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# Regulation of wound-responsive calcium-dependent protein kinase from maize (ZmCPK11) by phosphatidic acid

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**In plant cells, phospholipids are not only membrane components but also act as second messengers interacting with various proteins and regulating diverse cellular processes, including stress signal transduction. Here, we report studies on the effects of various phospholipids on the activity and expression of maize wound-responsive calcium-dependent protein kinase (ZmCPK11). Our results revealed that in leaves treated with n-butanol, a potent inhibitor of phosphatidic acid (PA) synthesis catalyzed by phospholipase D, a significant decrease of ZmCPK11 activity was observed, indicating contribution of PA in the kinase activation. Using lipid binding assays, we demonstrate that among various phospholipids only saturated acyl species (16:0 and 18:0) of phosphatidic acid are able to bind to ZmCPK11. Saturated acyl species of PA are also able to stimulate phosphorylation of exogenous substrates by ZmCPK11 and autophosphorylation of the kinase. The level of ZmCPK11 autophosphorylation is correlated with its enzymatic activity. RT-PCR analysis showed that transcript level of *ZmCPK11* in maize leaves increased in response to PA treatment. The influence of PA on the activity and transcript level of ZmCPK11 suggests an involvement of this kinase in a PA-mediated wound signal transduction pathway.**

**Keywords:** phospholipids, phosphatidic acid, calcium-dependent protein kinases, abiotic/wound stress signal transduction, *Zea mays*

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## INTRODUCTION

Phospholipids play an important role during growth, development and survival of plants. Phospholipids are not only essential components of membranes, but also crucial second messengers taking part in signal transduction. The large diversity of phospholipids synthesized and modified by various enzymes such as phospholipases, lipid kinases and phosphatases enables precise spatial and temporal regulation of production of specific lipid mediators in response to developmental changes and environmental conditions. Among the various phospholipids, phosphatidic acid (PA) is the most extensively studied. PA is a minor component of the membranes, representing only 1–2% of the total phospholipid pool of the cell. This phospholipid, synthesized quickly and transiently in response to various stimuli, is a perfect second messenger (Munnik, 2001). The cellular level of PA changes rapidly under various developmental conditions including plant growth, seed germination, leaf senescence, expansion of pollen tubes, and also in response to biotic and abiotic stresses and abscisic acid (ABA) treatment (Wang *et al.*, 2006b; Xue *et al.*, 2009). In response to numerous signals PA is produced from different phospholipid precursors by two pathways, one involving phospholipase D (PLD), and the second phospholipase C (PLC) and diacylglycerol kinase (DGK) (Munnik & Musgrave, 2001). Which pathway is activated depends on the particular signal, i.e., oxidative stress, ABA treatment and wounding activate PLD, whereas pathogen elicitors activate mainly PLC-DGK (de Jong *et al.*, 2004; Arisz *et al.*, 2009). Although many studies indicate that PA is involved in plant responses to different stresses, little is known about how PA works. Many modes of PA action are possible, but recruitment of interacting proteins to the plasma membrane and direct influence on the protein activity are the most likely (Testerink & Munnik, 2005). During the last decade, numerous plant proteins have been indicated as potential PA targets (Testerink *et al.*, 2004), but only few of them are proven to be PA targets *in vivo*. One of them is protein phosphatase 2C named ABI1, a negative regulator of ABA signaling, which is recruited to the membrane and inhibited by direct interaction with PA. In an ABI1 mutant which was unable to bind PA, translocation to the membrane was not observed, suggesting a role of PA in membrane trafficking in response to ABA (Zhang *et al.*, 2004). Another protein participating in ABA signaling in *Arabidopsis* identified as a direct target of PA is NADPH oxidase RbohD (respiratory burst oxidase homolog D). Interaction of RbohD with PA resulted in stimulation of NADPH oxidase activity which is essential for ABA-mediated ROS (reactive oxygen species) production and stomatal closure (Zhang *et al.*, 2009). AtPDK1, a protein kinase involved in root growth, also binds PA and its activity as well as the activity of its *in vivo* substrate (AGC2-1 kinase) are stimulated by PA (Anthony *et al.*, 2004; 2006). It has been reported that *in vitro* protein phosphorylation by calcium-dependent protein kinase (CDPK) from oat (*Avena sativa*) can be enhanced by crude lipid extract (Schaller *et al.*, 1992). Moreover, the activity of AtCPK1 from *Arabidopsis* can be enhanced by lysophosphatidylcholine (LysoPC), phosphatidylinositol (PI), or phos-

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**Abbreviations:** ABA, abscisic acid; BSA, bovine serum albumin; CDPK, calcium-dependent protein kinase; CLD, calmodulin-like domain; DAG, diacylglycerol; DGK, diacylglycerol kinase; GST, glutathione S-transferase; JD, junction domain; LysoPA, lysophosphatidic acid; MBP, myelin basic protein; PA, phosphatidic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PLC, phospholipase C; PLD, phospholipase D; PS, phosphatidylserine.

phatidylserine (PS) (Harper *et al.*, 1993), and activity of DcCPK1 from carrot (*Daucus carota)* can be enhanced by PS, PI and phosphatidylethanolamine (PE) (Farmer & Choi, 1999). Our previous studies demonstrated that the activity of one of maize CDPKs (ZmCPK11) was stimulated *in vitro* by PS, PI and PA (Szczegielniak *et al.*, 2000; 2005; Klimecka & Muszynska, 2007).

CDPKs are found only in plants and protists (Harmon *et al.*, 2000; Harper *et al.*, 2004), and are encoded by multigene families (Li *et al.*, 2008b). CDPKs consist of five domains: an N-terminal variable domain, a catalytic domain (CD), a junction domain (JD), a calmodulin-like domain (CLD), and a C-terminal domain. The N- and C-terminal domains are variable, differing in length and amino-acid composition. It has been suggested that these variable domains are responsible for the specific functions of individual CDPKs (Harmon *et al.*, 1994; Harper *et al.*, 1994). Because the activity of these protein kinases is strictly regulated by calcium ions, CDPKs are often considered as calcium sensors that can decode specific changes of cytoplasmic calcium ion concentration during growth and development of the plant as well as in response to various stresses, i.e., cold, drought, high salinity, wounding or pathogen attack (Knight & Knight, 2001; Ludwig *et al.*, 2004; Li *et al.*, 2008a).

We found that ZmCPK11 is involved in plant response to wounding (Szczegielniak *et al.*, 2005). The activity of ZmCPK11 increased within minutes after wounding, while induction of *ZmCPK11* expression in maize leaves was observed 3 h after wounding. Since it is known that wounding induces the synthesis of PA (Ryu & Wang, 1996) we decided to search for the possible role of PA in the regulation of ZmCPK11 in the wound signal transduction pathway. To reach this goal, we investigated the *in vivo* effects of PA on ZmCPK11 activity and its transcript level. Moreover, to find out whether the stimulation of ZmCPK11 activity is caused by a direct interaction of ZmCPK11 with PA or other phospholipids, lipid binding assay was performed.

## MATERIALS AND METHODS

**Reagents**. All reagents were of analytical grade. Glutathione-agarose beads, Hyperfilm MP, and Hybond-C membrane were from Amersham-Pharmacia Biotech (Sweden). BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate and *p*-nitroblue tetrazolium chloride), pGEM-T Easy vector and dNTPs were from Promega (USA). *Taq* DNA polymerase and molecular mass markers were from Fermentas International, Inc. (Canada). *Pfu* polymerase and QuikChange II site-directed mutagenesis kit were from Stratagene (USA). Primers were synthesized in the Institute of Biochemistry and Biophysics PAS (Poland). Anti-c-Myc antibodies (9E10) were from Santa Cruz Biotechnology (USA) and specific antibodies raised in rabbits against the C-terminal part of the calmodulin-like domain of ZmCPK11 (CMMRKGNAGATGRRTM) were ordered in BioGenes (Germany). PVDF was from Millipore (USA). [$\gamma$-$^{32}$P]ATP was from Hartmann Analytic (Germany). Proteinase inhibitor cocktail (Complete) was from Roche (Germany). Tri Reagent was from Molecular Research Center Inc. (USA). Enterokinase was from Novagen (USA). All phospholipids: PC — 8:0 (1,2-dioctanoyl-*sn*-glycero-3-phosphocholine); PA — 8:0 (1,2-dioctanoyl-*sn*-glycero-3-phosphate, monosodium salt); PA 16:0 (1,2-dipalmitoyl-*sn*-glycero-3-phosphate, monosodium salt); PA — 18:0 (1,2-distearoyl-*sn*-glycero-3-phosphate, monosodium salt); PA — 18:1 (1,2-dioleoyl-*sn*-glycero-3-phosphate, monosodium salt); PC — 18:1 (1,2-dioleoyl-*sn*-glycero-3-phosphocholine); PA — mixture of acyl species, from soybean (1,2-diacyl-*sn*-glycero-3-phosphate, monosodium salt,); PS — mixture of acyl species, from bovine brain (L-α-phosphatidyl-L-serine); PI — mixture of acyl species, from soybean (L-α-phosphatidylinositol sodium salt) were from Avanti Polar Lipids (USA). HSRT 100 kit and all basic chemicals were from Sigma Aldrich (Germany).

**Plant material.** Maize seeds (*Zea mays*, inbred line Rd 17-25, kindly provided by Dr. Roman Warzecha from the Plant Breeding and Acclimatization Institute, Radzikow, Poland) were soaked in water overnight at room temperature. Then, seeds were germinated and seedlings were grown at 26 °C on a mixture of vermiculite, perlite and soil in a growth chamber with a cycle of 14 h day (70–80 W/m$^2$)/10 h night. For PA and PC treatments, second leaves of 2-week-old plants were detached, preincubated overnight in water and then treated with 0.25 mM solutions of short chain, as appropriate for plant treatment, synthetic PA (8:0) or PC (8:0). At indicated time points the leaves were collected and frozen in liquid nitrogen. For n- or s-butanol treatment, leaves were detached and preincubated overnight in water. Next day the leaves were treated with 0.1% solution of n-butanol (or s-butanol as a control) and after 30 min were wounded (by rubbing the lamina of leaves with sandpaper). Fifteen minutes after the wounding the leaves were frozen in liquid nitrogen and stored at –80 °C.

**Expression and purification of recombinant ZmCPK11.** Expression and purification of recombinant ZmCPK11 was as described before (Szczegielniak *et al.*, 2005), with minor changes. ZmCPK11 was liberated from GST-ZmCPK11 immobilized on glutathione-agarose beads using enterokinase which recognized the amino-acid sequence DDDK introduced to the construct between GST and ZmCPK11. The cleavage was carried out overnight at 4 °C on a rocker, using 1 U of enterokinase per 50 μg of GST-ZmCPK11 in a buffer containing 20 mM Tris, pH 7.5 and 50 mM NaCl. Free ZmCPK11 was separated from GST-bound agarose beads by a short spin and immediately frozen in liquid nitrogen (for lipid binding assay) or mixed with glycerol added to a final concentration of 15% and then frozen (for activity assays).

**Preparation of *ZmCPK11* constructs for protoplast transient expression assay.** For protoplast transient expression assay pBlue-LNU vector (kindly provided by Professor Dirk Becker, University of Hannover) containing the *UBI* promoter was used. To amplify *c-Myc* from the pUC-Myc template, two *c-Myc* gene-specific primers, 5'-CCTAAGCTTGTTCACGGGGGATCCTCTAGA-3' and 5'-ACGCTCGAGTCATGTCGGCATATTCGAGGA-3' introducing *Hin*dIII and *Xho*I sites, respectively, were used. The amplified *c-Myc* was inserted in the pBlue-LNU vector. Two *ZmCPK11* gene-specific primers, 5'-TACTAGTATGCAGCCGGACCCGAGCGGGAAC-3' and 5'-AGGAAGCTTGGTTTTGCTGGGATTCAAGAG-3' were designed to introduce *Spe*I and *Hin*dIII sites, respectively, to amplify full-length *ZmCPK11* (accession no. AY301062) from pGEM-T Easy as previously described (Szczegielniak *et al.*, 2005). After digestion with *Spe*I and *Hin*dIII, the PCR product was inserted in the pBlue-LNU vector. The construct carrying a mutated inactive form of *ZmCPK11* (K74M) was obtained by site-directed mutagenesis with the QuikChange II

site-directed mutagenesis kit using the pGEM-T Easy-*ZmCPK11* construct as a template. At the C-terminus of *c-Myc* within the pBlue-LNU vector, various forms of *ZmCPK11* were fused. For amplification of the CD together with the N-terminal domain (called N+CD), primers 5'-GCGAAGCTTATCGTCAACAATCCATGC-GTGAC-3' (forward) and 5'-CACTAGTATGTACCG-CATCGGCAAGAAACTGG-3' (reverse), for amplification of the CLD together with the JD and C-terminal domain (called JD+CLD+C) primers: 5'-ACTAGTAT-GGTGGCACCTGATAAG-3' (forward) and 5'-AGAA-GCTTGGTTTTGCTGGGATT-3' (reverse) introducing *Spe*I and *Hin*dIII sites were used, respectively. In amplifications of all constructs *Pfu* polymerase was used. The correctness of the constructs' sequences was confirmed by DNA sequencing.

**Protoplast transient expression assay.** The preparation of maize leaf mesophyll protoplasts and transformation by electroporation was performed according to the protocol described by (Sheen, 1993). In each electroporation, about $2 \times 10^5$ protoplasts were transfected with 30 μg of plasmid DNA (containing various constructs of *c-Myc-ZmCPK11* or with *c-Myc* alone, as described above). The transfected protoplasts were incubated at 22 °C for 16 h, collected and frozen at –80 °C until further analysis. Expression of proteins was determined by immunoblotting and the activity of expressed ZmCPK11 was analyzed by an in-gel kinase activity assay.

**Immunoblot analysis.** At indicated time points after transformation with various constructs, proteins from maize mesophyll protoplasts were separated on 10% SDS/polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) membranes by electroblotting. The membranes were blocked for 2 h in room temperature in TBST buffer (10 mM Tris, 7.5, 100 mM NaCl, 0.1% Tween 20) containing 5% dry milk and then incubated for 1 h at room temp. with mouse anti-c-Myc antibodies in 1:2000 dilution. After extensive washing with TBST the blots were incubated with anti-mouse secondary antibodies conjugated with alkaline phosphatase for 1 h at room temp. After washing with TBST c-Myc-tagged proteins were visualized using BCIP/NBT.

**In-gel kinase assay.** Protein kinase activity was detected in maize protoplasts and leaves. At indicated time points after transformation with *c-Myc-ZmCPK11* constructs proteins from maize mesophyll protoplasts were separated on 10% SDS/polyacrylamide gels with immobilized 0.25 mg/ml MBP (myelin basic protein) as a substrate. The in-gel kinase assay was carried out as described later in this paragraph.

Maize leaves were ground in liquid nitrogen. Proteins were extracted with 200 μl of extraction buffer (50 mM Tris, pH 7.5, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 0.1 mM $Na_3VO_4$, 2 mM DTT, 500 μM PMSF, 0.5 mg/ml leupeptin, 1 mg/ml aprotinin, 0.7 mg/ml pepstatin). Suspensions were centrifuged at $20\,000 \times g$ for 30 min at 4 °C, and proteins of the supernatant (15–30 μg/lane) were separated on a 10% SDS/polyacrylamide gel with immobilized MBP.

After electrophoresis the gels were washed three times for 30 min at room temp. using buffer containing 25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM $Na_2VO_4$, 5 mM NaF, 0.5 mg/ml BSA, and 0.1% Triton X-100 (v/v). Next, the gels were washed overnight with three portions of buffer containing 25 mM Tris, pH 7.5, 5 mM NaF, 1 mM DTT, 0.1 mM $Na_2Vo_4$. After 30 min of preincubation in a buffer containing 50 mM Tris, pH 7.5, 15 mM $MgCl_2$, and 332 μM calcium acetate or 2 mM EGTA, kinase activity was assayed by addition of 0.05 mM ATP and 50 μCi of [γ-$^{32}$P]ATP for 90 min at room temp. After incubation unincorporated [γ$^{32}$P]ATP was removed by washing the gels with 5% TCA containing 1% sodium phosphate. After washing the gels were stained with Coomassie Brilliant Blue R250, dried and exposed to Amersham Hyperfilm MP. Markers of molecular mass were used to estimate the size of proteins.

***In vitro* protein kinase assay.** Approximately 1.5 μg of recombinant ZmCPK11 was incubated without any exogenous substrate (ZmCPK11 autophosphorylation) or with 6 μg of MBP in a buffer containing 20 mM Tris, pH 7.5, 0.1 mM EGTA, 0.11 mM calcium acetate, 5 mM $MgCl_2$, 0.05 mM ATP supplemented with 1 μCi of [γ$^{32}$P]ATP in the absence or presence of synthetic phospholipids at concentrations in the range of 0.25–1.0 μg/μl. The reaction was carried out for 8 min at 30 °C and was terminated by adding loading buffer and heating at 95 °C for 5 min. Proteins were separated on SDS/PAGE, then gels were stained with Coomassie Brilliant Blue R250, dried and exposed to autoradiography (Amersham Hyperfilm MP). Markers of molecular mass were used to estimate the size of analyzed proteins.

**Handling of lipids.** For lipid binding assay phospholipids were dissolved in chloroform (stock solutions 10–50 mg/ml) without heating (unsaturated lipids) or with heating above their transition temperature (saturated lipids), sealed tightly in glass test-tubes and stored at –20 °C.

For ZmCPK11 activity assay, appropriate amounts of each lipid (PA 8:0 or PC 8:0) were dried under a stream of nitrogen, and then 20 mM Tris, pH 7.5, was added and the mixture was sonicated for 5 min above the transition temperature of a particular lipid.

**Lipid binding assay.** Lipid binding assay was performed as described previously (Stevenson *et al.*, 1998) with minor modifications. Phospholipids (5 μg, or as indicated in Results) were spotted onto Hybond-C membrane and dried at room temp. for 1 h. Then, the membranes were blocked with 3% lipid-free BSA in TBST (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 4 h at room temp. and incubated overnight at 4 °C on a rocker with ZmCPK11 expressed in bacteria or with various forms of c-Myc-ZmCPK11 or c-Myc protein alone expressed in protoplasts. The incubation of ZmCPK11 or its fragments with phospholipids was carried out in a buffer composed of 50 mM Tris, pH 7.5, and containing proteinase inhibitor cocktail (Complete). After extensive washing with TBST, the membranes were incubated with specific antibodies for 1 h at room temp. in TBST containing 3% lipid-free BSA. Antibodies raised in rabbits against the C-terminal part of CLD of ZmCPK11 (CMMRKG-NAGATGRRTM) at the dilution of 1:1000 were used for detection of ZmCPK11 expressed in bacteria. Unfortunately, these antibodies were unable to recognize native or transiently expressed kinase, most probably due to the low concentration of the kinase, therefore for proteins expressed in protoplasts, mouse anti-c-Myc antibodies (1:2000) were used. After incubation with antibodies, membranes were washed in TBST (3 times for 5 min each) and incubated with secondary antibodies conjugated with alkaline phosphatase (anti-rabbit (1:2000) for ZmCPK11 expressed in bacteria or anti-mouse (1:2000) for c-Myc-ZmCPK11 expressed in protoplasts) in TBST containing 3% lipid-free BSA. After washing with TBST, proteins bound to the lipids

on the membrane and recognized by antibodies were visualized by reaction with BCIP/NBT.

**RT-PCR.** RT-PCR analysis was carried out as described earlier (Szczegielniak *et al.*, 2005), using total RNA isolated with Tri Reagent from maize leaves following the manufacturer's directions. Two micrograms of proper quality RNA was reverse-transcribed for 60 min at 47 °C in 20 µl of reaction mixture containing 1 unit of enhanced avian reverse transcriptase, 500 µM each dNTP, 3.5 µM anchored oligo(dT) primer, and 1 unit of RNase inhibitor (HSRT 100 kit). One microliter of the reverse transcription reaction was used for PCR in 20 µl of reaction mix containing 0.4 units of *Taq* DNA polymerase, 200 µM each dNTP, 1.5 mM $MgCl_2$, and 625 nM of the appropriate primers: for amplification of a 1296 bp *ZmCPK11* fragment 5'-CCAATGCAGCCG-GACCCGAGC-3' (forward), 5'-TTTGTCAAAAAAC-GAGAATGCTGAAACCAA-3' (reverse), for actin gene amplification 5'-GGCAGCTCGTAGCTCTTCTC-3' (forward), 5'-AACAGGGAGAAGATGACCCA-3' (reverse) (Chang *et al.*, 1999). Routine PCR conditions were: 3 min, 94 °C (first cycle); 30 s, 94 °C; 30 s, 55 °C; 1.5 min, 72 °C (25 cycles for actin and 30 cycles for ZmCPK11); and 10 min, 72 °C (final cycle).

## RESULTS

In order to establish if PA is involved in ZmCPK11 activation *in vivo*, activity of the kinase was monitored in leaves of 2-week-old maize plants treated with a solutions of PA or PC. PC, a structural phospholipid which *in vitro* does not influence the enzymatic activity of ZmCPK11 was used as a control. The activity of ZmCPK11 was determined at indicated time points by in-gel kinase assay (Fig. 1A). A maximal increase of ZmCPK11 activity was observed after 15 min of PA treatment. An increase of ZmCPK11 activity was also observed in leaves treated with PC, but the maximum activity was reached later, after 30 min of PC treatment. Only very weak phosphorylation of MBP by calcium independent protein kinases (most probably MAPK) was observed in the presence of EGTA after either treatment.

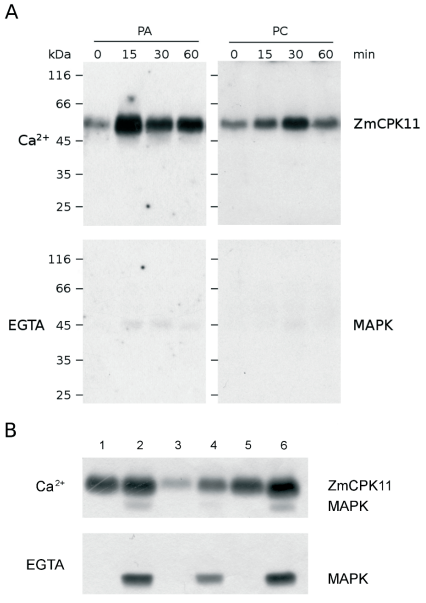


**Figure 1. Effect of treatment of maize leaves with PA, PC and n-butanol on endogenous ZmCPK11 activity.**
**(A)** Activity of ZmCPK11 in leaves treated at indicated time with 0.25 mM solution of PA (8:0) or PC (8:0); **(B)** Activity of ZmCPK11 in unwounded (lanes 1, 3, 5) and 15 min after wounding (lanes 2, 4, 6), non-treated (lanes 1, 2), treated for 30 min with 0.1% *n*-butanol (lanes 3, 4) or 0.1% **s**-butanol (lanes 5, 6); Activity of ZmCPK11 was determined in 2-week-old maize plant leaves by in-gel kinase assay using MBP as a substrate in the presence of 0.332 µM calcium ions or 2 mM EGTA.

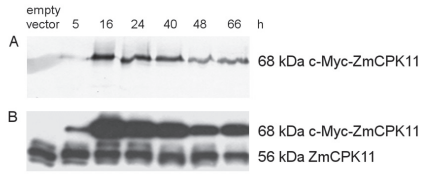


**Figure 2. Efficiency of transient expression of c-Myc-ZmCPK11 in maize protoplasts.**
The fusion protein was detected by immunoblot using anti-c-Myc antibodies **(A)** and activity of the protein kinase was determined by in-gel kinase assay using MBP as a substrate **(B)** in protoplasts transformed with c-Myc-ZmCPK11 or empty vector.

The obtained results indicate that the time course of ZmCPK11 activation after PC treatment is delayed relative to the PA treatment. The observed lag is probably due to the time required for the hydrolysis of PC to PA catalyzed by PLD. This would mean that the effect of PC is indirect and that PA is the final product responsible for the activation of ZmCPK11. To verify this suggestion, the effect of inhibition of PA synthesis on the wound-induced activity of ZmCPK11 was studied. For this purpose, before wounding plant leaves were treated with 0.1% n-butanol, which may substitute for water in the (PLD)-mediated formation of PA, causing production of an alcohol (phosphatidylbutanol) instead of PA (Andersson *et al.*, 2006; Yakimova *et al.*, 2007). As shown in Fig. 1B, the n-butanol treatment significantly decreased the ZmCPK11 activity in non-wounded and wounded leaves, and also inhibited significantly the activity of a $Ca^{2+}$-independent protein kinase, which was activated in response to wounding (Seo *et al.*, 1999). s-Butanol used as a control had no influence on the activities of studied kinases.

To evaluate whether the stimulation of ZmCPK11 activity by PA observed *in vivo* (Fig. 1A) is caused by its direct interaction with the protein kinase, lipid binding assay was carried out. For this purpose maize protoplasts expressing different forms of ZmCPK11 fused with the c-Myc epitope were used. Immunoblot analysis demonstrated that the transient expression of the fusion protein in mesophyll protoplasts was the most effective 16 h after transformation (Fig. 2A). Also an in-gel kinase assay showed that c-Myc-ZmCPK11 reached peak activity 16 h after transformation, whereas the activity of the native 56-kDa kinase was constant (Fig. 2B). Therefore, the protoplasts 16 h after transformation were used for binding with various phospholipids: PA (synthetic: 16:0, 18:0, 18:1, and mix of natural PA), PS, PI, and PC. Among the phospholipids studied only PA 16:0 and 18:0 were efficiently bound by ZmCPK11. The binding of a mix of natural PA and of synthetic PA 18:1 was much weaker. Rarely, very weak binding of PS was observed as well. In our experimental conditions no binding of PI or PC by ZmCPK11 was observed (Fig. 3A).

For an analysis of PA binding to ZmCPK11, an inactive form of ZmCPK11 and two fragments of ZmCPK11 were expressed in maize protoplasts. The structure of the expressed proteins or its fragments used for the lipid binding assay is illustrated schematically on the

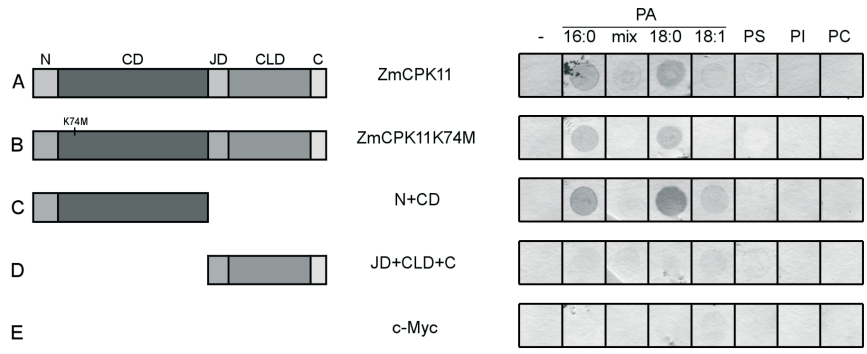


**Figure 3. Binding of various lipids to c-Myc-ZmCPK11 and its fragments.**
**(A)** Whole active form of ZmCPK11; **(B)** Whole inactive form of ZmCPK11 (ZmCPK11K74M); **(C)** N-terminal and catalytic domains fragment of ZmCPK11 (N+CD); **(D)** Junction, calmodulin-like and C-terminal domains fragment of ZmCPK11 (JD+CLD+C); **(E)** c-Myc as a control. Bound proteins were immunodetected using anti-c-Myc antibodies. (-) Without any lipid; PA: mix — natural mixture of soybean PA; 16:0, 18:0, 18:1 — synthetic PA species, PS — phosphatidylserine, PI — phosphatidylinositol, PC — phosphatidylcholine. Phospholipids (5 μg) were spotted on membrane and incubated with maize mesophyll protoplasts expressing different forms or fragments of c-Myc-ZmCPK11 (shown schematically on the left).

left of Fig. 3. A catalytically inactive form of ZmCPK11, in which lysine 74 from the ATP-binding site was mutated to methionine (ZmCPK11K74M), was used to elucidate if the inactive enzyme could also bind to various phospholipids. As shown in Fig. 3B, ZmCPK11K74M, like the active form of the enzyme, is able to bind efficiently only PA 16:0 and 18:0. This result shows that the mutation of lysine to methionine in the ATP-binding site of ZmCPK11 does not influence interaction of the kinase with PA. Two fragments of the enzyme were used to determine which region of ZmCPK11 is involved in PA binding. The first fragment consisted of the N-terminal variable domain and the CD (named N+CD), and the second fragment consisted of the JD, CLD and C-terminal variable domain (named JD+CLD+C). Similarly to the whole enzyme, PA 16:0 and 18:0 were efficiently bound by N+CD, the binding of PA 18:1 was weaker, whereas a mix of natural PA and the other phospholipids studied (PS, PI and PC) did not bind to N+CD (Fig. 3C). In the case of JD+CLD+C no binding to any of the phospholipids studied was observed (Fig. 3D). In the lipid binding assays c-Myc protein was used as a negative control (Fig. 3E).

To clarify whether the binding of PA depends on lipid concentration, the lipid binding assay was carried out with recombinant ZmCPK11 (expressed in bacteria) using increasing (2.5; 5; 10; 15 μg) amounts of PA (16:0; 18:0; 18:1; mix of natural PA), PS, PI and PC. In the conditions used only PA 16:0 and 18:0 were bound to ZmCPK11, and this binding was concentration-dependent (Fig. 4). The other studied phospholipids independently of their concentrations did not bind to ZmCPK11 (data not shown).

The effect of three different acyl species of PA and PC (as a control) on the autophosphorylation of the enzyme and phosphorylation of an exogenous substrate by recombinant ZmCPK11 was studied. In our experimental conditions PA 16:0 and PA 18:0 had the most pronounced effect on ZmCPK11 activity. Unsaturated PA (18:1) and PC (18:1) at the applied concentrations had no significant effect on the autophosphorylation of ZmCPK11 or phosphorylation of MBP (Fig. 5).

To determinate whether PA or PC influence the synthesis of mRNA encoding *ZmCPK11*, an analysis of its transcript level in maize leaves treated with PA or PC was performed by RT-PCR. The level of a 1296-bp fragment of *ZmCPK11* increased gradually, reaching a maximum at 6 h after PA treatment, and slightly declined after that time. The treatment with PC caused an increase of the transcript level later than did the treatment

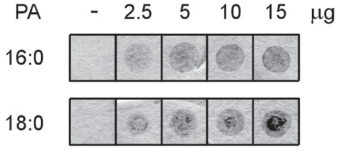


**Figure 4. Effect of different amounts of PA acyl species on binding to recombinant ZmCPK11.**
Various amounts (2.5–15 μg) of PA acyl species (16:0 or 18:0) were spotted on membrane, and incubated with 10 μg of recombinant ZmCPK11. Bound protein was immunodetected using specific antibodies against C-terminal part of CLD of ZmCPK11.

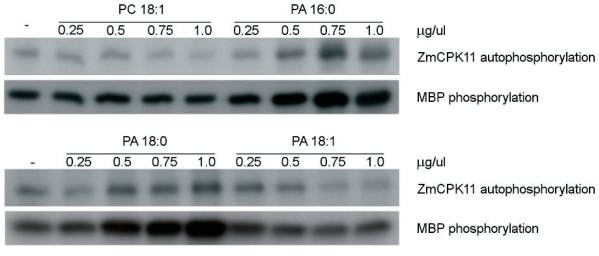


**Figure 5. Effect of various PA acyl species and PC on ZmCPK11 activity *in vitro*.**
For kinase assay, 1.5 μg of recombinant ZmCPK11 was used. Effect of three different PA acyl species (16:0, 18:0, 18:1) or of PC (18:1) in increasing concentrations (0.25–1.0 μg/μl) on ZmCPK11 autophosphorylation (without any exogenous substrate) and phosphorylation of MBP was determined.

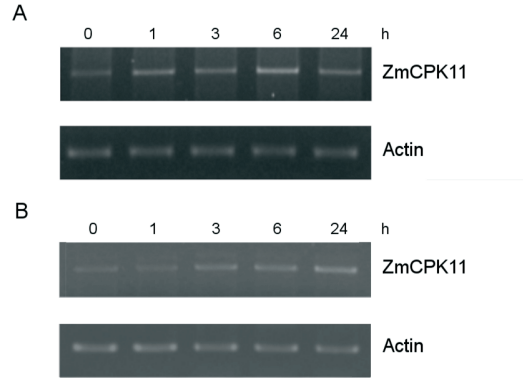


**Figure 6. Effect of PA and PC on *ZmCPK11* transcript level.**
Leaves of 2-week-old maize plants were treated with 0.25 mM solution of PA (8:0) **(A)** or PC (8:0) **(B)** and collected after 0, 1, 3, 6 and 24 hours. Transcript level was determined by RT-PCR using specific primers and total RNA isolated from leaves as template. *Actin* gene was used as a reference.

with PA. The level of the constitutively expressed actin mRNA was constant upon the both treatments (Fig. 6).

## DISCUSSION

Previously our results have shown that the activity of native and recombinant ZmCPK11 is efficiently stimulated *in vitro* not only by calcium ions but also by certain phospholipids (Szczegielniak *et al.*, 2005). In this work we have tried to elucidate the mechanism of this stimulation and whether the activation is caused by a direct interaction between ZmCPK11 and those phospholipids.

Studies carried out *in vivo* showed maximal stimulation of ZmCPK11 activity after 15 min of PA treatment, which was correlated in time with the stimulation of the enzyme activity by wounding reported earlier (Szczegielniak *et al.*, 2005). Because wounding causes rapid accumulation of PA (Ryu & Wang, 1996; Zien *et al.*, 2001), the above results suggest an involvement of ZmCPK11 in a PA-mediated wound signal transduction pathway. Most probably ZmCPK11 participates in the wound signaling pathway at two phases and two different levels: in the first, rapid phase (within minutes) stimulation of the enzyme activity is observed, while in the second phase (within 3–6 hours) *ZmCPK11* transcript is synthesized *de novo*. It is known that after wounding, the biosynthesis of PA occurs mostly due to activation of PLD, which uses mainly PC as a substrate. In *Arabidopsis thaliana* accumulation of PA was observed within minutes after wounding and was accompanied by simultaneous reduction of PC amount (Zien *et al.*, 2001). Moreover, analysis of the acyl species of PA formed 15 min after wounding showed that the newly synthesized PA had a composition similar to that of PC, and thus was most likely to have been derived largely from PC (Zien *et al.*, 2001). Also the present result showing delayed stimulation of ZmCPK11 activity by PC supports the observation that PC is the substrate for synthesis of PA. To confirm the assumption concerning the involvement of PLD in the PA-stimulated activation of ZmCPK11, plant leaves were treated with n-butanol prior to wounding (Andersson *et al.*, 2006; Yakimova *et al.*, 2006). Indeed, n-butanol greatly limited the increase of ZmCPK11 activity in response to wounding, suggesting that the PA affecting ZmCPK11 activity is mostly synthesized by PLD. Moreover, the substantial decrease of ZmCPK11 activity in non-wounded leaves suggests that n-butanol can inhibit production of PA (synthesized by PLD) also during normal growth and development of plants. It has been shown that n-butanol is able to perturb germination of *Arabidopsis* seeds and growth of seedlings (Gardiner *et al.*, 2003). Added to agar growth media at 0.2% n-butanol strongly inhibited the emergence of the radicle and cotyledons, while at 0.4% concentration it effectively blocked germination. Also in seedlings treated with 0.4% n-butanol root growth was significantly (about 90%) retarded. These results suggest that activity of PLD is required for normal growth of *Arabidopsis*. The residual activity of ZmCPK11 observed after n-butanol treatment in wounded leaves may be due to the PA formed by activation of PLC and DGK. Cooperation of PLD and PLC/DGK in PA formation has been shown in *A. thaliana* during defense responses to *Pseudomonas syringae*. Recognition of two bacterial type III effector proteins (AvrRpm1 and AvrRpt2) induced a biphasic accumulation of PA. The first wave of PA accumulation was caused by PLC/DGK activity, the second, delayed in time but higher, was caused by PLD activation (Andersson *et al.*, 2006). Similarly to the CDPKs, MAPK activation is also reduced by n-butanol. This is in agreement with the data demonstrating that a wound-induced MAP kinase in suspension-cultured soybean cells was efficiently inhibited by n-butanol (Lee *et al.*, 2001).

The present data indicate that ZmCPK11 is able to bind specifically PA in a concentration-dependent manner. The lack of an effect of other phospholipids is surprising, because it has previously been demonstrated that also PS as well as PI efficiently stimulate the activity of ZmCPK11 *in vitro*. This suggests that the binding of these lipids to the enzyme most probably is much weaker and therefore difficult to demonstrate.

The inactive form of the kinase was able to bind effectively the same PA acyl species as the active enzyme. This indicates that the mutation (K74M) at the ATP-binding site does not disturb the PA binding. Because no general motif has been identified to date as a potential PA recognition site (Stace & Ktistakis, 2006; Zhang *et al.*, 2009), we tried to localize the PA-binding region within the ZmCPK11 molecule. The lipid binding assay carried out with two fragments of ZmCPK11 shows that the PA-binding site is likely to be located in the catalytic domain of ZmCPK11. This finding corresponds with the results obtained for *Arabidopsis* MAPKK kinase CTR1 (constitutive triple response 1), where a PA-binding site has been identified within the catalytic domain (Testerink *et al.*, 2007).

The above results prove that regardless of which form of ZmCPK11 (whole active enzyme, inactive ZmCPK11K74M, N+CD fragment) is used for experiments, it binds the same PA acyl species. However, since no physiological function of ZmCPK11 is known, it is difficult to explain why ZmCPK11 binds only saturated (16:0; 18:0) species of PA. Both saturated and unsaturated fatty acids play important roles in plant metabolism. During growth and development of plants the content of various fatty acid species in glycerolipids, including phospholipids, is constantly changing (Wang *et al.*, 2006a). These changes are related not only to the maintenance of membrane functions during various internal and external disturbances, but also to the synthesis of several fatty acid derivatives that play important regulatory roles in plants (Weber, 2002). It has also been shown that in response to various stresses the content of particular acyl species of phospholipids increases (Welti *et al.*, 2002). For example, in *Arabidopsis* rosette leaves subjected to hyperosmotic stress (0.4 M NaCl treatment) the amount of 18:2 and 18:3 fatty acids in polyphosphoinositides, structural lipids (PC) and regulatory lipids (PA) increased gradually up to 2 h of treatment, whereas the amount of the 18:0 species remained unchanged. On the other hand, the amount of another saturated PA species (16:0) increased slightly after 15 min of NaCl treatment and decreased at 1 h (Konig *et al.*, 2007). A similar situation was observed during wounding of *Arabidopsis* leaves. A significant increase of unsaturated species of PA, mainly 18:2 and 18:3, was observed 15 min after wounding. Besides the unsaturated also saturated species of PA (16:0) increased after the stress (Zien *et al.*, 2001).

Similarly to the results of PA binding, the *in vitro* kinase assays showed that only saturated forms of PA (16:0 and 18:0) had a significant effect on ZmCPK11 activity as well as on its autophosphorylation. The degree of stimulation of the ZmCPK11 activity by PA was correlated with the autophosphorylation level of the enzyme, which suggests that the phosphorylation of ZmCPK11 stimulates its enzymatic activity.

In conclusion, the following scenario is proposed. Wounding of maize leaves leads to rapid activation of phospholipase D. PLD converts PC into PA. PA, but not other phospholipids, binds to the wound-responsive ZmCPK11 and stimulates its enzymatic activity and *ZmCPK11* expression. While the effect of PA on ZmCPK11 activity could be direct and require simply the lipid–protein interaction, the delayed response at the gene expression level must be due to a more complex and as yet unknown mechanism. The PA–ZmCPK11 interaction would stimulate the kinase autophosphorylation, thereby increasing its activity towards exogenous substrate as well. The presented results suggest that ZmCPK11 participates in a wound-signal transduction pathway mediated by phosphatidic acid.

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