Asian Journal of Plant Sciences

ISSN 1682-3974

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Asian Journal of Plant Sciences 2 (8): 585-592, 2003
ISSN 1682-3974

Localization of Calcium-Dependent Protein Kinase in Potato Microsomal Fraction and its Role in *Phytophthora infestans* Hyphal Cell Wall Component and Suppressor Signal Transduction

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Abstract: We investigated localization of calcium-dependent protein kinase (CDPK) in potato microsomal fraction (MF) and its role in relation to hyphal cell wall component (HWC) and suppressor glucan (suppressor) of *Phytophthora infestans* (Mont.) de Bary in hypersensitive resistance response (HR). Additionally, we studied activity of CDPK from MF in the absence and presence of staurosporine, a kinase inhibitor and okadaic acid, a phosphatase inhibitor. Stimulation of MF kinase activity by the addition of Ca^{2+} supported the presence of CDPKs in MF. This was confirmed when a 70 kDa CDPK was detected in potato MF using western immunoblot analysis. HWC and suppressor stimulated kinase activity of MF within 5-10 min after application. In the presence of staurosporine, HWC stimulated MF kinase activity but suppressor did not. In the presence of okadaic acid, HWC inhibited and suppressor stimulated kinase activity of MF. These results suggest that HWC and suppressor may interact with potato kinase(s) in the MF, initiating a signal leading to the occurrence of HR, or in the case of suppressor, to the inhibition of HR. We suggest that the signal cascades involving HWC and suppressor from *P. infestans* may be different in incompatible and compatible interactions, respectively, occurring between potato and *P. infestans*.

Key words: HWC, hypersensitive resistance response (HR), microsomal fraction, *Phytophthora infestans*, suppressor

Introduction

In our previous study, we reported that the hyphal wall component elicitor (HWC) and the suppressor glucan (suppressor), both from *Phytophthora infestans* (Mont.) de Bary, stimulated Ca^{2+} -dependent phosphorylation of several proteins of potato and bean cells (Furuichi *et al*., 1994). We also isolated from potato cells proteins related to β-lectin that recognize HWC (Furuichi and Suzuki, 1989). Furuichi *et al*. (1998) reported that the suppressor of HR from *P. infestans* bound to a CDPK in a biosensor analysis. Some kinase activity associated with the plasma membrane of higher plants has been reported to be Ca^{2+} -dependent (Klimczac and Hind, 1990; Klucis and Polya, 1988; Schaller and Sussman, 1988; Suzuki *et al*., 1992). A glycoprotein elicitor from *P. sojae* has been reported to cause a rapid influx of cytosolic Ca^{2+} (Zimmermann *et al*., 1997). Ca^{2+} effectively regulates superoxide anion (O_2^-) generation in the membrane fraction of potato (Doke and Miura, 1995; Furuichi *et al*., 1997). An oxidative burst is involved in initiation of HR triggered by an incompatible interaction of potato and *P. infestans* (Doke, 1983). It was suggested that Ca^{2+} may play a role in plant cells to activate protein kinases in response to some stimuli (Harmon *et al*., 1987). Recent studies have suggested a role for protein kinases and phosphatases in the activation of early defense responses *in vivo* phosphorylation assays in plants (Furuichi *et al*., 1994; Scheel, 1998; Yang *et al*., 1997).

We investigated the localization of CDPK in the MF from resistant and susceptible cultivars of potato by western immuno-blotting using anti-CDPK antibody. We also investigated the effect of HWC and suppressor on the kinase activity of the MF using staurosporine, a kinase inhibitor and okadaic acid, a phosphatase inhibitor. The interaction of CDPK with HWC and suppressor may regulate the signaling pathway leading to occurrence or inhibition of HR, respectively, in potato/*P. infestans* interactions. Here we report that a 70 kDa CDPK is localized to potato MF and may be involved in the signal transduction cascades of HWC and suppressor in potato/*P. infestans* interactions.

Materials and Methods

Preparation of suppressor: Fresh cultured fungal mat of

P. infestans was used for the isolation of suppressor, as reported previously (Doke and Tomiyama, 1980; Furuichi *et al*., 1997; Garas *et al*., 1979). *P. infestans* (Mont.) de Bary, race 0, was grown in rye-seed medium supplemented with 2% sucrose and 0.2% bacto yeast extract at 18°C (Doke and Tomiyama, 1980) prior to HWC and suppressor isolation.

All steps were performed at 4°C in an ice bath or a cold room, following the method of Garas *et al*. (1979) with slight modifications. Three-week-old hyphal mat of *P. infestans,* dried by desiccation and frozen in liquid nitrogen, was ground to powder with a dry-autoclaved pestle and mortar. The powder was homogenized with 0.05 M sodium acetate buffer, pH 4.5 (2 ml g^{-1} fresh weight of mycelia) containing 100 mM of phenylmethylsulphonylflouride (PMSF) until it turned into a viscous paste. The homogenate was sonicated twice for 1 and 1.5 min on ice. The sonicate was centrifuged at 12000 rpm using an RPR-20 rotor (Hitachi) for 20 min at 4°C. The supernatant was collected and the pellet was kept for HWC extraction. The supernatant was mixed with an equal volume of 80% phenol, incubated on ice for 3 h and centrifuged at 12000 rpm for 10 min at 4°C as above. The aqueous supernatant was collected and mixed with a chloroform:methanol (1:1 v/v) mixture in 1:1 v/v ratio. The components were mixed, incubated on ice for 30 min and the supernatant was collected. An equal volume of chloroform was added to the supernatant, the contents were mixed and incubated on ice for 30 min. The supernatant was collected and subjected to evaporation with an evaporator, adding water until the traces of phenol disappeared completely. Distilled water was added to the dried crystals at the rate of 2-2.5 ml g^{-1} of initial mycelial weight. The diluted sample was stored at –20°C and finally freeze-dried.

Preparation of HWC: Hyphal cell wall component elicitor (HWC) of *P. infestans* was prepared according to the method of Garas *et al*. (1979). The pellet, kept on ice during suppressor preparation, was dissolved in 100 mM sodium borate buffer, pH 8.8, in a 1:2 (w/v) ratio and homogenized mechanically on ice for 30 min. The homogenate was autoclaved at 120°C for 10 min. After cooling, the homogenate was centrifuged at 12,000 rpm using an RPR-20 rotor (Hitachi) at 4°C for 10 min. The supernatant was collected in another tube and the pellet was treated similarly as in the preceding step. The supernatant was collected and mixed with the previously collected aliquot. The supernatant was dialyzed in distilled water overnight, freeze-dried and stored at -20°C.

Preparation of potato MF: Potato MFs from cv. Eniwa and cv. Irish Cobbler were prepared according to the method reported by Furuichi and Suzuki (1989). Small pieces of potato tubers (100 g) were homogenized in 100 ml of homogenizing buffer in a blender for 30 at 4°C. The homogenizing buffer contained 35 mM Tris-HCl, 0.25 M sucrose, 2.3 mM O,O′-Bis (2-aminoethyl) ethyleneglycol-N, N, N′-N′- tetraacetic acid (EGTA, Wako), 100 ml of 0.4% polyvinylpyrrolidone (PVP), pH 8.0, 100 μl of 2-mercaptoethanol, 61 mg of dithiothreitol (DTT, Wako) and 25 μl of phenylmethylsulfonylflouride (PMSF, Sigma). The homogenate was filtered through four-layered cheesecloth and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was centrifuged at 27,000 rpm using an RP-30 rotor (Hitachi) at 4°C for 90 min to pellet crude MF. The MF was re-suspended in 10 ml of ice cold suspension buffer (1 mM Tris-MES, pH 7.2, containing 2.5 M sucrose) and stored at -80°C until used.

CDPK-peptides synthesis: CDPK peptides of the kinase domain-III of CDPK gene AK1 of *Arabidopsis thaliana* were synthesized by fmoc solid-phase peptides synthesizer (Millipore, Tokyo). All peptides were confirmed to be more than 95% pure by analytical reverse phase high performance liquid chromatography. Rabbit polyclonal CDPK antibodies were raised against the peptides as reported previously (Furuichi *et al*., 1998).

Kinase activity assay: Kinase activity was assayed using a microtiter plate with 155 μl reaction mixture according to the method reported by Furuichi *et al*. (1994). The reaction mixture contained 8.5 mM Tris-HCl, pH 7.1, 5 mM phosphocreatine (Sigma, St Louis), 0.4 units creatine phosphokinase (Sigma), 1 μM staurosporine or 1 μM okadaic acid, where appropriate, 1.5 μg MF and 2 μg each of HWC or suppressor from *P. infestans*. To determine the effect of Ca^{2+} on kinase activity in MF, kinase activity experiments were conducted in the absence or presence of Ca^{2+} (25, 50, 100 and 200 μM) alone or with EGTA (1.3 mM). Assays were initiated by adding 0.9 mM ATP, then incubated at 30°C for 10 min. Subsequently, 1-naphthol (0.2%, Wako, Tokyo) dissolved in stock alkali solution (1.5 M NaOH, 0.7 M Na_2CO_3) and 2,3-butane dione (0.06%, Wako, Tokyo) were added to each sample for color development. Absorbance was determined in the microplate reader (BioRad 3500) at 595 nm at 10 min intervals for 35 min. Temperature during the reading intervals was maintained at 30°C.

SDS-PAGE and western immunoblot analysis: MFs from potato cv. Eniwa and cv. Irish Cobbler were resolved (5 and 10 μg $lane^{-1}$) on a 12% SDS-PAGE gel according to the method described by Laemmli (1970). We used two

different concentrations of MF for both SDS-PAGE (Fig. 2A) and immunoblot analysis (Fig. 2B) to assess concentration dependent response. For western analysis, the MF was transferred onto polyvinylidene difluoride (PVDF) transfer membrane (0.45 μm pore size, Millipopre, Bedford, MA) by wet electroblotting following the method of Towbin *et al.* (1979). The membrane was blocked for 1 h in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) containing 3% BSA (w/v) and incubated for 12 h at 4°C with polyclonal anti-CDPK antibody at a 1:2000 dilution in TBST (TBS with 0.5% Tween 20) containing 1% bovine serum albumin (Sigma, Germany). After two washes with TBST, the membrane was incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (CAPPEL, Denmark) at 1:3000 dilution in TBS for 1.5 h at room temperature and visualized using an AP-conjugate substrate kit (BioRad, Tokyo).

Results

Effect of Ca^{2+} and EGTA on the kinase activity of MF: To investigate the effect of Ca^{2+} on kinase activity of MF, different concentrations of Ca^{2+} were applied to MF from potato cv. Eniwa (R_1-gene) and changes in kinase activity were measured. Kinase activity of MF was stimulated by Ca^{2+} addition (25, 50, 100 and 200 μM) in a concentration-dependent manner and inhibited by 1.3 mM EGTA (a Ca^{2+}-chelator). The highest concentration (200 μM) of Ca^{2+} stimulated kinase activity of MF ~6-fold as compared to the control (absence of Ca^{2+}) after 15 min of reaction (Fig. 1D). MF in the presence of a low concentration (25 μM) of Ca^{2+} also showed ~3-fold stimulation in kinase activity as compared to MF in the absence of Ca^{2+} after 15 of reaction (Fig. 1A). Stimulation of MF kinase activity in response to Ca^{2+} addition showed that the MF contained CDPK(s).

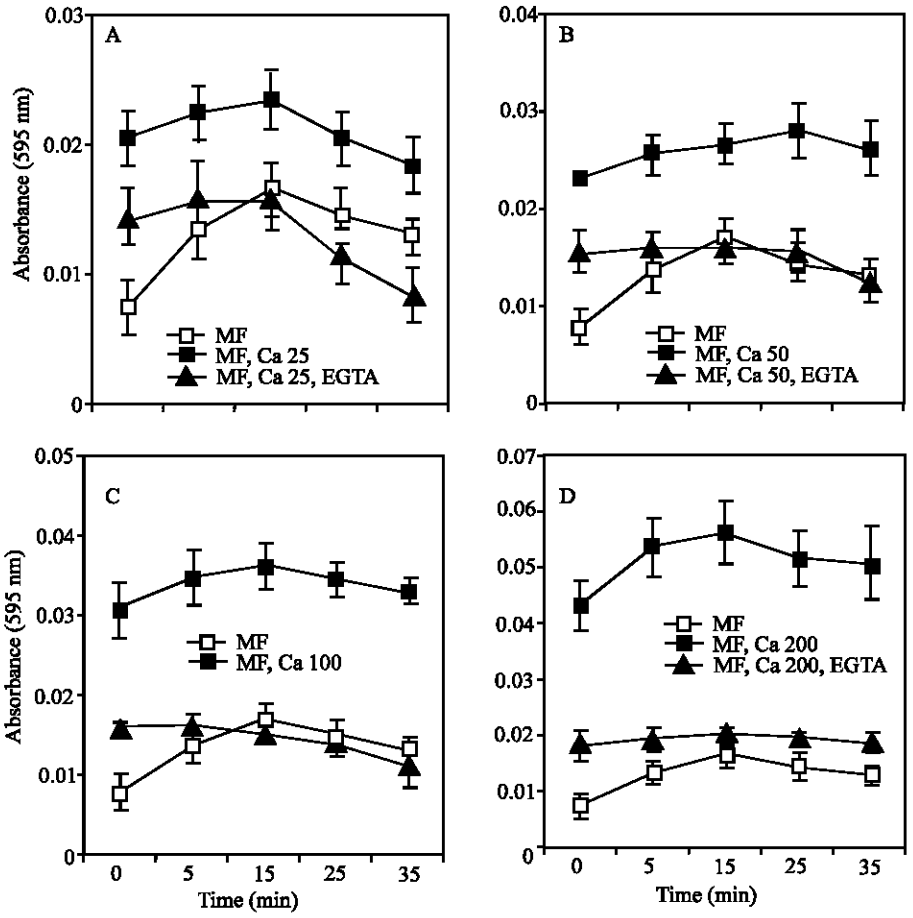

Fig. 1: Effect of Ca^{2+} and EGTA on the kinase activity of microsomal fraction (MF) from potato cv. Eniwa (R_1-gene). Kinase activity was determined in a microtiter plate. A, effect of 25 μM Ca^{2+}, B, effect of 50 μM Ca^{2+}, C effect of 100 μM Ca^{2+} and D, effect of 200 μM Ca^{2+}. Final concentrations were 1.5 μg, MF, 1.3 mM, EGTA. Control treatments (MF in the absence of Ca^{2+} and EGTA) were identical for all experiments in panels A-D. Data represent the mean of two separate readings ± SD

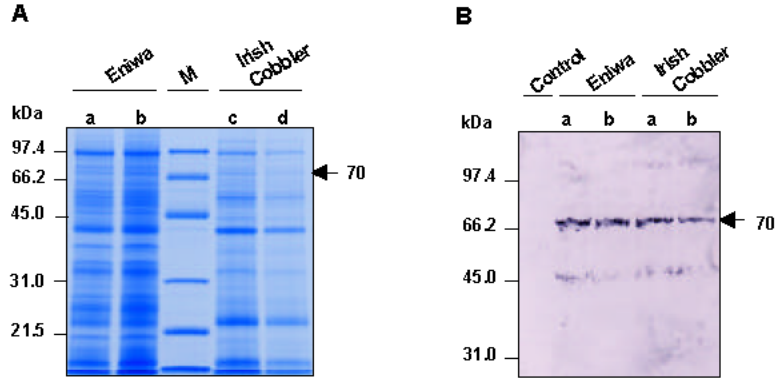

Fig. 2: Polyacrylamide gel electrophoresis (PAGE) and western immunoblot analysis of microsomal fraction (MF) from resistant potato cv. Eniwa (R_1-gene) and susceptible cv. Irish Cobbler (r-gene). A, SDS-PAGE pattern of MF stained with CBB. Concentrations of MF were: a, 5 μg; b, 10 μg; c, 10 μg and d, 5 μg. M denotes standard marker. B, Immunoassay of MF was carried out with anti-CDPK antibody (1:2000). Concentrations of MF were: a, 10 μg and b, 5 μg. Sizes are shown in kilodaltons (kDa). The control treatment suspension buffer (in the absence of MF)

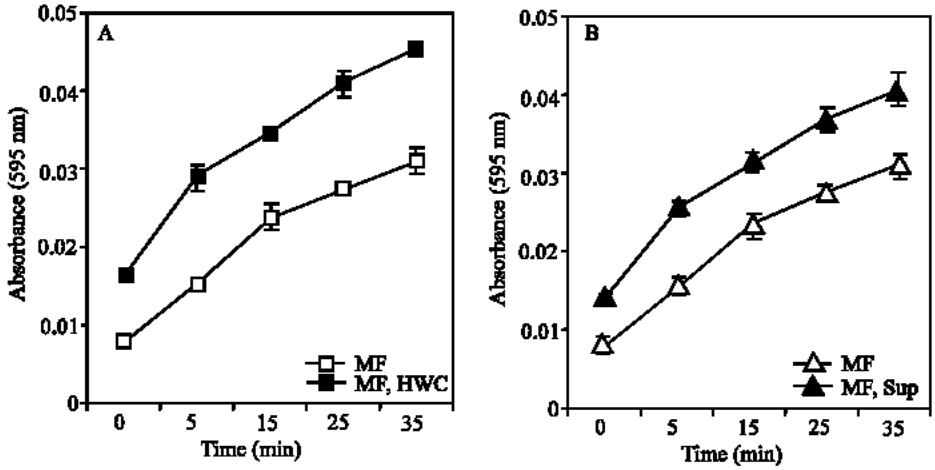

Fig. 3: Effect of HWC and suppressor (Sup) from *P. infestans* on the kinase activity of microsomal fraction (MF) from potato cv. Eniwa (R_1-gene). HWC and Sup were applied 2 μg well^{-1}, while MF was 1.5 μg well^{-1}. A, Effect of HWC and B, effect of Sup. Data represent the mean of two independent experiments ±SD

Localization of CDPK to potato MF: It has been reported that kinase active proteins exist in the plasma membrane of potato cells (Furuichi *et al*., 1994; Furuichi *et al*., 1998). To confirm the presence of CDPK in potato MFs from potato cv. Eniwa (R_1-gene) and cv. Irish Cobbler (r-gene), we used purified (more than 90%) anti-CDPK antibody, corresponding to a kinase domain-III of CDPK from *Arabidopsis thaliana* (AK1), against MF in immunoblot analysis. A 70 kDa protein band was observed in SDS-PAGE (Fig. 2A). The immunoblot showed that CDPK antibody strongly reacted with the 70 kDa protein in MFs from potato cv. Eniwa and cv. Irish Cobbler (Fig. 2B), containing R_1- and r-genes, respectively, showing that CDPK exists in both cultivars of potato. Two different concentrations of MF were used for SDS-PAGE and western immunobloting to confirm positive interaction of CDPK-antibody. The control treatment excluded MF. CDPK-antibody did not show any interaction in the control, but only in the presence of MF (Fig. 2B). It was evident from these results that CDPK exists in both resistant and susceptible cultivars of potato.

Effect of HWC and suppressor on MF: We studied *in vitro* effects of HWC and suppressor from *P. infestans* on MF from potato cv. Eniwa. HWC and suppressor addition to respective treatments of MF caused transient stimulation of kinase activity in MF. HWC caused 53% stimulation just after addition and 45% at 5 min (Fig. 3A).

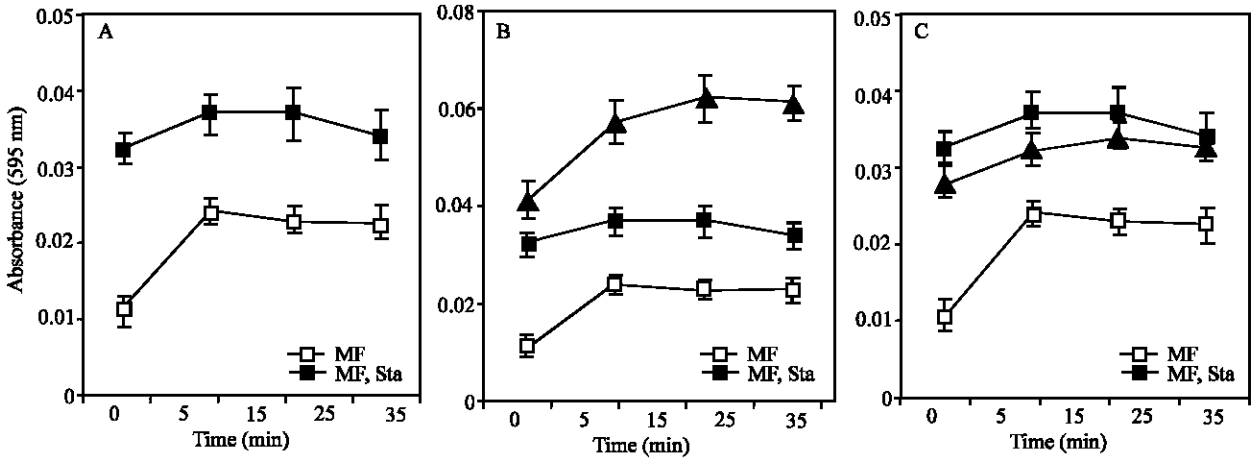

Fig. 4: Effect of HWC and suppressor (Sup) from *P. infestans* on the kinase activity of microsomal fraction (MF) from potato cv. Eniwa (R_1-gene) containing staurosporine (Sta). Concentrations of HWC, Sup and MF are the same as shown in Figure 2. Sta was applied at a final concentration of 1 μM. A, Effect of Sta, B, effect of HWC and C, effect of Sup. Data represent the mean of two independent experiments ± SD

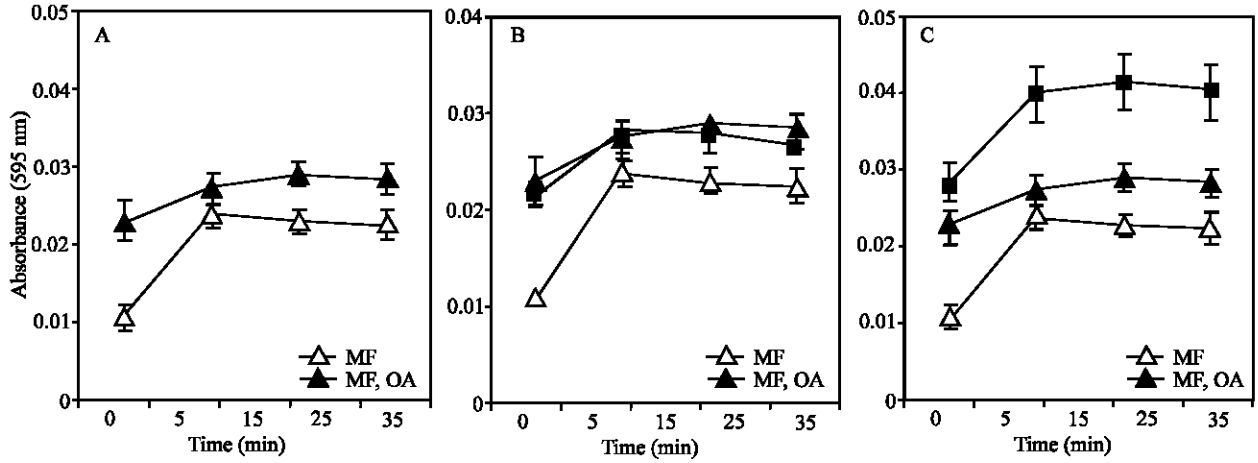

Fig. 5: Effect of HWC and suppressor (Sup) on the kinase activity of microsomal fraction (MF) from cv. Eniwa (R_1-gene) containing okadaic acid (OA). Concentrations of HWC, Sup and MF are as shown in Figure 2. OA was applied at a final concentration of 1 μM. A, Effect of OA, B, effect of HWC and C, effect of Sup. Data represent the mean of two independent experiments ± SD

On the other hand, suppressor addition also stimulated kinase activity in the MF up to 45, 39 and 23% at 0, 5 and 15 min, respectively (Fig. 3B). These results showed that HWC and suppressor effectively caused activation of MF kinase(s) in potato.

Effect of HWC and suppressor on MF containing staurosporine: To investigate whether HWC and suppressor stimulated different kinase(s), staurosporine, a kinase inhibitor, was added along with MF prior to HWC and suppressor treatment. Staurosporine did not inhibit, but stimulated kinase activity of MF up to 66% compared to untreated MF (Fig. 4A), immediately after the treatment; stimulation decreased to 35% by 15 min. When HWC was applied to MF treated with staurosporine, kinase activity was stimulated up to 26% at 0 min after treatment, 35% at 15 min, 41% at 30 min and 45% at 45 min after the treatment (Fig. 4B). Stimulation caused by HWC declined to 36% at 60 min. In contrast, suppressor caused inhibition up to 14% in kinase activity of MF with staurosporine just after treatment (Fig. 4C). The effect of suppressor became almost negligible at 45 min after treatment.

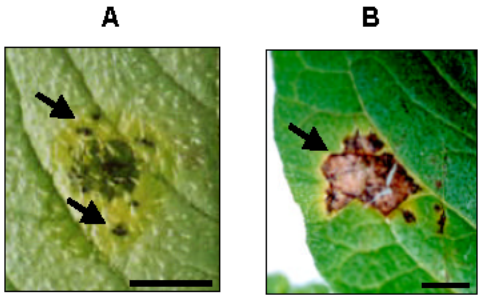

Fig. 6: A: Hypersensitive resistance response (HR) of potato cv. Eniwa (R_1-gene) to infection with *Phytophthora infestans* (Mont.) de Bary race 0, after 3 days of incompatible interaction. Arrows indicate the localized necrotic lesions of HR as a result of cell collapse that cause further progression of the pathogen to be restricted.
B: Symptoms of late blight disease in potato leaf from cv. Irish Cobbler (r-gene) caused by *P. infestans* in compatible interaction. Arrow shows brown necrotic spots with pale green margins that appeared on the upper surface of the leaves, resulting in characteristic disease symptoms. Scale bar is 1 cm.

Effect of HWC and suppressor on MF containing okadaic acid: Okadaic acid, a phosphatase inhibitor specific to serine/threonine phosphatase, PP1 and PP2A (Suganuma *et al*., 1992; Suganuma and Fujiki, 1993; Hernandez *et al*., 1991), stimulated kinase activity of MF up to 52% at 0 min (Fig. 5A). Stimulation caused by okadaic acid decreased to 13% by 15 min. HWC from *P. infestans* caused a little inhibition of okadaic acid-stimulated kinase activity of MF just after treatment (Fig. 5B). Addition of suppressor from *P. infestans* caused 19% stimulation of MF kinase activity in the presence of okadaic acid at 0 min, increasing to 31% at 15 min after application (Fig. 5C). This response of okadaic acid-treated MF toward HWC and suppressor was different from staurosporine-treated MF, where HWC stimulated and suppressor inhibited kinase activity.

Discussion

The present study suggests that potato MF contains Ca^{2+}-dependent protein kinases (Fig. 1) and that a 70 kDa CDPK that immunoreacted with anti-CDPK antibody was localized in MFs from potato cells with R_1- and r-genes (Fig. 2). One of the earliest cellular responses to stress stimuli is the change in cytoplasmic Ca^{2+} concentration in plant cells (McAinsh and Hetherington, 1998). The increase in cytosolic Ca^{2+} concentration, which occurs within seconds after elicitation, might be a primary regulator of various processes in defense reactions (Scheel, 1998) and also of potato CDPK activation. CDPK activation is accomplished by phosphorylation; its interconversion back to basal levels of unphosphorylated CDPK and thus inactivation, may be catalyzed by a protein phosphatase (Romeis *et al*., 2001). Some phosphorylation and dephosphorylation may be involved in HWC and suppressor signal transduction in the potato/*P. infestans* interaction. The large CDPK gene family implies that individual isoforms play different functions and participate in multiple distinct signaling pathways (Harmon *et al*., 2000). We have previously suggested that CDPK of potato plays a role in generation of active oxygen species (AOS) and inhibition of HR in potato cells (Furuichi *et al*., 1997; 1998). In a recent study it was reported that at CDPK stimulates NADPH oxidase activity in tomato protoplasts (Xing *et al*., 2001). Activation of membrane-bound CDPK of tomato in response to Avr9-elicitor from *Cladosporium fulvum* and the role of *Nt*CDPK in signaling pathways in plant defense response has also been reported (Romeis *et al*., 2000, 2001).

The HWC and suppressor are produced by *P. infestans* and were detected in germination fluid during the infection process (Furuichi *et al*., 1998). H.W.C. of *P. infestans* plays an important role as inducer of HR in incompatible interactions and suppressor serves as inhibitor of HR in compatible interactions of host and pathogen. It has been reported that suppressor from *P. infestans* binds to a plasma membrane associated CDPK of potato (Furuichi *et al*., 1998).

In the present study, immunoblot analysis showed that CDPK is present in the MF of both resistant and susceptible cultivars of potato. It was speculated that potato CDPK is involved in the signal cascade of HWC in incompatible interaction and of suppressor in compatible interaction of potato and *P. infestans*. The present results showed that kinase(s) affected by HWC treatment may not be inhibited by staurosporine. In contrast, since the suppressor did not stimulate kinase activity of MF containing staurosporine, it is suggested that suppressor-responsive kinase(s) may be inhibited by staurosporine. Staurosporine has been reported to be specific for protein kinase C and Ca^{2+}/calmodulim kinase-II (Yanagihara *et al*., 1991; Yuzuru, 1993). It appeared from the present results that MF from potato cv. Eniwa contained little staurosporine-sensitive kinase(s), but did contain kinase activity not affected by staurosporine (1 μM). We also observed that staurosporine did not inhibit kinase activity of the RiCDPK2 fusion protein, a CDPK from potato cv. Rishiri (R_1-gene) (data not shown). We speculate that

CDPK plays an important role in both incompatible (Fig. 6A) and compatible interactions (Fig. 6B) of potato and *P. infestans*. These data also show that the effect of HWC and suppressor on kinase(s) in the MF is different. We suggest that HWC and suppressor may transduce the initial signal via the same kinase and follow different pathways subsequently, leading to incompatible and compatible potato/*P. infestans* interactions, respectively.

Acknowledgments

We owe gratitude to Dr. M. Kojima (Niigata University), Dr. J. Harper (Scripps Institute, USA) and A. Shirata (BioResource Institute, Tukuba) for advice and discussion. We deeply acknowledge the critical review of the manuscript and valuable comments made by Dr. Martha (UNL, USA). We thank Dr. A. Suzuki (Niigata University) for use of the equipment. This work was supported by Grant-in-Aids from the Ministry of Education, Culture, Sports, Science and Technology, Japan to N. Furuichi and H. Oikawa and from Intelligent Cosmos Science Foundation (Sendai) to N. Furuichi. A. Hassan was supported by doctoral fellowship from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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