medium supplemented with 3 mmol·L^{-1} and 10 mmol·L^{-1} Ca^{2+}. Seedlings were collected and freeze-dried in a lyophilizer (Thermo, Electron Corporation, Heto, PowerDry, PL300, Freeze Dryer) for a period of 24 h. Calcium was determined by flame atomic absorption spectrometry (Avanta Sigma, GBC, Australia) using an acetylene–air flame at a wavelength of 422.7 nm. Lanthanum oxide in a

Table 1. The list of primers used in *CAX* cloning and gene expression analysis; full length (fl).

Gene name and accession No.		Forward (F) and reverse (R) primers	Product size (bp)
ZmAct81 (U60511)	F	5′-ACACAGTGCCAATCT-3′	316
	R	5′-ACTGAGCACAATGTTAC-3′	
ZCAX2 fl (AB044567)	F	5′-AATCGAGATGGGCGGTTTCAAGG-3′	1338
	R	5′-AGGTATTTGACGCATTACTTACAG-3′	
ZCAX3 fl (AY108295)	F	5′-CGGCGAAGGAGATGGATCGTCTGC-3′	1590
	R	5′-GACCGGATGCAAATTCGCTACCC-3′	
ZCAX2 (AB044567.1)	F	5′-ATCATTCTCCACTTCACGAC-3′	327
	R	5′-TGCCTTACTGAAGATTTGGT-3′	
ZCAX3 (AY108295.1)	F	5′-ATGAGCATCCAAGGCAGCAACG-3′	62
	R	5′-CATCAACCTTTGCACGGCATTG-3′	
GAPDH (Zm.3765)	F	5′-TGATCCGCCACATGTTCAAGACC-3′	79
	R	5′-CGGCATACACAAGCAGCAACC-3′	

concentration of 2 g·L^{-1} was used as a releasing agent to overcome common chemical interferences caused by phosphates. External calibration in the range of 0.1–10.0 mg·L^{-1} was used to evaluate the Ca^{2+} concentration in samples. Prior to the atomic absorption spectrometry determination, all samples were digested by a microwave digestion unit (Uniclever II, Plazmatronika, Poland) using a mixture of nitric acid and hydrogen peroxide following the method described in International Organization for Standardization 1986.

RNA extraction and cDNA synthesis

Five days after germination, coleoptiles and mesocotyls were harvested, frozen immediately in liquid nitrogen, and ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted from the tissues using a RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer instructions. An additional DNaseI treatment was performed using RQ1 RNA-free DNase (Promega, Madison, Wisconsin, USA) for 60 min at 37 °C and purification of the RNA was performed by a subsequent phenol–chloroform–isoamylalcohol (25:24:1) step. The reverse transcription reaction was performed from 1 μg of the total RNA by adding 4 μL of 5xRT buffer, 1 μL of 10 mmol·L^{-1} dNTP, 1 μL of 0.1 mol·L^{-1} DTT, 1 μL of 50 μmol·L^{-1} oligo(dT), 1 μL of RNasin Plus RNase inhibitor (Promega), and 1 μL of SuperScript III reverse transcriptase (Invitrogen, USA). The volume was filled to 20 μL using RNase-free water. The reaction mixture was incubated at 50 °C for 60 min followed by the heating deactivation of the reverse transcriptase at 70 °C for 15 min. The RNA was digested by 5 units of RNaseH (New England Biolabs, USA) for 20 min at 37 °C. The cDNA was subsequently diluted in RNase-free water before being used for PCR analysis. The integrity of the cDNA was confirmed by PCR using actin primers (*ZmAct81*, accession No. U60511). PCR reactions contained 1 μmol·L^{-1} of each primer, 1 mmol·L^{-1} dNTPs, 5 μL of cDNA (50 times diluted), 1x GoTaq Polymerase Buffer (Promega) and 1 μL of GoTaq Polymerase (Promega). The volume of the PCR reaction was filled to 20 μL. The DNA was denatured at 94 °C for 5 min followed by 35 cycles of amplification (30 s at 94 °C, 30 s at 45 °C, 45 s at 72 °C). The final incubation at 72 °C was extended to 7 min and the reaction was cooled and kept at 15 °C.

Database search, sequence analysis, and cloning

Blast algorithms available at the NCBI and maize GDB (http://www.maizegdb.org/) were used to identify AtCAX1 homologs in maize. As a reference, the sequence *A. thaliana AtCAX1* (accession No. AF461691.1) was used. Alignments were performed using CLUSTAL W version 1.8 program (Larkin et al. 2007). To amplify the full lengths of the *ZCAX2* (accession No. AB044567) and *ZCAX3* (accession No. AY108295) genes, specific primers were designed based on the sequences available in database (Table 1). The PCR reaction was performed as previously described with the following program: the DNA was denatured at 95 °C for 5 min, followed by 35 cycles of amplification (30 s at 95 °C, 30 s at 50 or 58 °C, 1 min. at 72 °C). The final incubation at 72 °C was extended to 7 min and the reaction was cooled and kept at 15 °C. PCR was performed in 20 μL volumes. The PCR products were isolated, cloned into the pGEM-T easy vector (Promega), and sequenced using a 3130 Genetic Analyzer (Macrogen Inc., Korea, Seoul). The prediction of transmembrane topology was done by the TMHMM program (Krogh et al. 2001).

Gene expression analysis

Expression analysis of both genes was first studied by semi-quantitative RT–PCR. Because the expression of *ZCAX3* was very weak and almost not detectable, expression analysis of *ZCAX3* was further investigated by quantitative RT–PCR (qRT–PCR). The primer combinations used and the size of the expected products are listed in Table 1. PCR for the *ZCAX2* expression analysis was performed under the following conditions. The DNA was denatured at 95 °C for 5 min, followed by 35 cycles of amplification (30 s at 95 °C, 30 s at 50 °C, 1 min. at 72 °C). The final incubation at 72 °C was extended to 7 min and the reaction was cooled and kept at 15 °C. PCR was performed in 20 μL volumes. PCR products were loaded into the 1.5% agarose gel and the picture was taken by a G:BOX Syngene Cold camera UV transilluminator. The intensity of signal was determined using the ImageJ processing program (Collins 2007). Normalization was assessed by the housekeeping gene *actin* and the gene expression was expressed as relative to the gene expression observed in WT. Amplification of target genes and real-time detection of amplicon production were monitored on an Mx3000P sequence detector (Stratagene, USA). qRT–PCR reactions contained 80 nmol·L^{-1} of each primer, 5 μL of cDNA template (diluted 1/50), and 12.5 μL of 2x absolute SYBR Green ROX Mix (ABGene, Epsom, UK). The volume was filled to 25 μL with sterile RNase-free water. PCR cycling conditions required an initial hot start activation of 15 min at 95 °C, followed by 45 cycles of 95 °C for 30 s, and 60 °C for 30 s. To confirm the product purity, a melting curve analysis was performed at the end of the PCR. The SYBR green fluorescent signal was standardized with a passive reference DYE (ROX). The target gene (*ZCAX3*) expression was quantified relative to the expression measured from the reference gene *GAPDH* (accession No. Zm3765) in the same sample. Differences in the cycle numbers during the linear amplification phase between the samples and the ΔΔCT method were used to determine the differential gene expression. Primers for qRT–PCR were designed using the program QuantPrime (Arvidsson et al. 2008) (Table 1). The gene expression in the *abp* mutants was expressed as relative to that obtained for WT plants.

Statistical analysis

When needed, statistical analysis was performed using Student's *t* test.

Results

Effect of exogenous calcium on the growth of maize seedlings

The effect of Ca^{2+} on the growth of maize seedlings was assessed in darkness using two concentrations of Ca^{2+}. Plants were able to grow in a medium without Ca^{2+} as well as in medium supplemented with 3 mmol·L^{-1} and 10 mmol·L^{-1} Ca^{2+}. In Ca^{2+}-free medium, the *abp4* mutant developed significantly shorter coleoptile compared with the WT. Mutation in the single *ABP1* gene and in both *ABP1* and *ABP4* genes did not have any significant effect on the coleoptile elongation (Fig. 1*a*). The data further showed that coleoptile growth of WT, and *abp1* and *double* mutants were insensitive to exogenous Ca^{2+} (3 mmol·L^{-1} and 10 mmol·L^{-1}). The elongation of the coleoptile in *abp4* mutant was slightly, but significantly stimulated by 3 mmol·L^{-1} Ca^{2+}. In Ca^{2+}-free medium, the mesocotyl length did not significantly differ between WT and *abp* mutants (Fig. 1*b*). Unlike in coleoptile, exogenous Ca^{2+} at both concentrations stimulated mesocotyl elongation in all genotypes. The greatest growth stimulation was observed for *abp4* mutant seedlings (Fig. 1*b*).

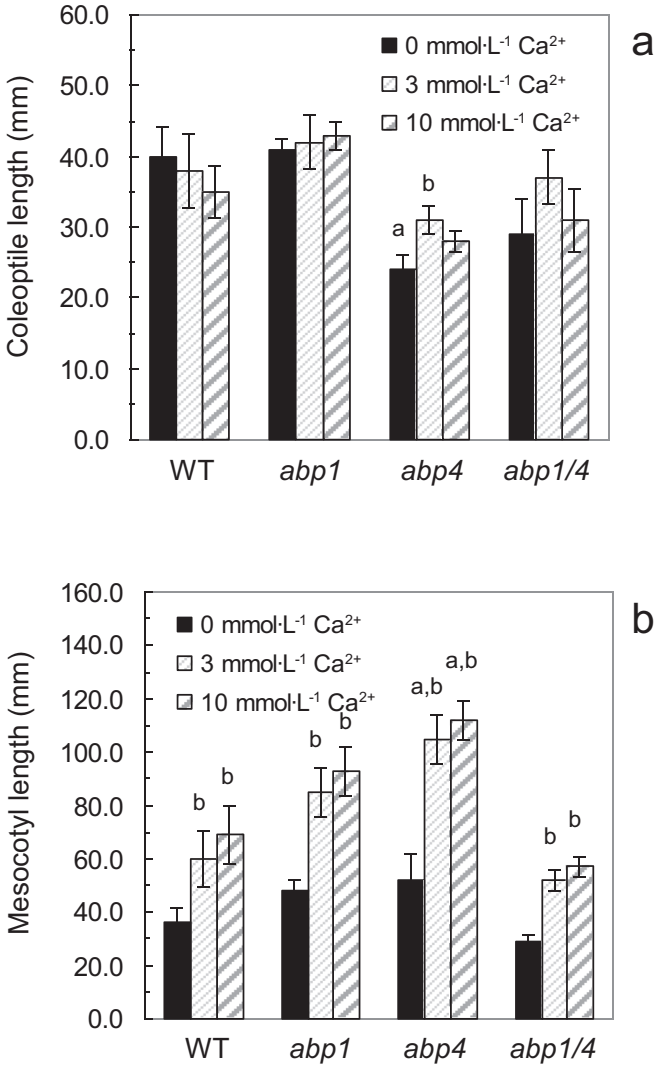
Calcium content in maize aerial parts (coleoptile and mesocotyl) of etiolated WT and *abp* mutant seedlings

The calcium content in maize aerial parts was determined in etiolated seedlings grown in conditions in vitro in medium without calcium or supplemented with 3 mmol·L^{-1} or 10 mmol·L^{-1} Ca^{2+}. In all genotypes, the Ca^{2+} content of the aerial parts of etiolated maize seedlings increased with the increase of the Ca^{2+} concentration in the medium. However, in the *abp4* mutant, significant increase of Ca^{2+} accumulation in the aerial parts of maize seedlings was observed in the presence of 10 mmol·L^{-1} Ca^{2+} (Fig. 2). Calcium accumulation in the mutant *abp1* or in double mutant was comparable with the calcium accumulation in WT plants (Fig. 2).

Search for *Arabidopsis* CAX1 homolog in maize in silico analyses of the sequences

To isolate and characterize the *AtCAX1* homolog in maize, NCBI and maize GDB databases were screened using the *AtCAX1* sequence (accession No. AF461691) as a reference. From this analysis, three sequences were identified in maize: *ZmHCX1* (accession No. AF256229), *ZCAX2* (accession No. AB044567), and *ZmPCO087284* (accession No. AY108295). The first sequence, *ZmHCX1*, was assigned to a CAX1-like protein sharing 65% of its identity with AtCAX1. The second sequence, *ZCAX2*, shares 72.4% of its identity with *Arabidopsis AtCAX6*. The third sequence, *ZmPCO087284*, encodes a protein sharing 71.8% similarity with *AtCAX3* from *Arabidopsis* and will be further reported as *ZCAX3*. Specific primers were designed to amplify the full length of *ZCAX3* and *ZCAX2* from our plant material (WT plants). *ZCAX3* is 1590 bp long, contains a 1389 bp open reading frame and encodes a 462 amino acid long protein with a calculated molecular mass of 48.9 kDa. *ZCAX2* is 1338 bp long, contains a 1260 bp open reading frame and encodes a protein containing 419 residues with a calculated molecular mass of 45.6 kDa. The maize sequences were introduced into an unrooted phylogenetic tree designed based on sequences from *Arabidopsis*, *Oryza sativa* L., and *Z. mays*. As described by Shigaki et al. (2006), the unrooted tree revealed a clustering of the sequences into two clades. The deeper analysis of the tree showed that *ZCAX3* is clustered into type IA together with *Arabidopsis AtCAX1* and *AtCAX3*, whereas *ZCAX2* was classified into type IB with other *CAXs* (Supplementary Fig. 1[1]). In addition, the analysis showed that on the basis of amino acid sequences, the *ZCAX3* gene is closely related to *OsCAX1a* from *O. sativa*, whereas *ZCAX2* is closely related to *OsCAX3*

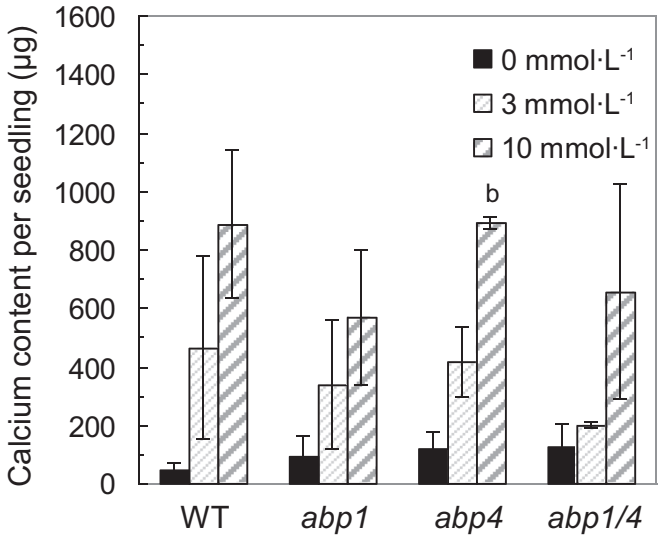
Fig. 1. Effect of exogenous Ca^{2+} on the growth of etiolated coleoptile (*a*) and mesocotyl (*b*) of WT and *abp* mutants. Maize seedlings were grown in vitro for 5 days on BM free of $CaCl_2$ or supplemented with $CaCl_2$ in concentrations of 3 or 10 mmol·L^{-1}. For each genotype, 5 to 10 seedlings were measured in each experiment. Values represent mean ± SE of 4 to 9 experiments. WT and *abp* mutants show a significant difference (Student's *t* test: $p \le 0.05$): a, significantly different from WT in the same concentration; b, significantly different from the control (0 mmol·L^{-1} Ca^{2+}) of the same genotype.



from the same species (Kamiya et al. 2005). The alignment of different CAXs shows that these proteins are highly conserved among monocots and dicots, except of the *N*-terminal domain of the protein (Supplementary Fig. 2[1]). The CaD, consisting of nine amino acids, was localized between the M1 and M2 spanning domains. Despite CaD itself was poorly conserved within the CAX family, the flanking regions were quite similar to each other (Supplementary Fig. 3*a*[1]). The prediction of the protein topology by TMHMM program (Krogh et al. 2001) determined that ZCAX2 and ZCAX3 contained 11 transmembrane domains with the long hydrophilic *N*-terminal part facing the cytosol (Supplementary Fig. 3*b*[1]). An acidic-amino-acid-rich region was found in the cyto-

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/cjb-2013-0262.

Fig. 2. Accumulation of calcium in the aerial part (coleoptile plus mesocotyl) of etiolated WT and *abp* mutant seedlings. Maize seedlings were grown in conditions in vitro for 5 days on BM free of $CaCl_2$ or supplemented with $CaCl_2$ in concentrations of 3 or 10 mmol·L^{-1}. Samples consisted of 4 to 11 seedlings collected from each calcium concentration. Calcium was determined by flame atomic absorption spectrometry using an acetylene-air flame at a wavelength of 422.7 nm. Values represent data ± SE from two independent experiments and are expressed as micrograms of Ca^{2+} per seedling. b, significantly different from the control (0 mmol·L^{-1} Ca^{2+}) of the same genotype (Student's *t* test: $p \leq 0.05$).



solic loop between the sixth and seventh transmembrane domain of both *CAX* candidates (Supplementary Fig. 2[1]).

Calcium-dependent expression of *ZCAX2* and *ZCAX3* in *abp* mutants

Because different responses to exogenous Ca^{2+} were observed among genotypes, the expression of *ZCAX2* and *ZCAX3* was investigated in coleoptiles and mesocotyls as a function of the Ca^{2+} supply (10 mmol·L^{-1}). The expression of *ZCAX2* and *ZCAX3* was investigated by semiquantitative RT–PCR and by qRT–PCR, respectively. The analysis of the *ZCAX2* expression did not show any significant difference between the gene expression in the absence or presence of Ca^{2+}, or in WT and *abp* mutants (data not shown); therefore, only the data on the *ZCAX3* gene expression are presented here.

In Ca^{2+}-free medium, the accumulation of *ZCAX3* transcripts in etiolated coleoptile did not differ between the WT and all *abp* mutants (Fig. 3*a*). Addition of Ca^{2+} (10 mmol·L^{-1}) to the culture medium did not significantly affect the expression of *ZCAX3* in WT and in all *abp* mutants (Fig. 3*b*). The expression of *ZCAX3* in the etiolated mesocotyl developed on calcium-free medium did not differ between the WT and *abp1* mutant but it was significantly lower in the *abp4* and in *double* mutant (Fig. 4*a*). Calcium (10 mmol·L^{-1}) slightly increased the expression of *ZCAX3* in mesocotyls of WT and *abp1*mutant, but it significantly up-regulated the expression in *abp4* and *abp1*/*abp4* mutants (Fig. 4*b*).

Auxin-dependent expression of *ZCAX2* and *ZCAX3* in *abp* mutants

Similar to the case of experiments with calcium, the analysis of the *ZCAX2* expression in the response to NAA was inconclusive (data not shown). Therefore, only the data on the *ZCAX3* gene expression are presented here.

We recently showed that *abp* mutants are significantly less sensitive than WT seedlings to the inhibitory effect of exogenous

Fig. 3. Expression of *ZCAX3* gene in etiolated coleoptiles of WT and *abp* mutants in the absence of Ca^{2+} (*a*) or in the presence (*b*) of Ca^{2+}. WT and mutant seedlings were developed in darkness, and on BM free of $CaCl_2$ (control) or supplemented with 10 mmol·L^{-1} $CaCl_2$. In 5-day-old seedlings, *ZCAX3* expression analysis was performed by qRT–PCR. Results are expressed relatively to the transcript accumulation in WT plants developed in the absence of calcium (0 mmol·L^{-1} $CaCl_2$). The *GAPDH* was used as housekeeping gene. The values represent the average ± SE of the data obtained from three independent experiments in each condition and genotype.

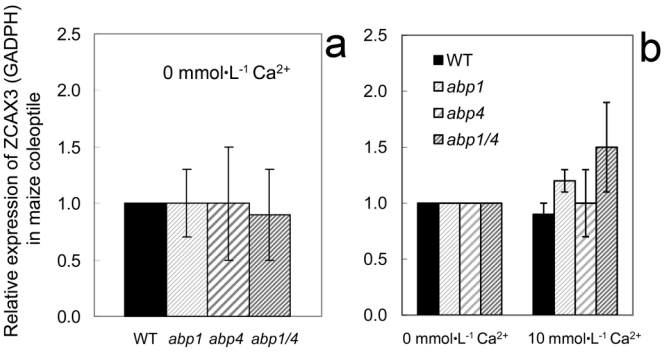
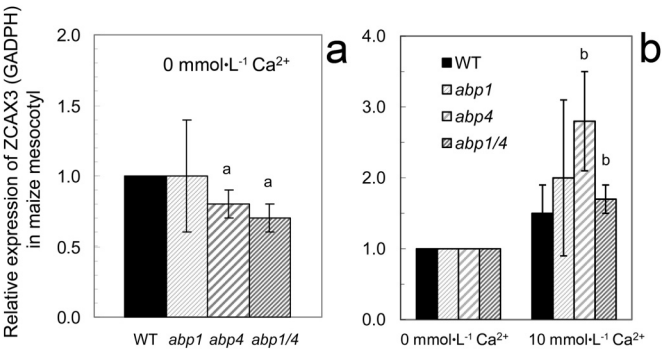
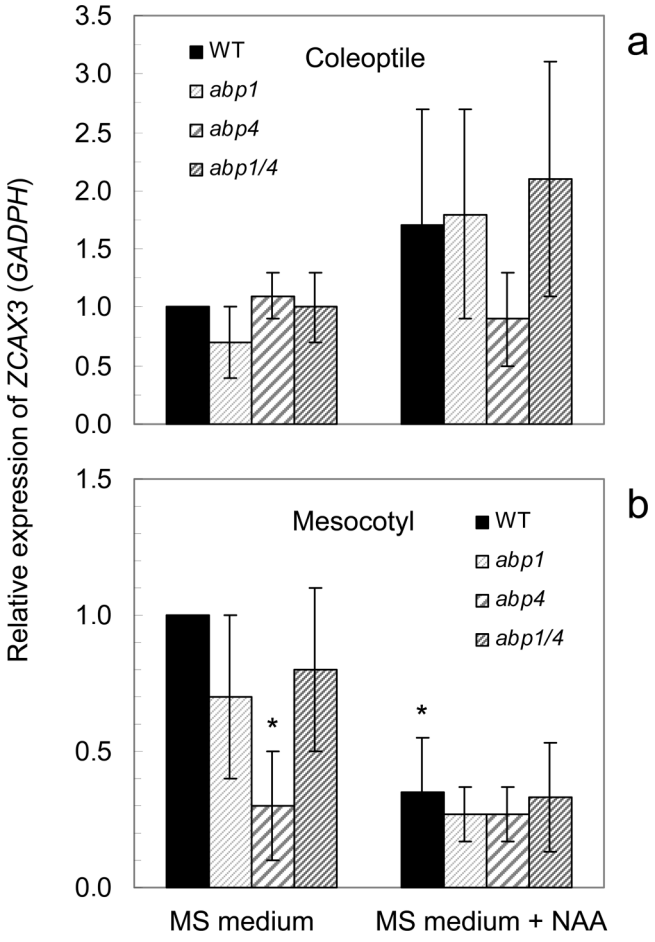


Fig. 4. Expression of *ZCAX3* gene in etiolated mesocotyls of WT and *abp* mutants in the absence of Ca^{2+} (*a*) or in the presence (*b*) of Ca^{2+}. WT and mutant seedlings were developed in darkness, and on BM free of $CaCl_2$ (control) or supplemented with 10 mmol·L^{-1} $CaCl_2$. In 5-day-old seedlings, *ZCAX3* expression analysis was performed by qRT–PCR. Results are expressed relatively to the transcript accumulation in WT plants developed in the absence of calcium (0 mmol·L^{-1} $CaCl_2$). The *GAPDH* gene was used as housekeeping gene. The values represent the average ± SE of the data obtained from three independent experiments in each condition and genotype. a, significantly different from WT (Student's *t* test: $p \leq 0.05$); b, significantly different from the same genotype at 0 mmol·L^{-1} Ca^{2+} (Student's *t* test: $p \leq 0.05$).



auxin (Jurišić-Knežev et al. 2012). The expression of *ZCAX3* in etiolated coleoptile and mesocotyl was investigated as a function of exogenous auxin (50 µmol·L^{-1} NAA). On the BM containing 3 mmol·L^{-1} Ca^{2+}, the transcript accumulation of *ZCAX3* in coleoptile did not differ between WT and *abp* mutants. Auxin treatment did not significantly alter the expression of the gene neither in coleoptile of WT nor in coleoptile of *abp* mutants (Fig. 5*a*). In the absence of NAA, the expression of *ZCAX3* in the etiolated mesocotyl was significantly lower in the *abp4* mutant compared with the WT, but not in *abp1* nor in double mutants (Fig. 5*b*). NAA significantly reduced the expression of *ZCAX3* in WT mesocotyl, and slightly in *abp1* and double mutant. However, in the *abp4* mutant the *ZCAX3* expression in the mesocotyl was comparable in the presence or absence of NAA (Fig. 5*b*).

Fig. 5. Auxin-induced expression of *ZCAX3* gene in etiolated coleoptile (*a*) and mesocotyl (*b*) of WT and *abp* mutants. WT and mutant plants were grown in darkness, and on BM in the absence or presence of 50 μmol·L^{-1} NAA. *ZCAX3* expression analysis was performed by qRT–PCR. Results are expressed relatively to the transcript accumulation in WT plants developed in the absence of NAA. The *GAPDH* gene was used as housekeeping gene. The values represent the average of the data obtained from three independent experiments in each condition and genotype. *, significantly different from WT in the absence of NAA (Student's *t* test: $p \leq 0.05$).



Discussion

It has been shown that calcium and auxin signaling cross talk in plant growth and development and it was proposed that ABP1 is involved in AtCAX1-mediated calcium transport (Shishova and Lindberg 2010). Using maize *ABP1* and *ABP4* loss-of-function mutants, we investigated the role of these two genes in Ca^{2+}/auxin-regulated growth of maize seedlings and in the regulation of expression of the two identified maize CAX genes as a function of Ca^{2+} and auxin. Our results first indicate that in the absence of exogenous calcium, ABP4 is positively involved in the elongation of etiolated coleoptile, whereas the loss of ABP4 does not affect mesocotyl growth. Our data further indicates that maize coleoptiles and mesocotyls show different requirements of exogenous Ca^{2+}, and that in both organs calcium and ABP4 signaling somehow interact with each other. Yamagami et al. (2004) proposed the existence of two signaling pathways participating in auxin-induced internode growth in pea: an ABP1-independent pathway, which depends on exogenous Ca^{2+} and an ABP1-dependent pathway independent of extracelullar Ca^{2+}. Here we show that the Ca^{2+}-regulated growth in etiolated maize seedlings involves ABP4.

To date, several Ca^{2+}/H^+ antiporters have been characterized at the biochemical and molecular level in different plant species. In *Arabidopsis* and in rice (*O. sativa*), six *CAX* genes and five *CAX*-homologs have been isolated, respectively (Shigaki et al. 2006; Kamiya et al. 2005). Using PCR strategy, we amplified two maize sequences, *ZCAX3* (accession No. AY108295) and *ZCAX2* (accession No. AB044567) homologous to *Arabidopsis AtCAX1*. The maize sequences encoded proteins sharing common features with the already known CAXs of various organisms (Ivey et al. 1993; Hirschi et al. 1996; Ueoka-Nakanishi et al. 1999; Kamiya and Maeshima 2004). Evolutional relationships among the characterized and uncharacterized CAXs can predict their potential roles. Based on their amino acid composition and substrate specificity, characterized and uncharacterized CAX sequences were described to cluster into two groups: the type 1A and the type 1B (Shigaki et al. 2006; Kamiya et al. 2005). The introduction of the two maize CAX sequences did not change the original phylogenetic tree and revealed that (*i*) *ZCAX3* belongs to type IA of CAXs, regrouping vacuolar Ca^{2+} transporters (Kamiya et al. 2005) and (*ii*) *ZCAX2* belongs to type IB of CAXs with its closest relative being rice OsCAX3, a vacuolar transporter responsible both for Ca^{2+} and Mn^{2+} transport (Kamiya et al. 2005). Nevertheless, solely based on the sequence similarity, physiological function of ZCAX2 and ZCAX3 cannot be ascertained based solely on sequence similarity.

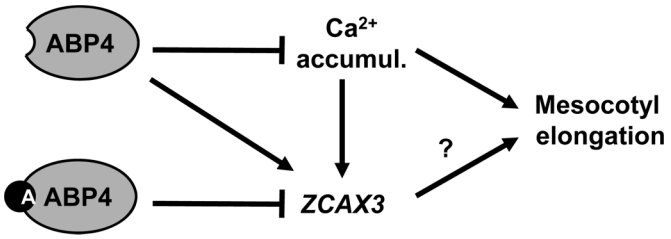
For example, complementation tests using yeast strains deficient in Ca^{2+} transport have to be performed to determine the substrate specificity of these two proteins.

So far, most of the characterized eukaryotic CAXs have been localized in the tonoplast, though some CAXs are also localized on the plasma membrane and the chloroplast thylakoid membrane (for review, see Manohar et al. 2011). In this study using the TmPred program (Hofmann and Stoffel 1993), we predicted that the preferred model for the *N* terminus localization for both *ZCAX3* and *ZCAX2* was a cytosolic localization similarly to AtCAX1 for which *N*-terminal regulatory region and the CaD are predicted to be located on opposite sides of the membrane (Shigaki et al. 2001). This suggests that the *N* terminus of the protein is cytosolic and that the CaD would be found inside the vacuole. Nevertheless, we do not have any evidence that ZCAX3 and ZCAX2 are tonoplastic proteins and a green fluorescent protein (or yellow fluorescent protein) fusion approach needs to be performed to determine their localization.

To study the possible relationship between the identified *ZCAXs* and ABPs, the expression of the *ZCAX2* and *ZCAX3* genes in the *abp* mutant was analyzed as a function of exogenous Ca^{2+} or auxin. However, since the results obtained in the three independent experiments did not show significant differences in the expression of *ZCAX2* in the absence or presence of Ca^{2+} and between WT and *abp* mutants, we therefore focused our attention only on the *ZCAX3* gene. Our results revealed that at calcium deficiency, the functional *ABP4* is partially needed for *ZCAX3* expression in etiolated mesocotyl. In addition, the distinct down-regulation of *ZCAX3* in *abp4* mutant in the presence of 3 mmol·L^{-1} Ca^{2+} (i.e., calcium content in the basal MS medium, Fig. 5*b*) and in opposite, the up-regulation of *ZCAX3* in *abp4* at 10 mmol·L^{-1} Ca^{2+} (Fig. 4*b*) may indicate the existence of a feedback in which high exogenous Ca^{2+} restrains the expression of *ZCAX3* via ABP4. The expression data are consistent with our previous conclusion that in etiolated maize seedlings, ABP4 may play a role in Ca^{2+} signaling. However, our data also suggest that the calcium accumulation observed in maize aerial parts is not likely mediated by ZCAX3.

Our results further indicate that in etiolated mesocotyls, functional ABP4 stimulates *ZCAX3* expression in the absence of auxin, whilst it mediates the inhibitory effect of NAA on *ZCAX3* expression. This is in agreement with our previous reports showing that the mesocotyl of etiolated maize seedlings knocked-out in *ABP4* gene or with reduced *ABP4* expression showed impaired sensitiv-

Fig. 6. Working model representing the cross talk between ABP, Ca^{2+}, and *ZCAX3* during elongation of etiolated maize mesocotyl in the absence or presence of exogenous auxin. The model shows that in the absence of exogenous auxin, the functional ABP4 regulates the expression of *ZCAX3* either directly or via calcium accumulation. The accumulation of Ca^{2+} promotes mesocotyl elongation. We further propose that the binding of NAA (A) to the ABP4 results in the suppression of *ZCAX3* gene expression. Our results do not allow the description of the role of ZCAX3 in mesocotyl elongation. Arrows and T-bars represent positive and negative effects, respectively.



ity to the inhibitory effect of exogenous auxin (Čudejková et al. 2012; Juršić-Knežev et al. 2012).

We previously reported that in comparison with old maize hybrid 307, expression of *ABP4* in etiolated mesocotyls is strongly reduced in the modern hybrid 3394 (Fellner et al. 2006). Moreover, in the old hybrid 307, but not in the modern hybrid 3394, the sensitivity of mesocotyl to exogenous auxin increased with the elevation of the calcium concentration in the growth medium (Supplementary Fig. 4[1]). These results on maize hybrids support the role of *ABP4* in the interaction auxin/Ca^{2+}. Moreover, they are in agreement with data of Shishova and Lindberg (2004) and Yang and Poovaiah (2000), who reported that higher concentrations of Ca^{2+} influence cell sensitivity to auxin and auxin-mediated signaling, respectively. The importance of a high external concentration of Ca^{2+} for an auxin-dependent reaction was also shown in experiments with tomato root hairs (Ayling and Clarkson 1996).

In conclusion, we provided evidence for a cross talk between *ABP4*, exogenous auxin, Ca^{2+}, and ZCAX3 during growth of etiolated maize mesocotyl. A working model of the interaction is shown in Fig. 6. The model proposes that, in the absence of exogenous auxin, functional ABP4 regulates expression of *ZCAX3* either directly or via calcium accumulation. Increased accumulation of Ca^{2+} promotes mesocotyl elongation. It is further proposed that binding of NAA (A in Fig. 6) to the ABP4 results in suppression of *ZCAX3* gene expression. Our results do not allow describing the role of ZCAX3 in mesocotyl elongation. Therefore, our future project is focused on localization of ZCAX3 in maize cell and determination of its function in mesocotyl growth.

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