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Possible Involvement of Ca^{2+}-Dependent Protein Kinases in Spore Germination of the Fern *Osmunda japonica*

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Fern gametophyte is a good model system to investigate signal transduction in plant cells. In this work, we examined whether CDPKs are involved in the mechanisms of spore germination of the fern *Osmunda japonica*. A protein extract from the spores included four CDPK isoforms with relative molecular weights of 56, 53, 49, and 47 kDa, as detected by immunoblot analysis, and they showed CDPK-like activities, as detected by in-gel protein-kinase assay. It was also found that the inhibitors effective on CDPKs, such as a general protein kinase inhibitor, K252a, and a calmodulin antagonist, W-7, largely suppressed the spore germination, and that many proteins of the spores were phosphorylated in vivo in a calcium dependent manner in the period when the spores require external Ca^{2+} for the germination. Furthermore, we showed that Sr^{2+} and Mn^{2+}, which could substitute for Ca^{2+} in the spore germination, were also able to activate the *Osmunda* CDPKs. From these results, we concluded that CDPKs would participate in the spore germination of *O. japonica*.

Keywords: CDPK, fern gametophyte, *Osmunda japonica*, spore germination

The gametophytes of a fern are free-living and directly exposed to the external environment, unlike those of a seed plant. This means that fern gametophytes have to adapt to the environmental changes to survive and achieve their final act, namely generation of sporophytes. Indeed, it is known that the gametophytes can sense and respond to a wide variety of environmental factors, such as light (Wada and Kadota, 1989), low temperature (Sato and Sakai, 1980; Sato and Sakai, 1981), dehydration (Sato and Sakai, 1981), and gravity (Edwards and Roux, 1998a, 1998b). Thus, it seems likely that fern gametophytes possess an advanced system for perceiving and responding to environmental changes that compare with those of the sporophytes and seed plants.

It is well known that Ca^{2+} plays an important role as a messenger in response to number of physiological stimuli, conducting developmental programs, controlling their metabolism, and coping with their environment (Bush, 1995; Sanders et al., 1999; Pandey et al., 2000). Studies indicate that CDPKs are involved in the mechanisms of Ca^{2+}-mediated signal transduction of plant cells (Harmon et al., 2000; Pandey et al., 2000; Cheng et al., 2002). CDPK is a monomeric enzyme with a calmodulin-like domain in its own molecule, which allows response to Ca^{2+} directly, and has been found in higher plants, algae, and protists, but not in animals or fungi (Roberts and Harmon, 1992). Many CDPK isoforms are also known to be present in a single species. In the model plant system *Arabidopsis*, the genome is predicted to encode 34 different CDPKs (Cheng et al., 2002), and each isoform is believed to have a distinct role in response to different Ca^{2+}-elevating stimuli (Hardie, 1999; Harmon et al., 2000).

From the findings that the Ca^{2+}-channel blocker La^{3+} or the impermeable Ca^{2+}-chelator EGTA inhibited the spore germination of *Onoclea sensibilis* (Wayne and Hepler, 1984) and *Dryopteris paleacea* (Scheuerlein et al., 1989), it has been believed that the external Ca^{2+} function as a messenger in fern spore germination (Hepler and Wayne, 1985). Furthermore, Haas et al. (1991) have shown that staurosporine, a protein kinase inhibitor, suppressed the spore germination of *Dryopteris filix-mas*. The phase-specific inhibitory effect of staurosporine in preventing germination was coincident with the phase-specific requirement of Ca^{2+}, suggesting that any Ca^{2+}-dependent protein kinase is the target of the inhibitor (Haas et al., 1991). In the present work, we examined whether CDPKs are involved in the mechanisms of fern spore germination using the chlorophyllous spores of *O. japonica*, which germinate rapidly in a highly synchronous manner (Inoue et al.,

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Abbreviations: CDPK, Ca^{2+}-dependent protein kinase; EGTA, ethyleneglycol bis(2-aminoethyl ether)-tetraacetic acid; MES, 2-morpholinoethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

1995) enabling us to easily evaluate the biochemical events that occur during the spore germination. The results obtained showed that at least four CDPK isoforms are present in the spores and may function in the germinating spores of *O. japonica*.

MATERIALS AND METHODS

Plant Material

Spores of *O. japonica* (Thunb.) were prepared according to Inoue et al. (1995). The spores were harvested from sporophylls collected in Toyama Prefecture, Japan, dried over silica gel for 2 days at room temperature, and then stored at −20°C. The spores were thawed at 24°C in a desiccator immediately before experimental use.

Germination Procedures

Spores were sterilized for 3 min with 0.05% (w/v) Triton X-100, 2 mM EGTA, and 0.6% Purelox, a commercially available 5-6% solution of NaOCl (Oyalox Co., Japan) and washed 4 times with 1 mM EGTA. The spores (1.7 mg) were added to 2 ml of a standard medium (10 mM MES-NaOH, pH 6.5, 3.45 mM KNO_3, 1 mM $MgSO_4$, 1 mM EGTA) supplemented with 2 mM of $CaCl_2$, $SrCl_2$, $MnCl_2$ or $MgCl_2$ and then cultured for 30 h at 24°C rotating at 105 cycles per min under a 20 W white fluorescent tube. The percentage of spore germination was determined by the nuclear staining method (Edwards and Miller, 1972) and by counting the number of germinations in 100 spores. Each spore was considered to have germinated when it had more than two nuclei.

Protein Extraction

Spores (6 mg) were homogenized with a glass-Teflon homogenizer in 0.5 ml of SDS-PAGE sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 15% (v/v) glycerol] that contained either 2 mM $CaCl_2$ or 2 mM EGTA. The homogenate was centrifuged at 8,000*g* for 5 min to remove debris, and the resultant supernatant was used as the protein extract.

SDS-PAGE and Immunoblot Analysis

The protein extract (15 μl) was applied to SDS-PAGE according to Laemmli (1970) with 12.5% separation gel. After the gel was equilibrated with transfer buffer (0.1 M Tris, 0.192 M glycine, 5% methanol), proteins were electroblotted onto a polyvinylidene difluoride membrane for 90 min at 2 $mA \cdot cm^{-2}$ with a semidry electroblotting system (AE-6675, ATTO, Japan). The blotted membrane was immersed overnight at 4°C with 1% bovine serum albumin in TBS-T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) and then incubated with a primary antibody at a 1:3,000 dilution for 1 h at 24°C. After being washed with TBS-T, the membrane was incubated with horseradish peroxidase coupled to anti-rabbit or anti-mouse IgG (Amersham Pharmacia Biotech, Sweden) at a 1:1,000 dilution as a secondary antibody. Finally, the proteins recognized with the antibodies were visualized by chemiluminescence with an ELC Western blotting analysis kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

In-Gel Protein-Kinase Assay

The assay was performed according to the method of Kameshita and Fujisawa (1989) with a slight modification. The protein extract (15 μl) was applied to SDS-PAGE with 12.5% polyacrylamide gel containing 0.15 $mg \cdot ml^{-1}$ histone III-S (Sigma, USA) as an exogenous substrate. After the SDS-PAGE, the gel was incubated for 1 h at 20°C in a mixture of 50 mM Tris-HCl, pH 8.0, and 20% (v/v) 2-propanol, and then washed with Buffer A (50 mM Tris-HCl, pH 8.0, and 5 mM 2-mercaptoethanol) for 1 h. Next, proteins in the gel were denatured for 1 h at 20°C with 6 M guanidine hydrochloride in Buffer A and then renatured overnight at 4°C with 10% (w/v) polyoxyethylene (20) sorbitan monopalmitate (Wako Pure Chemical Industries, Japan) in Buffer A. Finally, protein kinase activities in the gel were assayed for 1 h at 25°C in a mixture of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 20 μM $CaCl_2$ or 1 mM EGTA containing 20 μM [γ-^{32}P]ATP (NEN Life Science Products, USA) at the specific activity of 27,750 $MBq \cdot mmol^{-1}$. After the gel was thoroughly washed with 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate, it was applied to IP-autoradiography with a bio-imaging analyzer, BAS-1800 (Fuji Photo Film, Japan).

RESULTS

Ca^{2+} Requirement for Spore Germination

Table 1 shows the effect of external divalent cations

on the spore germination of *O. japonica*. There was not much germination when no divalent cations were supplemented in the standard medium. On the other hand, the spores fully germinated in the presence of external Ca^{2+}. The free Ca^{2+} concentration giving a half-maximal germination was 3 μM (data not shown), which is very similar to that obtained with other fern species (Wayne and Hepler, 1984; Scheuerlein et al., 1989; Kagawa and Sugai, 1991). Furthermore, it was shown that Sr^{2+} and Mn^{2+}, but not Mg^{2+}, could substitute for Ca^{2+} in the spore germination of *O. japonica*. Sr^{2+} was also reported to substitute for Ca^{2+} in the spore germination of *Onoclea sensibilis* (Wayne and Hepler, 1984).

Next, we examined when *Osmunda* spores require external Ca^{2+} to germinate. In the experiment of Fig. 1A, closed symbols, *Osmunda* spores were initially cultured with a Ca^{2+}-free medium, then exposed to Ca^{2+} at the designated times after imbibition, and further cultured for 30 h in total before the spore germination was determined. The result showed that the germination ability is rapidly lost as the addition of external Ca^{2+} is retarded. The time that gave a half-maximal effect on the spore germination ($t_{1/2}$) was 5 h. This time was much shorter than the half-maximal time of 21 h in the course of spore germination of *O. japonica* (Fig. 1B), indicating that *Osmunda* spores require external Ca^{2+} in the early stage of spore germination. On the other hand, a converse experiment was conducted (Fig. 1A, open symbols). The spores were initially cultured with the Ca^{2+}-containing medium, then placed into the Ca^{2+}-free medium at the designated times, and further cultured for 30 h in total before the spore germination was determined. The graph obtained was almost symmetrical to that of Fig. 1A in closed symbols, although the $t_{1/2}$ was slightly longer as 7 h.

CDPKs in *Osmunda* Spores

A protein extract from the quiescent spores was subjected to immunoblot analysis with the anti-*Os*CDPK7 antibody (Fig. 2A). Although 15 bands were recognized by the antibody (Fig. 2A, lane 1), only four bands designated by arrowheads showed a Ca^{2+}-dependent mobility gel shift (Fig. 2A, lane2), in which the protein extract was electrophoresed in the SDS-PAGE sample buffer containing 2 mM EGTA. Because it is well known that CDPKs show Ca^{2+}-dependent mobility gel shift in SDS-PAGE (Harmon et al., 1987), the four bands with relative molecular weights of 56, 53, 49, and 47 kDa were concluded to be CDPKs.

In order to confirm this conclusion, we further examined protein kinase activities in the protein extract using in-gel protein-kinase assay (Fig. 2B). The protein extract was separated by SDS-PAGE with a separation gel that contained histone III-S as a substrate, and then the protein kinase activities were assayed in the gel. Four bands derived from protein kinase activities were detected on the gel when the reaction was carried out in the presence of Ca^{2+} (Fig. 2B, lane 1). The relative molecular weights were 70, 54, 48, and 38 kDa, respectively. On the other hand, the 54- and 48-kDa bands were hardly detectable when the reaction was carried out in the absence of Ca^{2+} (Fig. 2B, lane 3), indicating that the protein kinase activities were dependent on Ca^{2+}. In addition, the protein kinases showed the Ca^{2+}-dependent mobility gel shift (Fig. 2B, lane 2). Thus, the 54- and 48-kDa protein kinases were suggested to be CDPKs. However, this result was inconsistent with that obtained from the immunoblot analysis

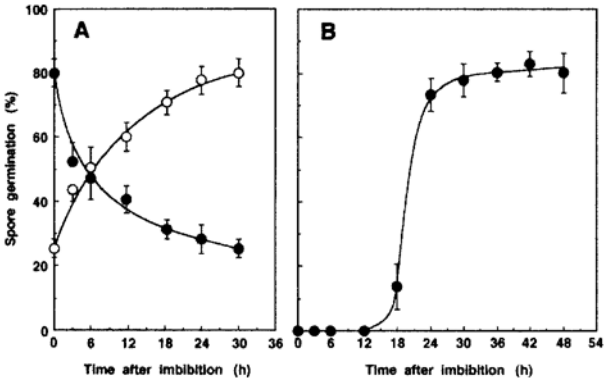

Figure 1. A. Temporal requirement for external Ca^{2+} during the spore germination of *O. japonica*. The culture was started with the Ca^{2+}-free (closed symbols) or Ca^{2+}-containing (open symbols) medium. At the designated times after imbibition, the spores were exposed to 1 mM Ca^{2+} (closed symbols) or placed in the Ca^{2+}-free medium (open symbols) and then further cultured for 30 h in total. **B.** Cumulative germination curve of *Osmunda* spores. The spores were imbibed in the Ca^{2+}-containing medium for the designated times, and then the spore germination was determined.

Table 1. Effect of divalent cations on the spore germination of *O. japonica*

Addition	% Germination
No addition	15 ± 1
Ca^{2+}	82 ± 7
Sr^{2+}	78 ± 5
Mn^{2+}	83 ± 3
Mg^{2+}	19 ± 3

The spores were germinated in the standard medium (10 mM MES-NaOH, pH 6.5, 3.45 mM KNO_3, 1 mM $MgSO_4$, 1 mM EGTA) supplemented with 2 mM of $CaCl_2$, $SrCl_2$, $MnCl_2$ or $MgCl_2$ for 30 h. Data are means ± 2SE of 4 measurements.

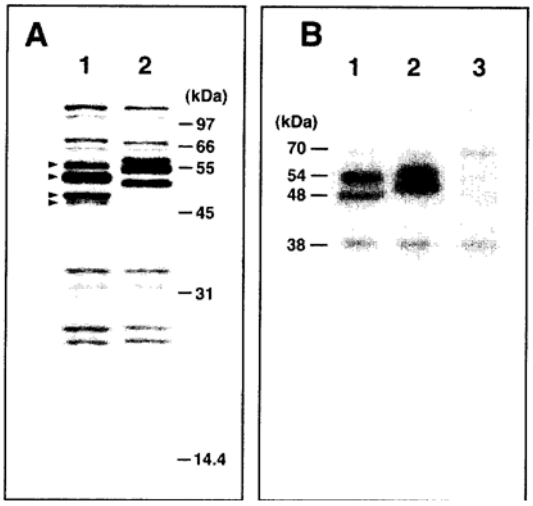

Figure 2. A. Immunochemical identification of CDPKs in the protein extract from the spores of *O. japonica*. The protein extract was prepared in the presence of 2 mM $CaCl_2$ (lane 1) or 2 mM EGTA (lane 2) (see Materials and Methods), separated by SDS-PAGE, and then blotted with anti-*Os*CDPK7 antibody from rice CDPK after transfer to a polyvinylidene difluoride membrane. Arrowheads indicate 56-, 53, 49-, and 47-kDa protein bands, respectively. **B.** In-gel protein-kinase assay of the protein extract. The protein extract was prepared from *Osmunda* spores in the presence of 2 mM $CaCl_2$ (lanes 1 and 3) or 2 mM EGTA (lane 2), and was separated by SDS-PAGE with a separation gel containing histone III-S as a substrate. After the proteins were renatured in the gel, protein kinase activities were assayed in the gel in the presence of 20 μM $CaCl_2$ (lanes 1 and 2) or 1 mM EGTA (lane 3).

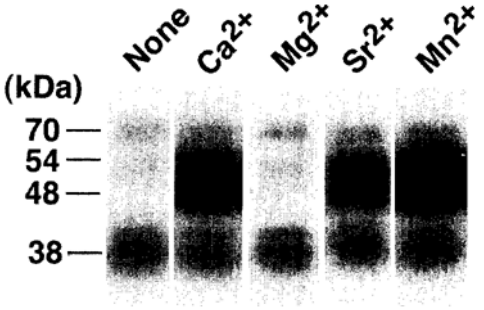

Figure 3. Effect of various divalent cations on the activation of the *Osmunda* CDPKs. The protein extract from *Osmunda* spores was separated by SDS-PAGE as described in Fig. 2B, and then protein kinase activities were assayed in the presence of 0.5 mM EGTA and 1 mM of various divalent cations.

Table 2. Effect of protein kinase inhibitors and calmodulin antagonists on the spore germination of *O. japonica*

Inhibitors	% Germination*
Noaddition	90 ± 6
K252a (2 μM)	21 ± 3
H-7 (50 μM)	90 ± 4
ML-7 (5 μM)	86 ± 1
KN-93 (5 μM)	84 ± 2
W-7 (0.1 mM)	24 ± 3
W-5 (0.1 mM)	84 ± 3

*Data are means ± 2SE of 3 measurements.

which showed four CDPK isoforms (Fig. 2A). Probably, only two CDPK isoforms could be detected by the in-gel protein-kinase assay. Alternatively, it seems that the CDPK isoforms diffused in the gel during the procedure of in-gel protein-kinase assay, resulting in two bands of 54- and 48-kDa.

Next, we examined whether Sr^{2+} and Mn^{2+} can activate the *Osmunda* CDPKs (Fig. 3), because Sr^{2+} and Mn^{2+} could substitute for Ca^{2+} in the spore germination of *O. japonica* (Table 1). The result showed that Sr^{2+} and Mn^{2+}, but not Mg^{2+}, fully activated the 54- and 48-kDa protein kinases as well as Ca^{2+} could. This implies that the *Osmunda* CDPKs may be targets of Ca^{2+}, Sr^{2+} and Mn^{2+} and are necessary to the spore germination process. However, the significance of Sr^{2+}- and Mn^{2+}-dependent phosphorylation is unclear at present.

Protein Kinase Inhibitors and Calmodulin Antagonists

Table 2 shows the effects of protein kinase inhibitors and calmodulin antagonists on the spore germination of *O. japonica*. The general protein kinase inhibitor, K252a, largely inhibited the spore germination to 21%. However, a protein kinase A inhibitor, H-7, and the calmodulin-dependent protein kinase inhibitors, ML-7 and KN-93, did not affect the spore germination. On the other hand, the germination was inhibited to 24% in the presence of a calmodulin antagonist, W-7, that was shown to inhibit soybean CDPKs (Harmon et al., 1987), while a less specific antagonist, W-5, had little effect on the germination. Thus, these results also imply that the *Osmunda* CDPKs may be involved in the germination processes of *O. japonica*.

Protein Phosphorylation in the Early Stage of Spore Germination

In order to examine whether the *Osmunda* CDPKs actually function during the spore germination, phosphoprotein levels were assayed by immunoblot analysis with a monoclonal antibody specific to phosphoserine (Fig. 4A). Protein extracts were prepared from *Osmunda* spores cultured for 0, 0.3, 1, and 3 h, respectively, in the presence or absence of external Ca^{2+}, and then applied to the immunoblot analysis. It was found that

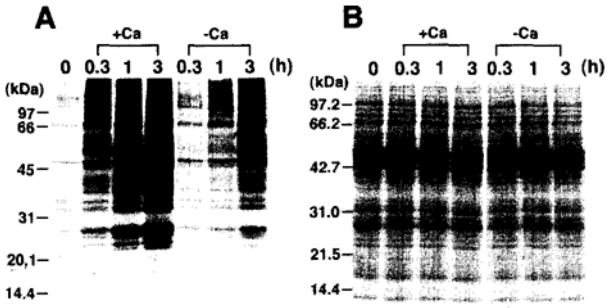

Figure 4. *In vivo* protein phosphorylation in the early stage of spore germination of *O. japonica*. The spores were cultured in the presence or absence of external Ca^{2+} for 0, 0.3, 1 and 3 h, and protein extracts were prepared from the spores as described in Materials and Methods. **A.** Phosphoproteins detected by the immunoblot analysis with monoclonal antibody specific to phosphoserine. **B.** Protein patterns stained with Coomassie brilliant blue.

the phosphoprotein levels largely increased with time in the presence of Ca^{2+} compared with the levels in the absence of Ca^{2+}. This result suggests that the *Osmunda* CDPKs may be responsible for a part of the protein phosphorylation *in vivo*. Figure 4B shows protein patterns stained with Coomassie brilliant blue. Because the protein levels and patterns did not change with time, the increase of the phosphoprotein levels observed in Fig. 4A was confirmed to be a post-translational event.

DISCUSSION

Fern spore germination has been believed to be under the control of external Ca^{2+} in some species, although the precise role of Ca^{2+} remains unknown (Roux et al., 1986; Tretyn et al., 1991). In this study, we showed that the spore germination of *O. japonica* is also under the control of external Ca^{2+}, and that the Ca^{2+} may be necessary to activate CDPKs for the germination. Four CDPK isoforms with relative molecular weights of 47-56 kDa were found in the quiescent spores, as detected by immunoblot analysis. Two of them were confirmed to have CDPK-like activities by in-gel protein-kinase assay. Several results obtained from the present study suggested that the *Osmunda* CDPKs may be necessary to the spore germination: (1) The general protein kinase inhibitor K252a and calmodulin antagonist W-7, which are reported to inhibit soybean CDPKs (Harmon et al., 1987; Lee et al., 1998), largely suppressed the spore germination, while protein kinase inhibitors specific to a protein kinase A, a myosin light chain kinase, and calmodulin-dependent protein kinases did not; (2) Sr^{2+} and Mn^{2+} could substitute for Ca^{2+} in the spore germination and also could activate the *Osmunda* CDPKs similar to Ca^{2+}, implying that the targets of Ca^{2+}, Sr^{2+} and Mn^{2+} are the CDPKs; and (3) Phosphoprotein levels increased rapidly immediately after imbibition in the presence of external Ca^{2+} during the period when *Osmunda* spores required external Ca^{2+} for the germination. From these results, we have concluded that CDPKs participate in the spore germination of *O. japonica*.

At present, the detailed roles of each isoform of the *Osmunda* CDPKs remain unknown. Nevertheless, we may speculate on the roles of the CDPKs from the findings reported so far. Wayne and Hepler (1984, 1985) have proposed that external Ca^{2+} may be acting as a second messenger in the phytochrome-mediated spore germination of *Onoclea sensibilis*. In the spores of *Adiantum capillus-veneris*, external Ca^{2+} was required for the entry to the S phase in the first cell cycle (Iino et al., 1989). Furthermore, chlorophylls were not synthesized in the absence of external Ca^{2+} during the spore germination of *Dryopteris paleacea* (Dürr and Scheuerlein, 1990). On the other hand, external Ca^{2+} has been demonstrated to control the position of asymmetric cell division sensing gravity in the germinating spores of *Ceratopteris richardii* (Chatterjee et al., 2000). Thus, it seems likely that many physiological events in germinating fern spores are controlled by external Ca^{2+}. In the future, it should be examined whether or not these physiological events are mediated by CDPKs.

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