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Two calcium dependent protein kinases are differently regulated by light and have different activity patterns during seedling growth in *Pharbitis nil*

Krzysztof Jaworski · Adriana Szmidt-Jaworska · Jan Kopcewicz

Abstract In this study using biochemical approaches we identified two calcium-dependent protein kinases (CDPKs) named PnCDPK52 and PnCDPK56 in soluble protein extracts from seedlings of *Pharbitis nil*. Both enzymes phosphorylated the specific substrate histone III-S in the presence of Ca^{2+} and cross-reacted with antibodies against the CDPK. PnCDPKs exhibited quite different activity and protein levels during germination and successive stages of seedling growth. PnCDPK52 protein level was high in seeds and during germination, whereas PnCDPK56 increased in the next stages of seedling growth, being the dominant enzymes in mature seedlings, of the light- and dark-grown plant. In all cases both activity and accumulation of protein PnCDPK56 was higher in dark grown plants whereas exposure to light reduced both factors. When etiolated cotyledons were exposed to light, the activity of PnCDPK56 was reduced to the basal level within 5 h. Conversely, increasing activity of PnCDPK56 in cotyledons of green plants shifted to darkness was extremely rapid, reaching the maximum level after just 1 h of darkness and then gradually decreased. Further lengthening of the darkness to 16 h resulted in a strong increase in activity at 12 h. These data indicate that at least two isoforms of CDPK are involved in germination and seedling growth of *P. nil*. The differences in PnCDPKs strongly argue for the pleiotropic role of these isoforms. It seems that PnCDPK52 is associated with the germination process and PnCDPK56 with seedling growth. Moreover it suggests that activity of PnCDPK56 is controlled by light via the photoreceptor-dependent pathway.

Keywords Calcium · Kinase · Light · *Pharbitis nil* · Seedling growth

Introduction

Throughout their life, plants are exposed to the action of numerous stimuli, both environmental and developmental, hence they have developed a complex machinery to respond and adapt to all these signals. In order to achieve specific physiological responses they activate a complex network analysis which consists of perception, transmission and processing of a specific signal. One of the early stages of the signal transduction pathway is the appearance of a large number of second messengers such as calcium ions and cyclic nucleotides (Pandey 2008).

Calcium is the main second messenger and calcium signaling is one of the best documented pathways in plants. It has been shown that many of the environmental stimuli including light, touch, osmotic and oxidative stress, fungal elicitors, shocks, temperature and nodulation factors act via a transient increase in cytosolic calcium concentration (Reddy 2001; White and Broadley 2003).

These changes in intracellular calcium, which occur in response to one of these factors, are called 'calcium signatures'. It was revealed that the spatial and temporal patterns of variations of Ca^{2+} concentration are characteristic to the stimuli that induce them, and each signal elicits a physiological response appropriate for the stimulus (reviewed in Rudd and Franklin-Tong 2001, Sanders et al. 2002). They are sensed by a specific set of calcium sensors that initiate a cascade of downstream effects, including

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differences in protein phosphorylation and gene expression patterns (Sanders et al. 2002; Scrase-Field and Knight 2003; Klimecka and Muszyńska 2007). One of the largest and most complex groups of calcium sensors are protein kinases, among which calcium-dependent protein kinases (CDPKs), which were identified only in plants and protists, play a superior role. CDPKs represent a novel class of enzymes that have an N-terminal variable domain, kinase catalytic domain, autoinhibitory junction domain and a C-terminal calmodulin-like regulatory domain, containing Ca^{2+}-binding motifs EF-hand. The CDPKs can directly bind calcium, so their calcium-stimulated kinase activities are independent of calmodulins (Klimecka and Muszyńska 2007).

Growing evidence indicates that CDPKs are involved in many aspects of plant growth and development as well as plant adaptation to biotic and abiotic stresses (Harmon 2003; Ivashuta et al. 2005; Jaworski et al. 2010). Expression of some CDPKs is induced by salt (Botella et al. 1996) and osmotic stress (Pestenacz and Erdei 1996), wounding (Tsai et al. 2007), fungal elicitors or pathogens (Romeis et al. 2001; Szczegielniak et al. 2005), changes in dark/light conditions (Jaworski et al. 2010; Giammaria et al. 2011), and phytohormones (Zhang et al. 2005; Khan et al. 2005). In addition CDPKs are involved in a wide variety of developmental processes. For example, CDPK participate in late stages of pollen development in maize (Estruch et al. 1994), in self-incompatibility in tobacco (Kuntz et al. 1996), tuberization in potato (Raíces et al. 2001), nodulation in soybean (Zhang and Chollet 1997), embryogenesis, seed development and germination in sandalwood (Anil and Rao 2001) and sexual organ development in liverwort (Nishiyama et al. 1999). These observations give CDPKs credibility as key intermediates in Ca^{2+}-mediated signaling in plants (Anil and Rao 2000).

Plant growth and development is flexible and subject to modulation by environmental factors such as light, water, and gravity. A light signal has particularly dramatic effects on the morphogenesis of seedlings regulating different aspects of plant growth and development, such as seed germination, stem elongation, and flowering time (Chory et al. 1996; Kaczorowski and Quail 2005; Roig-Villanova et al. 2006). For example, development patterns of seedlings grown under light are distinct from those observed in plants grown in darkness with respect to gene expression, cellular and subcellular differentiation, and organ morphology (Von Arnim and Deng 1996).

In light signaling, CDPKs are believed to be important regulators involved in various processes. For example, in plant organs grown in light, differences in expression and activity of some CDPKs were noted (Klimecka and Muszyńska 2007). In organs of zucchini seedlings, darkness elevated the expression level and protein activity of CpCPK1 (Ellard-Ivey et al. 1999). Significant differences between etiolated and light-grown tissues were also observed in maize and cucumber. Activity and the transcript levels were high in etiolated organs and dropped after light exposure (Barker et al. 1998; Ullanat and Javabaskaran 2002; Saijo et al. 1997). Also in wheat (Sharma et al. 1997), and Japanese morning glory seedlings (Jaworski et al. 2003), activity of CDPKs was higher in etiolated tissues.

In this paper we characterize at the biochemical level two soluble CDPKs from *Pharbitis nil*. Their protein accumulation and activity are differentially regulated in various organs of light- and dark-grown seedlings. We also provide evidence that these CDPKs differently participate in seedling development showing distinct spatio-temporal accumulation during seed imbibition, seed germination, and subsequent stages of growth in various light regimes. These observations strongly indicate the involvement of these two CDPKs in seedling growth in response to light conditions.

Materials and methods

Plant material

Seedlings of Morning glory (*P. nil* Chois. cv. Violet) (Marutane Co., Kyoto, Japan) were grown for 5 days in various light conditions (white light, dark). CDPK was assayed at daily intervals in whole seedlings during different developmental stages: dry seeds (Ds—0 h), imbibed seeds (Ib—24 h), the stage of germination (Gr—48 h), stages of seedling growth (D1–D4—72–144 h) (Fig. 1a–b). Seeds before imbibition were soaked in concentrated sulphuric acid for 45 min and then washed with running tap water for 3 h. They were left in ddH_2O for 24 h at $25 \pm 2°C$ in continuous white light (130 µmol m^{-2} s^{-1}; cool white fluorescent tubes, Polam, Poland) or dark. The imbibed seeds were planted on a mixture of vermiculite and sand (2:1) in plastic pots, covered with Saran Wrap to maintain high humidity and grown at $25 \pm 2°C$ in continuous light or dark for 5 days.

Roots, hypocotyls and cotyledons were harvested from 5-day-old seedlings growing in continuous light and dark. Some of these plants grown in the light were exposed to a 16-h-long night to promote flowering. Thereafter the plants' organs were collected, frozen in liquid nitrogen and stored at $-80°C$ for later use in protein extraction.

Protein extraction

Tissues from dormant seeds and different stages of *P. nil* growth were frozen in liquid nitrogen and homogenized using a mortar and pestle. The homogenate was then suspended in extraction buffer (20 mM, Tris–HCl pH 7.5,
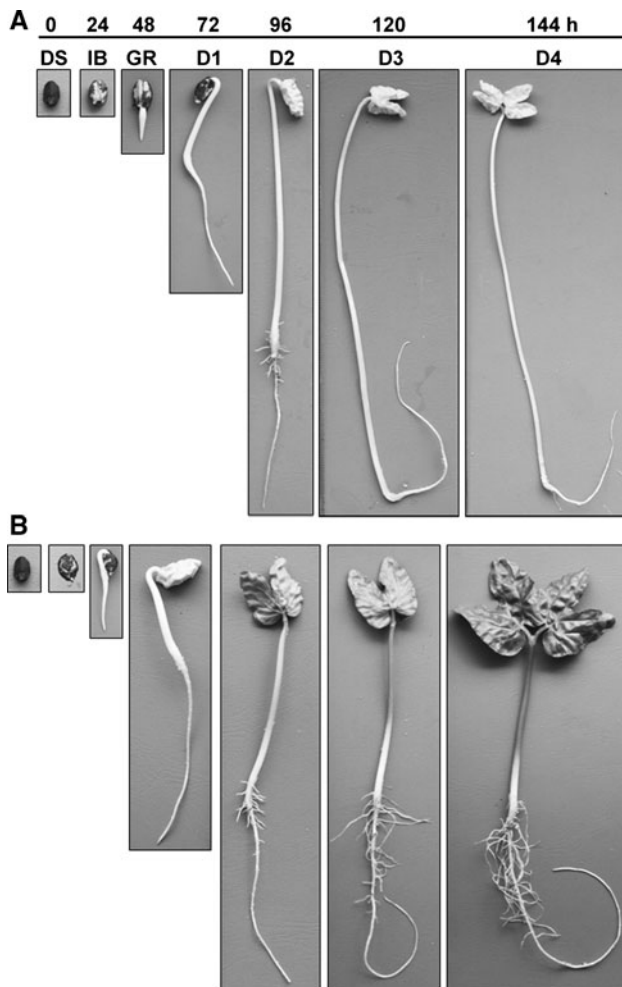
Fig. 1 Stages of germination and seedling growth of *P. nil*. Plants were grown in two light regimes: *darkness* (**a**) and *continuous light* (**b**). Seedlings were harvested every 24 h: dry seeds (*DS*), imbibed seeds (*IB*), germinating seeds (*GR*), and growing seedlings (*D1–D4*)

2.5 μM EDTA, 5 mM NaF, 10 μM aprotinin, 10 μM leupeptine and 1 μM PMSF) and held on ice for 15 min. The crude protein extracts were centrifuged at 16,000g at 4°C for 30 min. The pellet was discarded and the supernatant containing the soluble proteins was used for further experiments. Protein concentration was determined by the method of Bradford (1976) using BSA as standard.

In vitro enzyme assay

Activity was determined in vitro by measuring the incorporation of ^{32}P from $[\gamma\text{-}^{32}P]$ ATP (Polatom, Poland) into endogenous histone III-S. Calcium-dependent protein kinase assays were performed in a total volume of 50 μL containing 50 mM Tris–HCl (pH 7.5), 10 mM MgCl$_2$, 0.5 mg mL^{-1} histone III-S, 1 mM CaCl$_2$ or 1 mM EDTA. Reactions were initiated by the addition of 10 μM $[\gamma\text{-}^{32}P]$ ATP (1,000 cpm pmol^{-1}) and assay mixtures were

incubated for 10 min at 30°C. Spotting 40 μL on P-81 filter terminated the reaction. The filters were washed with 5% (w/v) phosphoric acid and 95% ethanol, dried and added to scintillation vials containing 4 mL scintillation cocktail and counted in a liquid scintillation counter (Wallac 1407).

In a SDS–polyacrylamide gel protein kinase assay

In-gel phosphorylation of histone III-S was carried out according to the method described by Jaworski et al. (2003). Aliquots of soluble fraction of each homogenate (40 μg) were subjected to SDS–PAGE in 10% gel with histone III-S (0.5 mg mL^{-1}) added to the separation gel just prior to polymerization. Soluble proteins were incubated in protein sample buffer at 95°C for 10 min and resolved on the gel. After electrophoresis, SDS was removed by washing the gel for 30 min at room temperature with 20% (v/v) isopropanol in 50 mM Tris–HCl (pH 8.0) and then 2 × 30 min in 50 mM Tris–HCl (pH 8.0), 5 mM β-ME (buffer A). Proteins were denatured by treating the gel with 6 M guanidine-HCl in buffer A for 1 h at room temperature and then renatured with 0.04% Tween 40 in buffer A at 4°C over night. Next, the gel was preincubated with 25 mL 50 mM Tris–HCl (pH 7.5) containing 5 mM MgCl$_2$, 2 mM MnCl$_2$ and 2 mM DTT for 1 h at 30°C and then in 4 mL of the same buffer 50 μCi $[\gamma\text{-}^{32}P]$ ATP (3,000 Ci mmol^{-1}) and 1 mM CaCl$_2$ or 2 mM EGTA for 1 h at 30°C. The reaction was stopped by washing the gel with 1% (w/v) sodium pyrophosphate in 5% (w/v) trichloroacetic acid. The washed gels were dried and exposed to X-ray film (Foton). Signals were also scanned with phosphoimager Fuji 5000 and quantified with ImageQuant software.

Immunodetection of CDPK using anti-soybean CDPK

Soluble protein extracts from different developmental stages of *P. nil* were resolved on a 10% (w/v) SDS–PAGE as described by Laemmli (1970). Proteins were transferred to PVDF membrane by the semi-dry system (BioRad) (15 min at 15 mA) using 25 mM Tris, 192 mM glycine and 20% (v/v) methanol (pH 8.3). Membrane was blocked in TBS containing 3% non-fat dry milk and then incubated overnight at 4°C with primary polyclonal antibodies against the CLD domain of CDPK from soybean 1:2,000 in TBS. After washing three times in TBS, the membrane was incubated for 1–2 h with secondary horseradish peroxidase conjugated to goat anti-rabbit IgG diluted to 1:10,000 in TBS buffer and bands were visualized by chemiluminescence using the ECL plus system (GH Healthcare).

The computer application used for the analysis was Quantity One (BioRad), and for the calculations and graphs we used SigmaPlot 2001 v. 7.0 (SPSS Inc.)

Results

Soluble protein extracts of Morning glory (*P. nil* Chois. cv. Violet) grown under different light conditions were analyzed to determine calcium dependent phosphorylation. When protein kinase activity was assayed in vitro phosphorylation of histone III-S, used as a substrate in the presence of a micromolar concentration of calcium, was observed. The in-gel protein kinase assayed for cotyledons, roots, stem and stages of seedling development showed two protein bands at 50 and 56 kDa that phosphorylated histone III-S in a Ca^{2+}-dependent manner, which indicated that enzymes have CaM-independent nature. Furthermore, both of these proteins cross-reacted with anti-soybean CDPK antibodies, indicating that they are isoforms of CDPK family.

Monitoring of PnCDPK accumulation and activity during germination and seedling growth

To answer the question whether various CDPK isoforms are involved in growth processes and if their level is under light control, enzyme accumulation and activity were measured during seed germination and seedling growth in two light regimes: darkness or continuous white light.

The protein kinase assay revealed that, despite the fact that in protein fractions from etiolated plants two CDPK isoforms, named PnCDPK52 (52 kDa) and PnCDPK56 (56 kDa), were active (Fig. 2a–b) during germination and seedling growth, the activity of each of the enzymes significantly changed. In the dry seeds the percentage of both isoforms is different and amounts to 85 and 15% in the case of PnCDPK52 and PnCDPK56 kDa protein, respectively. However, the activity of 50 kDa protein was predominant in dry and swelling seeds and decreased gradually during the sprouting process and seedling growth. The activity of PnCDPK56 was on a low level in dry and imbibed seeds and during germination, afterwards it gradually increased, reaching its highest level in 5-day-old seedlings (90%).

For the same scheme protein gel blot analysis was carried out using polyclonal antibodies made against the CLD-domain of CDPK. Figure 2c shows that two clear bands corresponding to a molecular mass of 52 and 56 kDa were recognized. A 52 kDa protein was predominant in dry, imbibed and germinated seeds, while a 56 kDa protein was at a very low level in seeds and gradually increased during seedling growth, reaching the highest level in 5-day-old seedlings.

Studies of the PnCDPKs activity in dark- and light-grown plants showed a generally similar pattern of phosphorylation, and only small differences were noted. Both in dry and imbibed seeds two main bands, stronger PnCDPK52 and weaker PnCDPK56 were observed
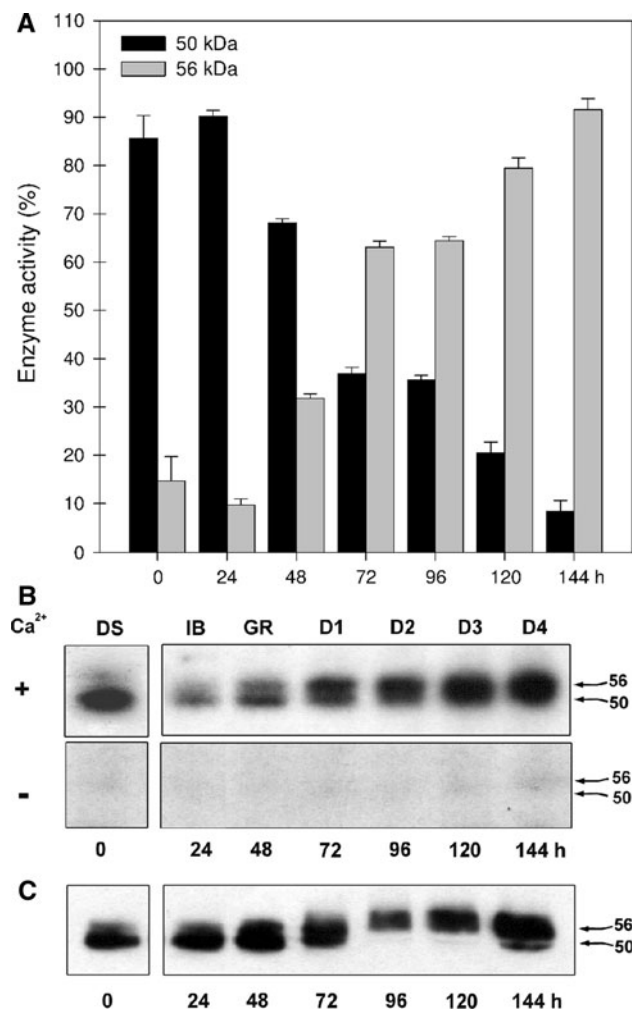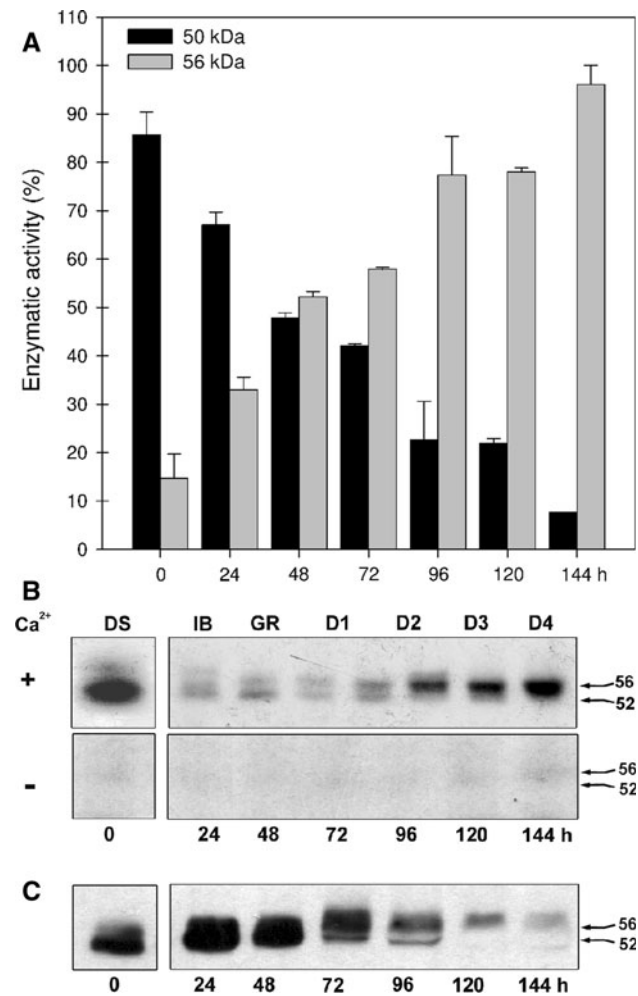

Fig. 2 CDPK activity and protein level in *P. nil* seedlings grown in the darkness. **a** Ratio of 52 and 56 kDa CDPKs activity during etiolated seedling growth. **b** In-gel Ca^{2+}-dependent phosphorylation assay of proteins from seeds through different stages of seedling growth : dry seeds (*Ds*—0 h), imbibed seeds (*Ib*—24 h), the stage of germination (*Gr*—48 h), stages of seedling growth (*D1–D4*—72–144 h). Protein extracts were electrophoresed in 10% (w/v) SDS–PAGE containing histone III-S as a substrate. After gel renaturation CDPK activity was detected in the presence of 1 mM of $CaCl_2$ (+) or 1 mM EDTA (−). **c** Gel blot analysis of protein extracts using anti-soybean CDPK

(Fig. 3a). Then, during seedling growth PnCDPK52 activity gradually decreased, whereas PnCDPK56 activity increased.

Analysis of PnCDPK concentration revealed that PnCDPK52 protein was at a very high level in dry seeds and during the germination process, and gradually decreased to a poorly visible or undetectable amount during the next stages of seedling growth, whereas PnCDPK56 protein was present in all analyzed samples, reaching the highest level in cotyledons lifted above the ground (Fig. 3b); afterwards the amount decreased slightly.

Fig. 3 CDPK activity and protein level in *P. nil* seedlings grown in the light. **a** Ratio of 52 and 56 kDa CDPKs activity in seedlings growing in the light. **b** In-gel Ca²⁺-dependent phosphorylation assay of proteins from seeds through different stages of seedling growth : dry seeds (*Ds*—0 h), imbibed seeds (*Ib*—24 h), the stage of germination (*Gr*—48 h), stages of seedling growth (*D1–D4*—72–144 h). Protein extracts were electrophoresed in 10% (w/v) SDS–PAGE containing histone III-S as a substrate. After gel renaturation CDPK activity was detected in the presence of 1 mM of CaCl₂ (+) or 1 mM EDTA (−). **c** Gel blot analysis of protein extracts using anti-soybean CDPK

Changes in Ca²⁺-dependent phosphorylation activity of the exogenous substrate in the soluble protein extracts from different seedling's organs are shown in Fig. 4a. It was found that in the presence of calcium ions, intensity of the phosphorylation in vitro was differentially regulated by light in all analyzed organs. In contrast to the high activity detected in roots and hypocotyls grown in darkness, in light grown plants the enzyme was less active. The highest activity was observed in etiolated hypocotyls (157 pmol min⁻¹ mg⁻¹) and it was 1.4-fold and 8.5-fold higher when compared to etiolated roots and cotyledons, respectively.
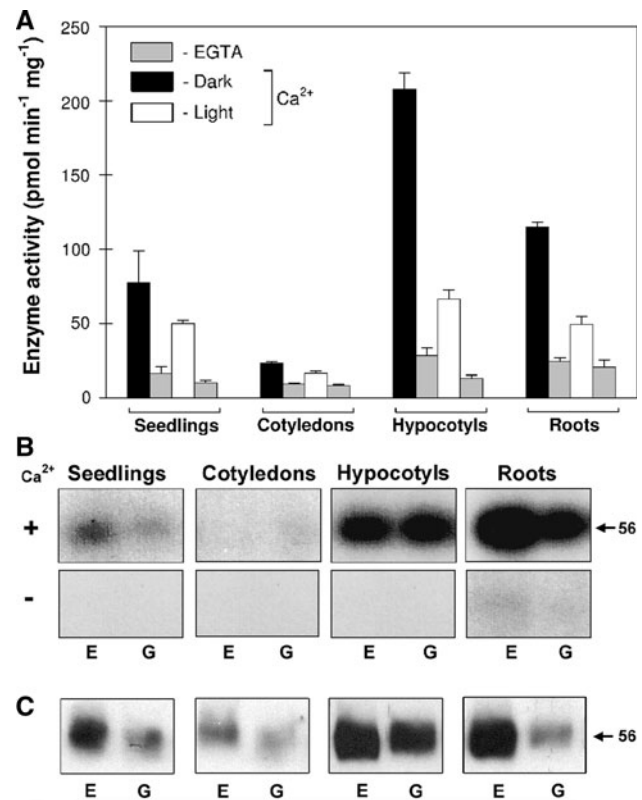

Fig. 4 Effect of different light conditions on CDPK in vegetative organs of 5-day-old seedlings. **a** In vitro Ca²⁺-dependent phosphorylation of histone III-S by soluble protein extract in the presence of 1 mM of CaCl₂ or 1 mM EDTA followed by counting of incorporated ³²P in a liquid scintillation counter. **b** In-gel Ca²⁺-dependent phosphorylation assay of proteins from etiolated (*E*), green (*G*) plants. Protein extracts were electrophoresed in 10% (w/v) SDS–PAGE containing histone III-S as a substrate. After gel renaturation CDPK activity was detected in the presence of 1 mM of CaCl₂ (+) or 1 mM EDTA (−). **c** Protein gel blot analysis of proteins using anti-soybean CDPK. *Bars* represent SE

In addition, in-gel assays were undertaken to confirm results received from in vitro studies. Kinase activity was observed for PnCDPK56 protein only (Fig. 4b). It was noted that in roots and hypocotyls PnCDPK56 activity corresponded to in vitro assays and in cotyledons was under the detection limit. However, when time of exposure was longer, a three-fold increase in enzyme activity in the cotyledons of plants grown in the darkness was observed (Fig. 5).

Anti-soybean CDPK cross-reacted with PnCDPK56 band only and no reaction with PnCDPK50 band was observed (Figs. 4, 5).

Analysis of PnCDPK in cotyledons under various light conditions

To determine the role of light conditions on PnCDPK kinase activity in cotyledons of *P. nil* seedlings were grown
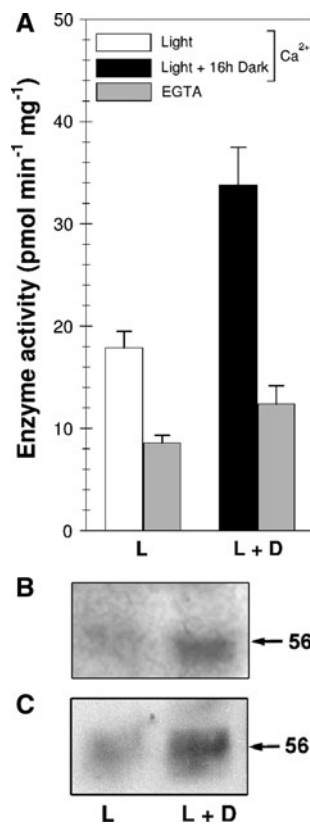
Fig. 5 Light-mediated regulation of CDPK in cotyledons. **a** In vitro Ca^{2+}-dependent phosphorylation of histone III-S by soluble protein extracts prepared from cotyledons removed from plants growing in the light (L) and after exposure to 16 h of darkness (L + D). Analysis was performed in the presence of 1 mM of $CaCl_2$ or 1 mM EDTA followed by counting of incorporated ^{32}P in a liquid scintillation counter. **b** In-gel Ca^{2+}-dependent phosphorylation. **c** Protein gel blot analysis using anti-soybean CDPK. *Bars* represent SE

for 5 days in the presence or absence of light, then light conditions were reversed. Etiolated plants were moved to white light and green plants to darkness.

The obtained results clearly show that in etiolated cotyledons shifted to light both activity and protein accumulation of PnCDPK56 is strongly reduced, reaching the level observed in green cotyledons in 5 h (Fig. 6a).

By contrast, darkness caused a rapid fourfold increase in activity and protein levels after 1 h of dark exposure. In the next hours, a gradual reduction was observed, reaching the lowest level in the 5th hour of darkness. However, both protein level and enzyme activity were on a higher level than that observed in the cotyledons of seedlings growing in continuous light (Fig. 6b).

In the last experiments the role of light conditions on kinase activity and PnCDPK protein level was examined with regard to the flowering process. Both parameters were analyzed in green cotyledons exposed to darkness (L + D) (16-h-long night promoting flowering) or continuous light (L) (Fig. 7). Kinase activity and protein level were

measured at 1-h intervals. In cotyledons transferred to darkness two peaks of PnCDPK56 activity were noted. The first maximum was observed just 1 h after moving and activity was fourfold higher than light treated cotyledons. In the next hours activity gradually decreased, reaching the initial level at 8 h. The second peak of the same value as at 1st h was noted at 12 h. In cotyledons kept in continuous white light the enzyme activity was under the detection limit (data not shown).

Discussion

In the soluble protein fractions from *P. nil* seedlings grown in light or dark, we identified two proteins of 52 kDa (PnCDPK52) and 56 kDa (PnCDPK56), which phosphorylate histone III-S in a Ca^{2+}-dependent manner, and react with antibodies raised to soybean CDPK, which confirms that both proteins are calcium-dependent protein kinases.

Regardless of the light growing conditions protein accumulation and enzyme activity significantly changed in seeds and during seedling growth. High activity of PnCDPK52 presents both in dry and imbibed seeds and during germination strongly decreased when PnCDPK56 activity gradually commenced to increase during stages of seedling growth. The obtained results demonstrate that these two CDPKs differ in their temporal and special distribution to accomplish their diverse physiological role. Available data show that most CDPK isoforms are expressed constitutively and neither organ nor tissue specificity were observed (Morello et al. 2006). However for some of them a unique, very restricted expression pattern and enzyme activity in different organs or tissues at different stages of growth and development were noted. For example in maize, a specific CDPK isoform is expressed only in the late stages of pollen development (Estruch et al.1994) and the other one in very rapidly growing tissue (Abbasi et al. 2004).

The presence of this particular 52 kDa PnCDPK in seeds only shows its high organ specification. In the literature we can find information that some CDPKs are restricted for seeds only. The rice *spk* gene was specifically expressed in developing seeds and little of its mRNA was detected in other organs like leaves and roots (Kawasaki et al. 1993). The expression of *spk* occurred exclusively in the endosperm of immature seed, and was not detected in the embryo or the aleurone layer (Asano et al. 2002). In rice, two isoforms OsCDPK2 and OsCDPK11 showed expression profiles which diverged significantly during seed development. OsCDPK2 protein was expressed at low levels during early seed development and then increased to a high level that was maintained in later stages. Conversely, OsCDPK11 protein levels were high at the

Fig. 6 PnCDPK56 activity and protein level in cotyledons of seedlings growing in different light regimes. Mature seedlings (5-day-old) grown in continuous light (**a**) or dark (**b**) were exposed to dark and light conditions, respectively. Protein kinase activity (A2; B2) and protein level (A3; B3) were assayed in soluble protein extract from cotyledons at the indicated time points. Relatively PnCDPK56 activity level (average from three independent experiments) is plotted as A1 and B1
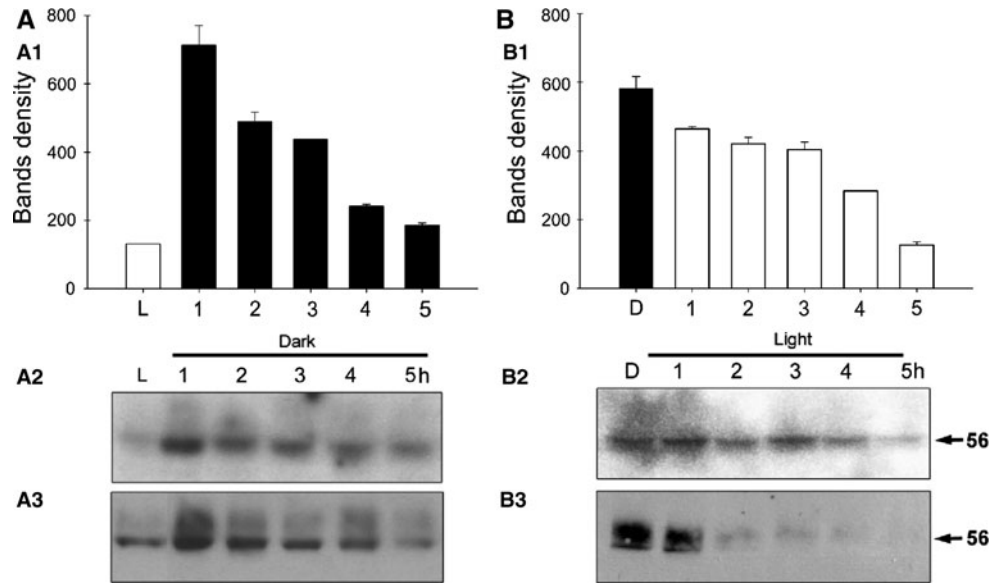
Fig. 7 Effect of darkness on PnCDPK56 in cotyledons of *P. nil*. Five-day-old seedlings grown under continuous light were placed in darkness for 16 h and then moved to the light. Protein kinase activity (**b**) and protein level (**c**) were assayed in soluble protein extract from cotyledons at the indicated time points. **a** Relatively PnCDPK56 activity level
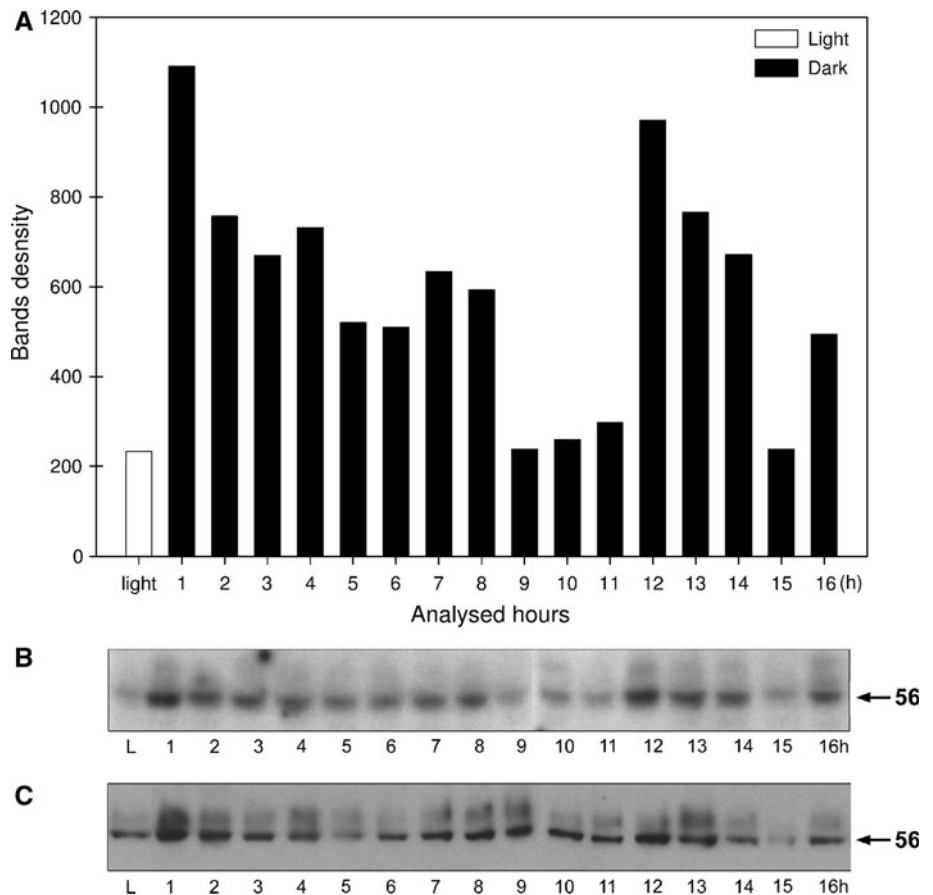
beginning of seed development, but fell rapidly from 10 days after fertilization onwards (Frattini et al. 1999). In sandalwood high activity/expression of swCDPK were observed in zygotic embryos, endosperm, during germination and early stages of seedling growth only, which provides evidence for a post-regulation of this enzyme

during seed maturation and early stages of germination (Anil and Rao 2000, 2001).

The presence of CDPKs in dry seeds proves that these proteins have already been synthesized during embryogenesis. In *P. nil*, as in many dicots, the cotyledons are storage organs and represent the majority of the weight of

the seed. There is a possibility that the activity of PnCDPK52 during imbibition and germination only may be associated with activation of enzymes involved in structural changes and metabolic processes known as embryo activation, such as activation of starch hydrolyzing enzymes or phosphorylation of transcription factors that trigger transcription of genes encoding proteins involved in cardinal enzymatic processes for the growth and development of seedlings. Earlier reports implicate some isoforms of CDPKs in the regulation of starch synthesis or break down (McMichael et al. 1995; Huber et al. 1996; Iwata et al. 1998).

Growing evidence indicates that CDPKs modulate transcription factors by the phosphorylation process in response to various stimuli. It was found that AtCPK32 from *Arabidopsis* phosphorylates ABA-induced transcription factor ABF4 in a calcium-dependent manner (Choi et al. 2005), while AtCPK4 and AtCPK11 may regulate stomata aperture by phosphorylation of ABF1 and ABF4 (Zhu et al. 2007). Also, the bZIP factor of shoot growth (RGS), activator of GA biosynthesis, interacts with NtCDPK1 in tobacco (Ishida et al. 2008). AtCPKs 3 and 13 are directly involved in transcriptional activation of a heat shock transcriptional factor (HsfB2a) in herbivore-infested plants (Arimura and Maffei 2010). AtCPK3 phosphorylates also JA/ethylene-inducible APE/ERF domain transcription factor 1 (ERF1) (Lorenzo and Solano 2005) and the wound-inducible CZF1/ZFAR1 transcription factor (Cheong et al. 2002).

In this study we showed that during seedling growth there occurs protein accumulation and activity increase of a PnCDPK56 isoform, which is dominant in the later stages of seedling growth as well as in all organs of a well-formed 5-day-old seedling (Figs. 2, 3). PnCDPK protein (56 kDa) was mainly present in all organs of both light- and dark-grown plants. Activity was higher in dark-grown than in their light-grown counterparts. The protein level was highest in the hypocotyls and roots, whereas in cotyledons it was barely detectable. During seedlings' growth organs like hypocotyls and roots are capable of fast growth, providing enlargement of the plant body, by division of meristematic cells at the tips of roots and shoots, while the cotyledons' expansion is achieved mainly by water uptake. This clearly gives evidence that this isoform is specifically expressed primarily in rapidly proliferating cells and growing tissue, suggesting that it might be related to cell differentiation and particular metabolic function (Klimecka and Muszyńska 2007). It was reported that some CDPKs are expressed in intensively dividing and growing tissues. For example, in *Nicotiana tabaccum* NtCDPK1 is expressed in rapidly proliferating tissue of shoot and root meristem while *NtCDPK4* is present in rapidly growing tissues like root tip and lateral root primordia (Lee et al. 2003;

Zhang et al. 2005). The highest level of expression in rapidly growing young seedlings' tissue was also detected for *OsCDPK13* from rice and maize *ZmCPK11* (Abbasi et al. 2004; Szczegielniak et al. 2005).

In entire seedlings and organs such as hypocotyls and roots grown in continuous light, reduction of PnCDPK56 protein level and enzyme activity was observed, clearly indicating that both protein accumulation and its activity is down-regulated by light. Many reports have demonstrated that some CDPKs exhibit different expression and activity in organs growing in the light or the darkness (Klimecka and Muszyńska 2007). The level of *CpCPK1* mRNA in etiolated and light-grown zucchini seedlings demonstrate that expression of this gene is differentially regulated in various organs and expression is suppressed by light. In dark-grown tissue, expression was the highest in the hypocotyls followed by hooks and roots, whereas little or no expression was detected in dark-grown cotyledons. In light-grown tissue, expression was greatly reduced (Ellard-Ivey et al. 1999). Also in maize, the expression of *ZmCDPK7* and *ZmCDPK9* was higher in etiolated leaves than in light-grown leaves (Saijo et al. 1997). A detailed study on another maize CDPK (p67^{cdpk}) revealed particularly high levels in expanding tissues of both etiolated and light-grown plants and mature etiolated leaves, roots, coleoptiles. However, mature light-grown tissues had barely detectable levels. When etiolated plants were exposed to 8 h light the activity of CDPK decreased to a background level confirming the reducing effect of light (Barker et al. 1998). In rice plants, after overexpressing *OsCDPK2* in etiolated leaves, stems and flowers the higher level of the transcript was detected (Morello et al. 2000). In wheat seedlings (Sharma et al. 1997) as well as in seedlings of morning glory (Jaworski et al. 2003), activity of CDPKs was also higher in etiolated tissues. However, exposure to light was found to differentially regulate the transcript level of *CsCDPK3* isolated from various organs of cucumber (*Cucumis sativus*). The transcript level decreased in light-grown hypocotyls and roots, whereas in cotyledons light had an up-regulatory effect (Ullanat and Javabaskaran 2002). The higher protein accumulation and gene expression of StCDPK2 was in plants and sprouts of potato exposed to light when compared to dark-treated ones (Giammaria et al. 2011). Light different regulation was also noted for other classes of enzymes such as the small family of plant calcium-independent Ser/Thr protein kinase PEPC-k (phosphoenolpyruvate carboxylase kinase) encoded by PPCK genes. In maize the PPCK1 orthologue (ZmPPCK1) is light-dependent and specifically expressed in mesophyll tissue, while the PPCK2 orthologue is specifically expressed in the dark in bundle sheath tissue (Shenton et al. 2006). This means that light-regulated transcription of CDPKs is organ-specific.

The degradation is crucial to activation and deactivation of regulatory proteins involved in different signalling pathways (Belizario et al. 2008). The light-induced instability of protein concentration may result from the presence of a specific proteolytic site in N-terminal PEST motif. Such a motif has been found in many rapidly degraded proteins for instance, light sensitive labile phytochrome where it contributes to their degradation (Vierstra, 1996; Frattini et al. 1999; Morello et al. 2000; Belizario et al. 2008). In many deletions of the PEST motif reduced ubiquitination, leading to proteins stabilization, was observed (Lukov and Goodell 2010; Ramakrishna et al. 2011). Previously, we found a PEST sequence in the N-terminal domain of the recombinant protein PnCDPK1 from an etiolated plant, so it is possible that PnCDPK protein could be regulated by PEST-dependent degradation (Jaworski et al. 2010). Analysis of CDPKs proteins from different plants using the program EMBOSS 6.3.1:epestfind (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=pestfind#forms::epestfind) (data not shown) revealed that some of them are also negatively regulated by light. For instance ZmCDPK7 (D87042.1), CsCDPK5 (AY027885.2), CpCPK1 (U90262.1) possess PEST motif, whereas AtCPK9 (AAB03242.1), OsCDPK2 (X81394), ZmCDPK9 (D85039) do not. These data suggest that rapid destruction of CDPKs without PEST region could occur by the proteasom pathway. The miscellaneous degradation pathways of these two groups of CDPKs may be due to their high identity in the protein construction. Rice OsCDPK2 is very similar to *Arabidopsi* AtCPK9 and maize orthologue ZmCDPK11 with more than 90% identity (Morello et al. 2000; Hong et al. 1996; Saijo et al. 1997).

Despite the fact that cotyledons and leaves are such important bodies in relation to photosynthesis, gene expression level and enzyme activity of CDPK is lowest in the cotyledons and leaves; in the case of seedlings or young plants these are unquestionably the most important organs. Cotyledons are the first organs that until the leaves appear carry out photosynthesis or as in the case of *P. nil* are responsible for the perception of a photoperiodic stimulus leading to flowering. Hence, we examined how activity and protein level of PnCDPK56 change in etiolated and green cotyledons exposed to light or darkness, respectively. As shown in Fig. 5 the exposure of green seedlings to a long period of darkness (16 h) resulted in a twofold increase in the level of protein. Detailed analysis carried out every hour showed that the CDPK undergo oscillation changes during the dark, reaching two peaks (Fig. 7). A rapid, significant increase in enzyme activity was observed at first after moving the plants to darkness (after 1 h) and after 12 h of dark treatment. Such high enzyme activity (fivefold) in the first hour of darkness may suggest involvement of this isoform of CDPK in the perception of changes in

light/dark conditions, while a further increasing of enzyme activity can suggest participation of CDPK in metabolic processes proceeding in the dark such as etiolation, length growth or flowering (Jaworski et al. 2003, 2010). Similar light-dependent regulation was also observed in light-harvested leaves of rice. Rice OsCDPK2 protein level was almost undetectable in leaves exposed to light, but sharply increased within 2 h after the beginning of the dark period and remained high up to 12 h (Frattini et al. 1999).

It should be noted that the presence of PnCDPK56 in different plant organs in various light conditions indicates the pleiotropic role of this enzyme. For example, two main enzymes, Suc-phosphate synthase (SPS) and nitrate reductase (NR), are inactivated in the dark by CDPK phosphorylation on specific Ser residues (McMichael et al. 1995; Douglas et al. 1998). It might coordinate the supply of carbon skeletons and ammonia needed for starch synthesis or amino acids when photosynthesis is off (Cheng et al. 2002; Morello et al. 2006; Klimecka and Muszyńska 2007).

Summing up, in our research we are endeavoring to find the elements of the calcium pathway involved in selected photomorphogenesis processes. The present results not only indicate the existence of two isoforms of CDPK, but also that each of them is the dominant form of CDPK at different stages of the growth and development of seedlings. Moreover, changes in the activity of these enzymes in plants grown in different light conditions suggest their involvement in various photomorphogenetic processes.

The future research in this area should be driven towards identification of protein targets emphasis on their spatial and temporal cellular dynamics and protein–protein interactions to help to delineate CDPKs functions. So, we initiate a systematic yeast two hybrid screening program to identity CDPK substrates or interactive regulatory proteins that appear in *P. nil* seedlings following plant cultivation in certain light conditions.

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