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Ca^{2+}-Dependent Protein Kinase in Tomato is Stimulated by Host-Selective Toxin from *Alternaria solani*

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ABSTRACT

We demonstrated that a fusion protein with a calcium-dependent protein kinase (CDPK) activity from the potato *RiCDPK2* gene, purified from *Escherichia coli*, was stimulated by two toxins from tomato early blight (*Alternaria solani*). The kinase activity of the RiCDPK2 fusion protein was stimulated by a host-specific toxin (HST), alternaric acid, and a non host-specific toxin, solanapyrone A, both produced by *A. solani*. The addition of Ca^{2+} and Mg^{2+} was required for the stimulation of kinase activity. We suggest that the HST from *A. solani* may stimulate RiCDPK2 kinase activity during the infection process as part of the process that leads to the compatible interaction between tomato and *A. solani*, comparable to the role of suppressor from *Phytophthora infestans* which inhibits the occurrence of hypersensitive cell death during the potato-*P. infestans* interaction.

Keywords: alternaric acid, CDPK, compatible interaction, host selective toxin, suppressor

INTRODUCTION

Host specific toxins (HSTs) are known to play an important role in disease development (Furuichi *et al.* 1992). Toxins cause physiological changes in host cells either by affecting the permeability of the cell membrane, through a rapid increase in electrolyte loss (Nishimura and Kohmoto 1983; Otani 2000; Coffeen and Wolpert 2004) or by inhibiting enzymes, interrupting the corresponding enzymatic reactions (Langsdorf *et al.* 1990; Furuichi *et al.* 1992). Treatment of potato tuber slices with alternaric acid (AA), from *Alternaria solani* (Ellis & Martin) Jones & Grout, resulted in delayed cell death when infected with an incompatible race of *Phytophthora infestans* and it was presumed that AA acted like a fungal suppressor (Furuichi *et al.* 1992). In this study we investigated (1) the effect of *A. solani* toxins on the kinase activity of purified RiCDPK2 protein, a calcium-dependent protein kinase gene from potato cv. 'Rishiri' (Furuichi *et al.* 1998; Okuta *et al.* 1999); and (2) the role of Ca^{2+} and Mg^{2+} in the toxin-RiCDPK2 protein interaction. Our aims were: (1) to understand the role of HSTs in the hypersensitive response (HR) in potato, its relevance to HR suppressor in the potato-*P. infestans* interaction and (2) whether HSTs and suppressors determine the compatibility of the pathogen. We report here the direct interaction of His-CDPK from potato with toxins *in vitro*. We also report that AA from *A. solani* affects the kinase activity of His-RiCDPK2 and that Ca^{2+} and Mg^{2+} may play an important role in the regulation of the toxin-CDPK interaction.

MATERIALS AND METHODS

Toxins and plants

AA and solanapyrone A (SpA) (**Fig. 1**) used in this study were purified from cultured fluid of *A. solani* as reported previously (Langsdorf *et al.* 1989) and chemically synthesized as reported by Tabuchi and Ichihara (1992) and Tabuchi (1994). For the bioassay of the effect of toxins from *A. solani*, fully expanded compound leaves from tomato (*Solanum lycopersicum* L., syn. *Lycopersicon esculentum* Mill.) cv. 'Fukuju II' were disinfected with 0.05% sodium hypochlorite and rinsed several times with distilled water. The leaves were treated with 30 µl of AA (25, 2.5, 0.25 and 0.1 µM) and incubated in aseptic moist conditions at 23°C under a 14-hr light period. In the case of SpA, tomato leaves were treated with 0.25 µM toxin (30 µl) and incubated under the same conditions as those for AA. Mock treatments were done with water, as a control. The assessments for necrosis were made after 24 hr of the treatment.

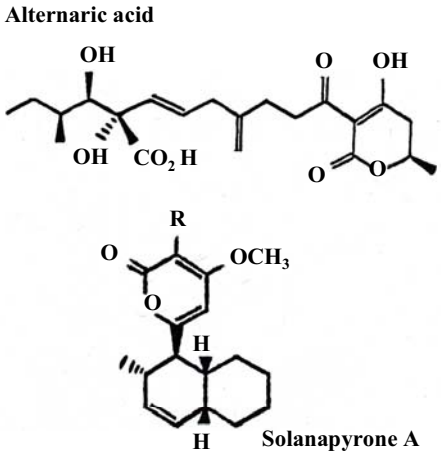

Fig. 1 Chemical structure of alternaric acid [12-(5,6-dihydro-4-hydroxy-6-methyl-2-oxopyran-3-yl)-4,5-dihydroxy-3-methyl-9-methylene-12-oxododec-6-ene-5-carboxylic acid] and solanapyrone A used in this study. Both the toxins are produced by *Alternaria solani*, the causal fungus of early blight of potato and tomato crops.

CDPK protein

The full length (1488 bp) of RiCDPK2 cDNA (DDBJ accession

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Research Note

No. AB0551809) was cloned into pCR-expression vector. The recombinant plasmid DNA was transformed into *E. coli* (BL21 pLysS) cells for expression according to the method described by Sambrook and Russell (2001). Recombinant protein was purified as described in the Xpress™ System protein purification protocol by using a His-bind resin column (Invitrogen) according to the manufacturer's instructions. The purified protein of RiCDPK2 was used for the subsequent kinase activity assay.

Purification of His-CDPK2

Fusion proteins were purified using the XpressTM System protein purification protocol (Invitrogen). The six-His-tagged fusion protein was loaded onto a ProBond His-bind resin column equilibrated with lysate buffer. The column was washed consecutively with 8 ml of denaturing binding buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8) followed by 8 ml of the same buffer at pH 6.0 or pH 5.3. The protein was eluted with 5 ml of the same buffer at pH 4.0. The eluate was dialyzed against 1000 ml of 10 mM Tris-HCl, pH 8.0, 0.1% (w/v) Triton X-100 overnight at 4°C to remove urea (the dialysis buffer was replaced four times). The concentration of the purified protein was determined using the BCA protein assay kit (Pierce) using bovine serum albumin (BSA) as a standard.

Kinase assay

A study to ascertain the effect of toxins on His-RiCDPK2 fusion protein in the presence of Ca^{2+} and Mg^{2+} was performed. The kinase activity assay was carried out using the method reported by Furuichi *et al.* (1994a) and (Furuichi and Yokokawa 2008), in a 96-well micro titer plate with a total volume of 155 µl per treatment per well. The reaction mixture contained 33 mM Tris-HCl pH 7.1 (40 µl), 20 mM phosphocreatine (40 µl, Sigma, St. Louis), 0.4 units creatine phosphokinase (25 µl, Sigma, St. Louis), and 1.5 µg (20 µl) of purified fusion proteins of RiCDPK2. To determine the effect of toxins on the kinase activity of the fusion protein of RiCDPK2, we applied AA and SpA (20 µl each) to the respective fusion protein treatment keeping the final concentration of the toxins at 20 µM. For the effect of toxins on the kinase activity of the fusion protein in the presence of metal ions, we added 10 µl each of $CaCl_2$ (100 µM final) and $MgCl_2$ (0.9 mM final) solution to the reaction mixture. Assay reactions were initiated by the addition of 10 µl of 15 mM ATP. The sample was allowed to incubate at 30°C for 10 min. Subsequently 60 µl of 1-naphthol (0.66%, Wako, Tokyo) dissolved in stock alkali solution (1.5 M NaOH, 0.7 M $NaHCO_3$) and 40 µl of 2,3-butane dione (0.26%, Wako, Tokyo) was added to each treatment (**Fig. 2**). Readings were made in the microplate reader (BioRad 3500) at 595 nm at 10 min intervals for 50 min. The temperature during the reading intervals was maintained at 30°C.

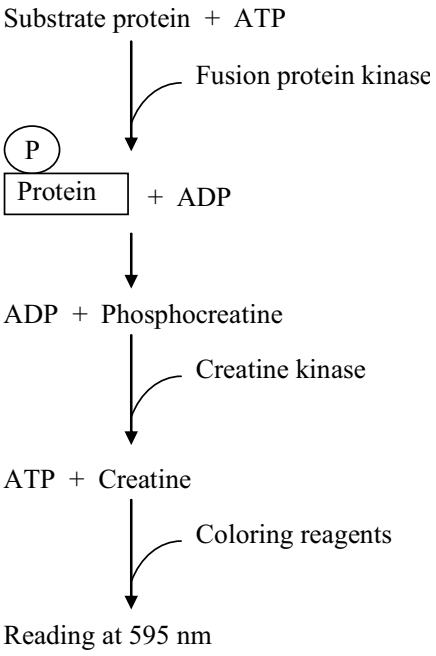

Fig. 2 Schematic presentation of kinase activity assay.

RESULTS

The effect of two *A. solani* toxins (AA and SpA) on tomato leaves is shown in **Fig. 3A** and **3B**. Both toxins induced necrosis on the leaf blade, although that caused by AA was in a concentration-dependent manner (from 0.1 to 2.5 µM). Leaf veinal necrosis was visible at 0.25 µM AA, 24 h after the application of the toxin (**Fig. 3A, c**). A more severe veinal necrosis of tomato leaf blade was observed with 25 µM AA (**Fig. 3A, a**). AA at 0.1 µM also caused yellowing of the treated site on the leaf (**Fig. 3A, d**). These necrotic symptoms were similar to the symptoms caused by infection with *A. solani* on potato leaves. In the case of SpA, symptoms did not appear on the leaves after 24 h, but only after 5 days (**Fig. 3B, a**).

We investigated the effect of toxins from *A. solani* on RiCDPK2 in a microplate kinase assay. Just after addition, AA stimulated the kinase activity (47%) of RiCDPK2, unlike the control, i.e. no AA at 0 min (**Fig. 4**). The stimulation of RiCDPK2 caused by AA increased until 10 min in contrast to the activity of RiCDPK2 without AA, which had started to decrease by that time. The effect of AA on RiCDPK2 added with Ca^{2+} and Mg^{2+} was also tested. AA showed different effects on the kinase activity of RiCDPK2 in the presence of Ca^{2+} (**Fig. 4**) or Ca^{2+} and Mg^{2+} together (**Fig. 4**). The kinase activity of RiCDPK2 was inhibited by AA up to 53% in the presence of Ca^{2+} early on (0 min) in the reaction (**Fig. 4**). When His-RiCDPK2 containing Ca^{2+} was supplemented with Mg^{2+}, 46% stimulation in the activity of RiCDPK2 was observed just after the reaction (**Fig. 4**). The addition of AA to RiCDPK2 containing Ca^{2+} and Mg^{2+} resulted in the stimulation (12%) of kinase activity after 10 min (**Fig. 4**). The stimulation tendency of the kinase activity of RiCDPK2 caused by AA was 22% higher in the presence than in the absence of Ca^{2+} and Mg^{2+} at 10 min (**Fig. 4**), indicating that the presence of both Ca^{2+} and Mg^{2+} in the reaction mixture played a role in the stimulation of the kinase activity of RiCDPK2. In another experiment, suppressor glucan of hypersensitivity from *P. infestans* also stimulated the kinase activity of RiCDPK2 containing Ca^{2+} and Mg^{2+} just after the addition of AA under the same conditions (Furuichi *et al*., unpublished).

Our results indicated that the stimulation of kinase activity of RiCDPK2 caused by the addition of Ca^{2+} and Mg^{2+} and RiCDPK2 resulted from the addition of AA, and was higher (**Fig. 4**) than AA treatments with RiCDPK2 with Ca^{2+} or RiCDPK2 only (**Fig. 4**). In contrast, the kinase activity of RiCDPK2 was adversely affected by the addition of AA in the absence of Mg^{2+}, resulting in an 18% decrease (**Fig. 4**) after 10 min.

DISCUSSION

So far, the physiological role of host specific toxin, alternaric acid has been reported from our and other laboratories. We have reported that AA inhibited the occurrence of hypersensitive cell death in tomato and potato (Langsdorf *et al*. 1990b; Furuichi *et al*. 1994a). In this report, our aims were to investigate the signaling mechanisms of AA cascades, especially CDPK signalling in host cells.

The Ca^{2+} and Mg^{2+} requirements for AA and SpA to interact with RiCDPK2 were different. AA inhibited RiCDPK2 kinase in the presence of Ca^{2+} (**Fig. 4**) but stimulated it in the presence of Ca^{2+} and Mg^{2+} together (**Fig. 4**) while SpA inhibited the RiCDPK2 kinase activity in the presence of Ca^{2+} alone and showed little stimulation in the presence of Ca^{2+} and Mg^{2+} together at 50 min (**Fig. 5**). We thus assumed that AA, and not SpA, plays an important role in the signal transduction of the inhibition of HR in host cell, interacting with both Ca^{2+} and Mg^{2+} in a role in the sti-

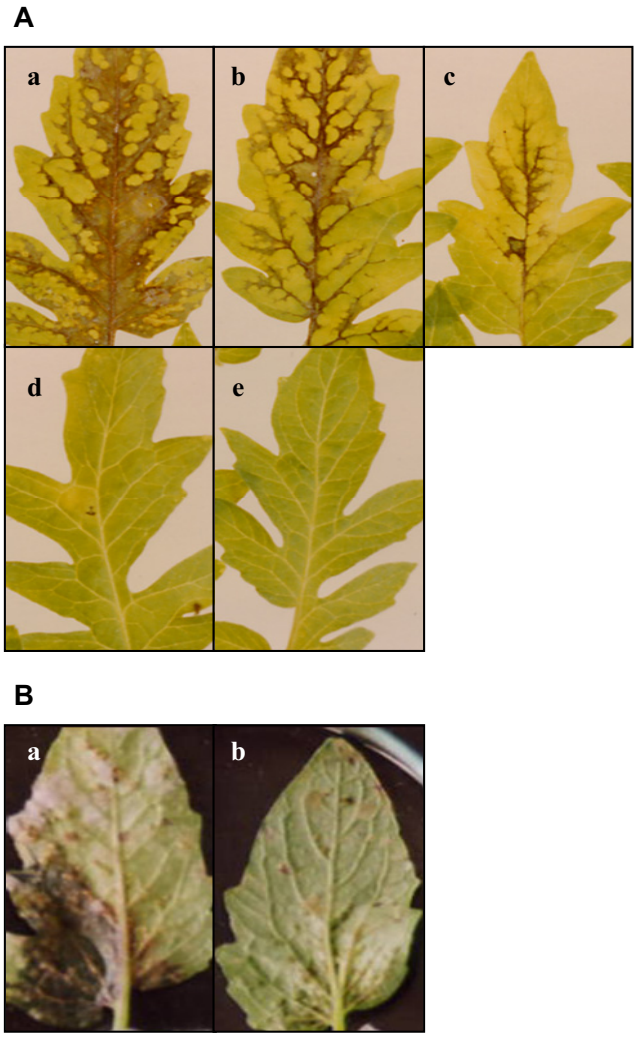

Fig. 3 Bioassay of tomato leaves treated with micromolar concentrations of host-specific toxin, alternaric acid (AA) (A) and solanapyrone A (SpA) (B), both produced by *Alternaria solani*. The observations for AA were made 24 h and for SpA 5 days after treatment. The treated leaves were incubated under aseptic moist conditions at 23°C with a 14 h light period. For AA: a, 25 µM; b, 2.5 µM; c, 0.25 µM; d, 0.1 µM; and e, control treatment (water). For SpA: a, 0.25 µM; and b, control treatment (water).

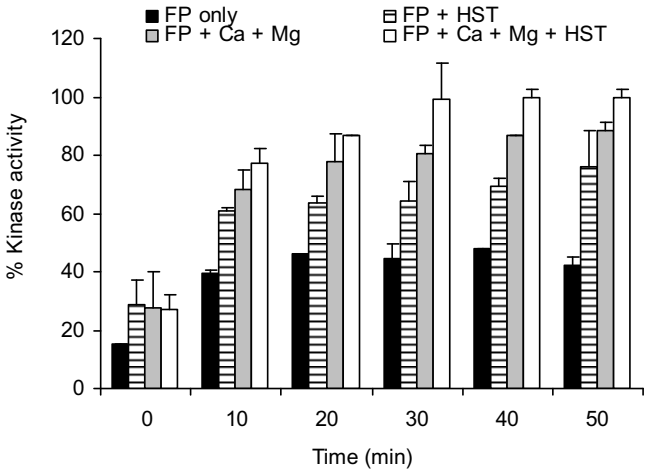

Fig. 4 Effect of alternaric acid (AA), on the kinase activity of the affinity purified His-RiCDPK2 fusion protein (FP) containing Ca^{2+} and Mg^{2+}. The final concentrations were as: FP (1.5 µg), AA (20 µM) and Ca^{2+} (100 µM) and Mg^{2+} (0.9 mM). For **A**: T1, FP only; T2, FP treated with AA; T3, FP containing Ca^{2+}; and T4, FP containing Ca^{2+} and treated with AA. For **B**: T1, FP only; T2, FP treated with AA; T3, FP containing Ca^{2+} and Mg^{2+}; and T4, FP containing Ca^{2+} and Mg^{2+} and treated with AA. Each bar represents the mean ± SD of two independent experiments.

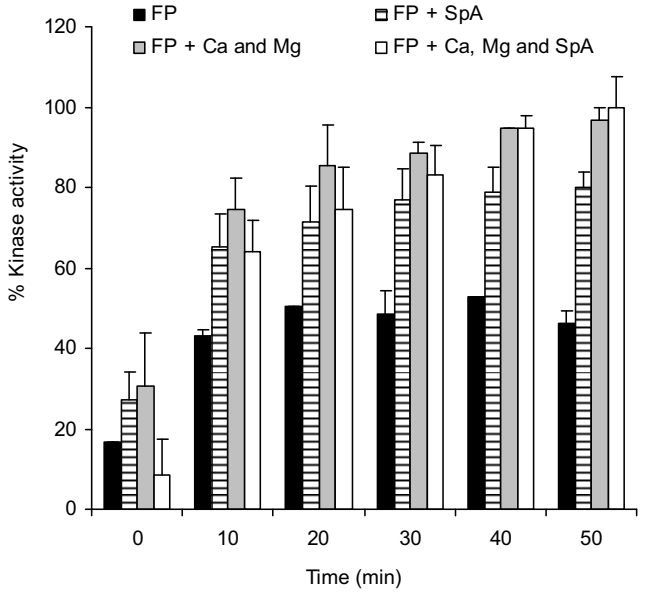

Fig. 5 Effect of solanapyrone A (SpA) on the kinase activity of the affinity purified His-RiCDPK2 (FP) containing Ca^{2+} and Mg^{2+}. The final concentrations used were as shown in **Fig. 4**. For **A**: T1, FP only; T2, FP treated with SpA; T3, FP containing Ca^{2+}; and T4, FP containing Ca^{2+} and treated with SpA. For **B**: T1, FP only; T2, FP treated with SpA; T3, FP containing Ca^{2+} and Mg^{2+}; and T4, FP containing Ca^{2+} and Mg^{2+} and treated with SpA. Each bar represents the mean ± SD of two independent readings.

mulation of RiCDPK2. The large variability, which is as large as the activation may be as a result of only two measurements. Few measurements did not permit us to conduct tests of significance.

AA has been reported to act as an HST in disease development (Furuichi and Nishimura 1984; Langsdorf 1991; Furuichi *et al*. 1994) and shows an early effect on the plasma membrane of the host cell (Langsdorf 1991; Otani 2000). The key role of HSTs in pathogenesis is assumed to be similar to that of suppressor of host resistance mechanisms (Furuichi 1984; Langsdorf *et al*. 1989; Otani 2000). AA (Furuichi 1984; Langsdorf 1991; Tabuchi and Ichihara 1992; Tabuchi 1994) is a primary determinant of the pathogenicity of the fungus in its host (Langsdorf *et al*. 1990; Furuichi *et al*. 1994) and is thought to play a role in the infection process leading to the suppression of hypersensitive cell death (Furuichi *et al.* 1992).

The present study suggests that toxins directly influence the kinase activity of RiCDPK2. AA has previously been reported to delay significantly hypersensitive cell death like a suppressor in the potato-*P. infestans* interaction (Furuichi *et al*. 1992). Since AA caused a significant delay in defense responses of host cells, it was presumed that AA is a member of suppressors of the hypersensitive defense reaction and it may down-regulate RiCDPK2 resulting in the inhibition of HR in the host plant.

On the other hand, in this study, SpA showed little stimulation of RiCDPK2 in the presence of Ca^{2+} and Mg^{2+} at 10-30 min after the reaction (unpublished data; Furuichi and Ichihara). It should be noted that SpA has neither been checked in germination fluid of *A. solani* nor has its physiological role been ascertained in HR, unlike AA which has been reported to delay HR (Furuichi *et al*. 1992).

We have identified the signal transduction pathway for the toxins from *A. solani*. We have also purified the recombinant protein of a kinase gene *RiCDPK2* from potato cv. 'Rishiri' localized in the potato plasma membrane (Furuichi and Yokokawa 2008). It may be possible that AA produced in the germination fluid from the fungus at an early period of infection could bind to the plasma membrane kinase of the host cell and then transduce the signal for inhibiting the

hypersensitive death of the host cells. This is the first report on the identification of the target kinase of pathogenesis factors in a plant-pathogen signal transduction system.

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