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PiP Elicitor and Suppressor from *Phytophthora infestans* Regulate Ca^{2+}-Dependent Protein Kinase (CDPK) in the Plasma Membranes of Potato

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ABSTRACT

The relationship between the effect of Ca^{2+}-dependent protein kinase (CDPK) domain-III peptide-antibodies (Abs) raised against CDPK from *Arabidopsis thaliana* on the hypersensitive response of plant cells was investigated. The effect of peptide elicitor (PiP) and suppressor glucans from *Phytophthora infestans* on the kinase activity of membrane protein (MP) from potato (R_1 gene) was investigated. Stimulation of the kinase activity of the MP with CDPK-Abs but not with pre-immune sera was assumed to be caused by the interaction with CDPK suggesting the presence of the kinase in the MP. It was assumed that the interaction of kinase domain-III peptide-Abs with CDPK in MP might have disengaged the active site of CDPK resulting in an increase in the kinase activity of MP. The PiP and suppressor from *P. infestans* inhibited the kinase activity of MP, which contained CDPK-Abs. It was suggested that PiP and suppressor might have interacted with the active site resulting in the inhibition of the CDPK activity of MP. We suggest that PiP and suppressor may interact with CDPK of MP initiating the signal, which, in the case of PiP, leads to the occurrence and/or inhibition of HR.

Keywords: hypersensitive cell death, *in vitro* kinase assay, plant resistance, potato, *Solanum tuberosum* x *S. demissum*
Abbreviations: Abs, antibodies; **CDPK**, Ca^{2+}-dependent protein kinase; **HR**, hypersensitive response; **PiP**, peptide elicitor of *P. infestans*; **TBS**, Tris-buffered saline

INTRODUCTION

It was reported previously that the PiP (*P. infestans* peptide) elicitor of hypersensitivity isolated from *Phytophthora infestans* stimulated Ca^{2+}-dependent phosphorylation of several proteins of potato and bean cells (Furuichi 1993; Harper and Harmon 2005). The predominant kinase activity associated with the plasma membrane of plants is Ca^{2+}-dependent (Romeis *et al*. 2000; Harper and Harmon 2005; Kobayashi *et al*. 2007), including MAP3/MAP6 cascades rather than the CDPK cascade (Romeis *et al*. 2000; Ren *et al*. 2008).

Ca^{2+} effectively regulates the generation of reactive oxygen species (ROS) in the membrane of potato (Furuichi *et al*. 1997; Cheeseman 2007), which is involved in the initiation of a resistance response triggered by an incompatible interaction of potato and *P. infestans*. Ca^{2+} may also act as a secondary messenger in plant cells to activate protein kinases in response to various stimuli (Nurnberger *et al*. 1994; Dyachok *et al.* 1997; Lamb and Dixon 1997; Harper and Harmon 2005). It was reported that elicitor from *Phytophthora* spp. caused rapid influx of cytosolic Ca^{2+} (Zimmermann *et al.* 1997; Romeis *et al*. 2000). The increase of cytosolic Ca^{2+} concentration, which occurs within seconds after elicitation, might be a primary regulator of various processes in defense reactions (Scheel 1998; Scheel *et al*. 1999; Kawano *et al*. 2003).

In the present experiments Ca^{2+} strongly stimulated the kinase activity of potato microsomal proteins (MP) which was inhibited by EGTA, showing the presence of Ca^{2+}-dependent protein kinases (CDPK) in MP. Protein kinase and phosphatase have been shown to be crucial for the activation of early defense responses in potato *in vivo* phosphorylation experiments (Taylor and Low 1997; Yang *et al.* 1997; Scheel 1998; Hematy and Höfte 2008). Two autophosphorylation sites, Thr38 and Ser198 have been reported which are required for AvrPto-Pto resistance gene-mediated hypersensitive response (HR) in tomato and in the *Pseudomonas syringae* pv.-tomato interaction (Sessa *et al*. 2000). In this case, only two phosphorylation sites are important for the occurrence of HR in tomato. We also studied the junction domain in CDPK for possible phosphorylation sites.

A potential autophosphorylation site is also present in the putative autoinhibitory domain of CDPKs (Harmon 2000; Harper and Harmon 2005) which may have a role in the hypersensitive response.

In other studies (Furuichi *et al*. 1997, 1998) we reported that glucans from *P. infestans*, which suppressed the hypersensitive response, bound to the kinase domain of CDPK in a biosensor analysis. The interaction of CDPK with either elicitor or suppressor may regulate the hypersensitive defense reaction in the signaling pathway of potato. To investigate what CDPK exists in MP and whether PiP and suppressor from *P. infestans* can interact with the kinases, we searched the effect of CDPK domain-III Abs, PiP and suppressor on the kinase activity of the MP and performed immune blot-analysis of MP using CDPK Abs.

We report here that CDPK is localized in the membranes (including the plasma membrane) of potato. Moreover, CDPK Abs of kinase domain-III stimulated the CDPK of MP, and PiP elicitor and suppressor from *P. infestans* inhibited the kinase in MP with CDPK-Abs. A part of this report was reported at the International Congress of Molecular Genetics of Host Specific Toxins in Plant Disease, Tottori, Japan (1998).

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Original Research Paper

Table 1 Comparison of kinase domain III synthetic peptides with CDPKs from three different plant species.

CDPK peptides	Kinase domain-III											Homology (%)	
CDPK synthetic peptide		R	E	I	Q	I	M	H	H	L	A		
AK1 (*Arabidopsis thaliana*)	197[b]	R	E	I	Q	I	M	H	H	L	A	206[b]	100
SK5 (soybean)	81[b]	R	E	I	Q	I	M	H	H	L	S[a]	90[b]	90
RiCDPK2 (potato cv. 'Rishiri')	90[b]	K[a]	E	I	Q	I	M	H	H	L	S[a]	80[b]	80

[a] The underlined amino acids are different from the synthetic CDPK peptides. The synthetic CDPK peptides were derived from *AK1* of *Arabidopsis thaliana*.
[b] Numbers show the position of the amino acid in each protein from the respective genes.
Different letters indicate amino acids: A = Ala; E = Glu; I = Ile; H = His; L = Leu; Q = Gly; R = Arg; S = Ser.
AK1 (Harper *et al*. 1993), SK5 (Harper and Sussman 1991; Harper *et al*. 1993), RiCDPK2 (DDBJ AB0551809)

MATERIALS AND METHODS

Plant and oomycete strain

Phytophthora infestans (Mont.) de Bary isolate DN101 (race 0) grown in rye-seed medium (Furuichi *et al*. 1997) was used for the isolation of PiP and suppressor as described by Furuichi and Suzuki (1990). The purity of both was verified by TLC (Furuichi and Suzuki 1992). Microsomal membrane protein from potato cv. 'Eniwa' (R_1) was prepared according to our method reported previously (Furuichi *et al*. 1997).

Kinase assay

Kinase activity assay was performed using our previous method (Furuichi 1993; Furuichi *et al.* 1994; Furuichi and Yokokawa 2007). In brief, the assay was performed in a 96-well microtitre plate in a total volume of 155 µl per treatment. The mixture contained 33 mM Tris-HCl, pH 7.1, 20 mM phosphocreatine (Sigma), 25 µl of creatine phosphokinase (0.4 units, Sigma), and 1.5 µg of membrane protein (MP). To determine the effect on the kinase activity of the MP, we applied 100 µg/ml each of the elicitor and suppressor from *P. infestans*, race 0. The CDPK polyclonal antibodies and pre-immune serum at a 1:500 dilution were applied in 10 µl volumes. Assays were initiated by the addition of 10 µl of 15 mM ATP (Sigma) and were incubated at 30°C for 10 min. After incubation, 80 µl of 0.5% 1-naphthol (Wako) dissolved in stock alkali solution (1.5 M NaOH, 0.7 M $NaHCO_3$) and 40 µl of 0.26% of 2,3-butane dione (Wako) was added to each treatment. The readings were taken in a microplate reader (BioRad 3500, BioRad) at 595 nm at 10 min intervals. The temperature during the reading intervals was 30°C. In the inhibition of kinase assay, EGTA (*O*,*O'*-bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N'*,*N'*-tetraacetic acid, Dojindo, Wako, Tokyo) solution was added to PiP elicitor or suppressor.

Controls did not contain ATP or protein kinase.

Antibodies against CDPK domain-III

Peptides were synthesized by a FMOC solid-phase peptides synthesizer (Millipore). All peptides were confirmed to be more than 95% pure by analytical high performance liquid chromatography. The CDPK Abs were raised against CDPK from *Arabidopsis thaliana AK1* as reported previously (Furuichi *et al*. 1997, 1998). Control sera were obtained from a rabbit before injection with antigen.

SDS-PAGE and western immunoblotting

Microsomal MP (whose purity was not assayed) from potato cvs. 'Eniwa' (resistant to *P. infestans*) and 'Irish Cobbler' (susceptible to *P. infestans*) diluted in sample buffer, was resolved (5 µg per lane) in 12% SDS-gel (*ATTO*, Tokyo, mini PAGE system) according to the method described by Laemmli (1970). The separated protein was transferred onto polyvinylidene difluoride (PVDF) transfer membrane (0.45 µm pore size, Millipore, Bedford, MA) by wet electroblotting (Milliblot-SDS system) following the method of Towbin *et al.* (1979). SDS low range molecular weight standards (BioRad) were used for size determination. For Western analysis of the MPs with anti-CDPK Abs, the blots were blocked for 1 hr in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) with 3% BSA (w/v) at room temperature with gentle agitation. After two washes with TBST (TBS + 0.5% Tween 20) the blot was incubated with polyclonal anti-CDPK antibody (No. 825) in a 1:2000 dilution in TBST with 1% BSA for 12 hr at 4°C. After two further washes with TBST, the blot was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000 dilution, CAPPEL) for 1.5 hr at room temperature. The blot was washed twice with TBST, once with TBS and then rinsed two times with AP buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5) and then transferred electrophoretically to 0.45 µm PVDF transfer membrane. The signals were visualized by using AP-conjugate substrate kit (BioRad) following the manufacturer's instructions.

RESULTS AND DISCUSSION

Activity of MP by the treatment of CDPK Abs

To further investigate what type of kinase is involved in signaling cascade of PiP and suppressor in MP of potato, we used CDPK-Abs (No. 825), raised against kinase domain-III of CDPK gene (*AK1*) from *A. thaliana* (**Table 1**) for the treatment of MP. The synthetic peptides have high homology with *AK1* from *A. thaliana* (100%), *SK5* from soybean (90%) and *RiCDPK2* from potato cv. 'Rishiri' (80%) (**Table 1**). We observed that the CDPK Abs stimulated the kinase activity of the MP from potato cv. 'Eniwa' up to 44% after 35 min of the treatment as compared to the untreated MP (**Fig. 1**). On the other hand, pre-immune serum inhibited the kinase activity of MP up to 22% (**Fig. 1**). We assumed that the CDPK Abs recognized the CDPK antigen existing in the MP of potato and caused some conformational change in it. Stimulation of the kinase activity of MP may have been caused by the binding of CDPK-Abs to the kinase domain-III of CDPK in MP resulting in the availability of an enzymatically active part of CDPK that would have not have otherwise been available. Since the synthetic peptides have high homology with the domain-III of RiCDPK2 (80%) from potato cv. 'Rishiri' (DDBJ AB0551809), it was presumed that cv. 'Eniwa' may have the same CDPK sequence in MP as cv. 'Rishiri'. Abs alone showed little kinase activity.

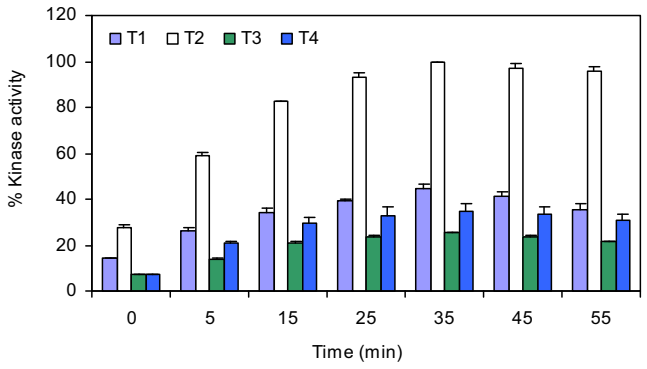

Fig. 1 Effect of PiP and suppressor (S) of *P. infestans* on the kinase activity of membrane protein (MP) from potato cv. 'Eniwa' with the CDPK-Abs. MP was added to a final concentration of 1.5 µg while PiP (2 µg) and suppressor (2 µg) were applied in a 20 µl volume. Abs diluted at 1:500 was applied in 10 µl volumes. T1: MP only; T2: MP with Abs; T3: MP with Abs mixed with PiP; T4: MP with Abs mixed with suppressor. Data are shown as the mean ± SD of two independent readings. Data for 'Irish Cobbler' published in Furuichi *et al*. (2002).

Effect of PiP and suppressor from *Phytophthora infestans* on the kinase activity of membrane proteins

PiP and suppressor glucans are produced by *P. infestans*. PiP plays an important role in incompatible and suppressors in compatible interaction of host and pathogen. The water-soluble nature of the elicitor and suppressor enhances the likelihood of their intracellular signal transduction of the host cells. In the present experiment when MP with CDPK-Abs was treated with PiP or suppressor in the absence of Ca^{2+}, the response of MP was different from the control MP containing Abs only and without Ca^{2+} in the reaction mixture (**Fig. 2**). We observed 20–25% stimulation of the MP activity after 5 min of PiP or suppressor treatment (**Fig. 2**). The stimulation of the kinase activity of MP caused by PiP increased to 29% and that by suppressor 21% after 35 min of treatment as compared to the MP without PiP or suppressor treatment. On the other hand we observed that PiP and suppressor can stimulate the kinase activity of

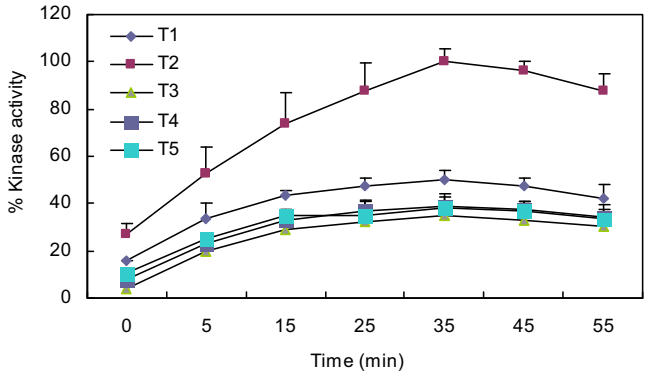

Fig. 2 Effect of CDPK-Abs of kinase domain-III (Abs) on the kinase activity of membrane protein (MP) from cv. 'Eniwa'. Abs and pre-immune sera were applied in a 1:500 dilution. MP was added at a final concentration of 1.5 µg. T1: MP only (20 µl); T2: MP (20 µl) with Abs (10 µl); T3: Pre-immune sera only (10 µl); T4: MP (20 µl) with Ps (10 µl); T5: Abs only. Data are shown as the mean ± SD of two independent replicates. Data for 'Irish Cobbler' published in Furuichi *et al*. (2002).

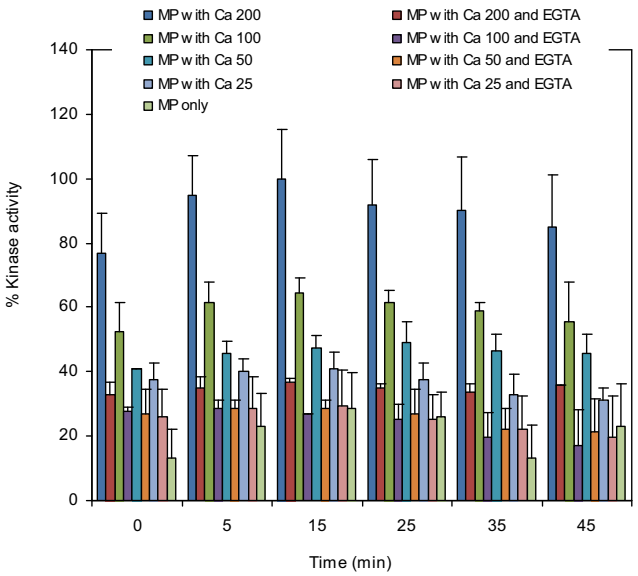

Fig. 3 Effect of exogenous Ca^{2+} and EGTA on the kinase activity of membrane protein (MP) from cv. 'Eniwa'. The concentration of MP was 1.5 µl and that of EGTA, 1.3 mM per well. MP was mixed with (**A**) Ca^{2+} (200 µM), (**B**) Ca^{2+} (200 µM) and EGTA, (**C**) Ca^{2+} (100 µM), (**D**) Ca^{2+} (100 µM) and EGTA, (**E**) Ca^{2+} (50 µM), (**F**) Ca^{2+} (50 µM) and EGTA, (**G**) Ca^{2+} (25 µM), (**H**) Ca^{2+} (25 µM) and EGTA; (**I**) MP only. Data are shown as the mean ± SD of two independent replicates. Data for 'Irish Cobbler' published in Furuichi *et al*. (2002).

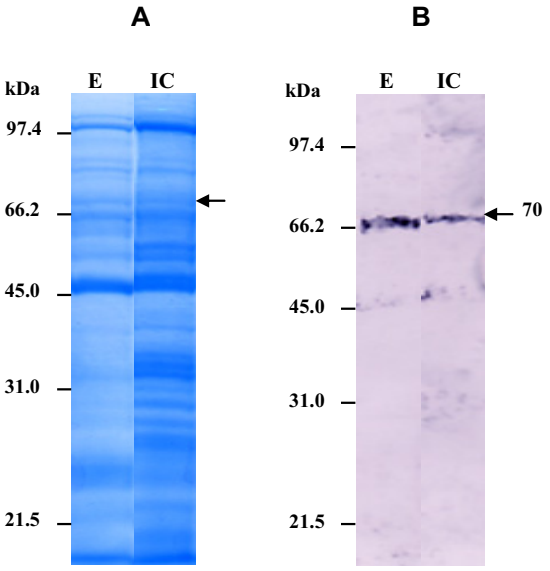

Fig. 4 SDS-PAGE and western immunoblot analysis of MP from potato cvs. 'Eniwa' (E) and 'Irish Cobbler' (IC). The concentration of the samples (5 µg per lane) of MP in **A** and **B** is the same. (**A**) SDS-PAGE pattern of MP stained with CBB. (**B**) MP immunoassay was carried out with anti-CDPK antibody. Sizes are shown in kDa.

RiCDPK2 in the presence of Ca^{2+} in the early period of treatment (Furuichi *et al.* 2002; Furuichi and Yokokawa 2007). In this study the stimulation of kinase activity of MP with PiP and suppressor treatment was attributed to the absence of Ca^{2+} and the presence of CDPK-Abs in the reaction mixture. It was also assumed that the interaction of suppressor from *P. infestans* with CDPK of MP stimulates the kinase activity.

Fig. 3 shows that the effect of exogenous Ca^{2+} and EGTA (1.3 mM) on the kinase activity of membrane proteins (MP) of cv. 'Eniwa' was dependent on the concentration of Ca^{2+} (200, 100, 50 and 25 uM). Ca^{2+} at 200 uM strongly activated CDPK kinase activity, but the addition of EGTA in the wells inhibited CDPK activity. These results suggested that the potato plasma membranes contained CDPKs in the signal cascade, and that EGTA had the critical effect on kinase activity in the plasma membranes of potato cells.

Through immunoblotting it is shown that potato microsomes contain the CDPK, 70 KD, protein band, showing the presence of CDPK 1 or CDPK 2 of potato in the plasma membranes of cells (**Fig. 4**). Both the resistant and susceptible cultivars contained CDPK1 and CDPK2 in their membrane fractions. This suggested that CDPK signal cascades of the membranes played a role in the early period of the infection process, and then the signals were transduced in downstream pathways in both cultivars.

CONCLUSION

Our results suggest that potato MP contain CDPKs (**Fig. 1**). The stimulation of kinase activity of the MP with CDPK-Abs but not with pre-immune serum may be caused by the binding of the Abs to kinase domain-III of CDPK in MP. PiP and suppressor treatment of potato MP with CDPK-Abs and without Ca^{2+} resulted in the inhibition of kinase activity of the MP. This inhibition might be the result of the interaction of PiP and suppressor with the kinase domain of RiCDPK2.

We have demonstrated that a 70 kDa CDPK from potato plasma membrane immunoreacted with anti-CDPK Abs and that CDPKs are localized in the plasma membrane of potato cells with an *R*- and *r*-gene to *P. infestans* as shown in **Fig. 3**, supporting findings from our previous data (Furuichi *et al*. 1997). Membrane-bound CDPKs have been reported in tomato and are activated in response to Avr9-elicitor from

Cladosporium fulvum (Sheen 1998; Harmon 2000; Romeis *et al*. 2000; Harper and Harmon 2005) which substantiates our present report of the localization of CDPK in the potato membranes (including the plasma membranes). The *Arabidopsis* cell death program reported by Dangle *et al*. (2006) did not contain CDPK signals in the signal cascades of cell death. Rather, we paid attention to the pathways following elicitor treatment, in which CDPK controls NADPH oxidase for the generation of reactive oxygen in plant cells.

In the present study PiP and suppressor from *P. infestans* inhibited the kinase activity of MP in the presence of CDPK kinase domain-III Ab and without Ca^{2+} in the reaction mixture. We assume that kinase domain-III of CDPK may play an important role in the activation of CDPK. We suggest that CDPK is involved in the signal transduction pathway of PiP and suppressor leading to switching between in the incompatible or the compatible interaction of potato and *P. infestans*.

To verify the findings of this study, we are now conducting a comprehensive dose response and interaction effect. Moreover, we reported elsewhere that GFP-CDPK2 is only localized in the plasma membrane of 'Irish Cobbler' to determine whether CDPKs are located in the host cells (Furuichi *et al*. 2002).

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JAPANESE ABSTRACT

Ca^{2+} 依存型タンパク質キナーゼ（CDPK）キナーゼドメインIIIのペプチド抗体（CDPK-Abs）を作成し、ジャガイモの過敏感反応における役割を検討した。疫病菌の生産するペプチドエリシター（PiP）および過敏感反応抑制因子であるグルカンサプレッサーのCDPKキナーゼ活性における影響を生化学的活性測定により評価した。CDPK-Absは、添加によりジャガイモ細胞膜中のCDPK活性を高めたが、非免疫抗体は影響しなかった。このことはまた、細胞膜中にCDPK-Absが認識する抗原の存在を示唆する。本実験結果は、CDPKキナーゼドメイン（I-XI）中でもドメインIIIが、CDPKキナーゼの活性の調節にかかわる事を示唆した。PiP とグルカンサプレッサーは、CDPK-AbsによるジャガイモCDPKの活性化を阻害した。これらの結果は、細胞膜CDPKの活性部位にPiPあるいはグルカンサプレッサーが直接作用する可能性を示唆した。PiPエリシターとグルカンサプレッサーは、CDPKと相互作用を行い、過敏感反応の誘導あるいは阻害作用をCDPKによる調節を介して行うことを示唆した。