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Signaling of CPK2, Ca^{2+} Dependent Protein Kinase, from Potato by Alternaric Acid, a Host-Selective-Toxin in the Suppression of HR, and Solanapylon A Produced by *Alternaria solani*

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Abstract

Effect of alternaric acid (AA), a host-specific toxin (HST) and the suppressor of hypersensitive cell death (HR) produced by *Alternaria solan*i, on a plasma membrane and cytosolic kinase, CPK2 (Ca^{2+}-dependent protein kinase), of potato and on HR of host cells was investigated. We report here that AA in the presence of Ca^{2+} stimulates *in vitro* phosphorylation of His-CPK2, a CPK from potato cv. Rishiri. Ca^{2+} played an important role in the interaction between AA and CPK2. These results suggest that AA regulate CPK2 kinase during the early infection process in a compatible interaction between potato, tomato and *A. solani*, leading to the inhibition of HR in the host cells. Solanapylone A, a non-host specific toxin of *A. solani*, also stimulated the CPK2 in host cells. We suggest that AA is a primary HR suppressor by which *A. solani* may stimulate CPK activity in the host suppressing the hypersensitive cell death in host cells.

Keywords: *Alternaria solani; Alternaric Acid; Phosphorylation; CPK; Host-Selective Toxin; Hypersensitive Cell Death*

Introduction

Recently, Furuichi., *et al*. [1,2] and other groups [2-5] have been reported that CPK regulated the AOS generation in resistant and compatible interaction of potato-Phytophthora by using the single molecule signal analysis. CPK1, plasma membrane binding kinase, and CPK2, a cytosol localizing one, played a role in AOS regulation in host cell.

Host-selective toxins (HST) and a suppressor of hypersensitive response (HR) are low molecular weight, secondary metabolites belonging to various classes of chemical compounds [5-10]. HSTs have been reported as primary determinants of pathogenesis in the recognition of host cells and in disease development, similar to the suppressor of *Phytophthora infestans*. Toxins cause physiological change in host cells altering cell membrane permeability resulting in the rapid increase of electrolyte loss [6,7,11,12] and decrease the membrane potential of potato cell [1,13-15]. Alternaric acid (AA) was reported to play a role to determine host specificity and to contribute to disease development caused by *A. solani* [16,17]. Treatment of potato tuber slices with AA resulted in delayed HR when infected with an incompatible race of *P. infestans*, suggesting that AA is a fungal suppressor [17,18]. Tabuchi and Ichihara [9] reported complete stereochemistry and full synthesis of AA. It was also reported that biological Diels-Alder reaction is involved in the polyketide pathway for the production of AA [19,20].

Suppressor molecules from compatible pathogens cause inhibition of HR [4,5]. Suppressors isolated from *P. infestans*, are soluble glucans containing units bonded via β-1, 3 and β-1, 6 linkages [1,13,21,22]. Ca2+-dependent phosphorylation of various proteins of potato was reported after treatment with the elicitor, hydrogen peroxide, salicylic acid and suppressor glucan from *P. infestans* [17,23], showing the role of Ca2+-dependent protein kinase(s) (CPKs) in response to various stimuli.

CPKs are multifunctional with several isoforms providing specific pathways to control transcription, metabolic enzymes, membrane transport and cell structure [4,24-26].

In this study we investigated the effect of AA on the phosphorylation activity of the purified His-CPK2 (DDBJ accession number AB051809), a new isoform of CPK gene family from potato cv. Rishiri, a highly resistant cultivar to *P. infestan*s [27]. We further examined the role of Ca^{2+} and Mg^{2+} in the interaction of AA and His-CPK2.

Purpose of the Study

The purpose of this report is to ascertain 1) the role of AA as an HST and its effect on HR in potato and tomato and to compare the effect of HST with that of suppressor of HR in the host-*P. infestans* interaction and 2) the role of AA and solanapylone A [9] in the CPK signaling of AOS (Active Oxygen Species) generation in NADPH oxidase regulation in the host-parasite interaction.

Materials and Methods

Leaf bioassay

AA used in this study was purified from cultured fluid of *A. solani* as reported previously [28]. The fungus was grown in potato glucose medium at 25oC for 25 days. The cultured fluid was fractionated by silica gel column chromatography and AA was eluted with a mixture of chloroform and ethanol (19:1, v/v) as reported by Furuichi [1,13,20]. To study the biological effect of AA on plant leaves, fully expanded compound leaves from tomato cv. Fukuju II were used. Leaves were disinfected with 0.1% sodium hypochlorite and rinsed several times with distilled water. The surface of two leaves per plant was gently punctured uniformly over an area of ~15 mm2 with a needle before AA treatment. Leaves were treated with 30 µl AA (0.1, 0.25, 2.5, and 25 µM, each on different leaves) and incubated under aseptic moist conditions at 23°C for 14h under light. The leaves were assessed for necrosis beginning 24h after AA treatment.

Preparation of a cDNA library and DNA templates for the polymerase chain reaction (PCR)

Total RNA was isolated from potato leaves and tuber disks according to the method of Nagy [29] and was purified by chromatography on oligo (dT) cellulose, as described [29]. Double-stranded cDNA was synthesized from poly (A)+-RNA using a cDNA synthesis System Plus (Amersham Bioscience, Tokyo).

Preparation of Fusion CPK2

CPK2 was isolated from potato cv. Rishiri with R1-resistance gene to *P. infestans* [1,30]. CPK2 contains a kinase domain followed by a 31 amino acid putative auto-inhibitory domain that presumably functions as a pseudosubstrate inhibiting phosphorylation reactions in the absence of Ca^{2+} [24,25,31] and Ca^{2+}-binding regulatory domain to the C-terminus. The autoinhibitory domain in CPK2 contains a potential autophosphorylation site (Lys-Gln-Phe-Ser) [24]. A BLAST search of DDBJ database revealed that the amino acid sequence corresponding to the serine/threonine protein kinase active site and the autophosphorylation site within CPK2 are 100% identical to the respective sites in AK1 from *Arabidopsis thaliana* [25]. The CPK2 possesses 33 serine and 21 threonine residues with TGA stop codon at base pair 1500 from the ATG start codon in N-terminal priming site.

Full length of CPK2 cDNA (1488 bp) was cloned into the pCR-expression vector (Invitrogen, Carlsbad, USA) and transformed into *E. coli* (BL21 pLysS) for expression according to the reported method [29]. Protein expression was induced by adding 0.6 mM IPTG to Luria-Bertani (LB) culture medium containing transformed *E. coli* cells with 70 µg ml-1 ampicillin, incubated shaking for 24h at 25°C and harvested at 4000 rpm for 10 minutes at 4°C. The protein was isolated in guanidinium lysis buffer by centrifugation using RPR-20 rotor (Hitachi). The supernatant was collected and purification was achieved using a histidine affinity column according to the manufacturer's instructions (Invitrogen XpressTM System). The protein concentration was determined using the Bio-Rad protein assay kit according Bradford (1976) [32] using bovine serum albumin (BSA) as standard. The final concentration of the His-tagged protein ranged from 80 to 100 µg ml^{-1}.

Phosphorylation assay

The purified His-CPK2 was used for the phosphorylation assay. Effect of AA on His-CPK2 phosphorylation in the presence of Ca^{2+} and Mg^{2+} was studied *in vitro* using the assay reported by Furuichi., *et al*. [17] and Furuichi., *et al* [13]. Assays were performed in a 96-well microtiter plate with a total volume of 155 µl per well. The reaction mixture contained 8.5 mM Tris-HCl, pH 7.1, 5 mM phosphocreatine (Sigma), 0.4 unit creatine phosphokinase (Sigma), and 1.5 µg His-CPK2. To determine the effect of AA on the phosphorylation of CPK2, 25 µM AA was applied to the respective sample.

To determine the effect of AA on the phosphorylation of his-CPK2 in the presence of Ca^{2+} ions, the phosphorylation experiments were conducted in the absence or presence of 100 µM Ca^{2+} and 0.9 mM Mg^{2+}. Assays were initiated by adding 0.9 mM ATP and then incubated at 30°C for 10 minutes. Subsequently, 1-naphthol (0.2%, Wako, Tokyo) dissolved in stock alkali solution (1.5M NaOH, 0.7 M $NaCO_3$) and 2, 3-butane dione (0.06%, Wako) was added to each sample for color development. The absorbance was determined in the micro plate reader (BioRad) at 595 nm at 10 minutes interval for 40 minutes.

Results

Alternaric acid bioassay

A leaf-puncture on the center of the leaves with needle was employed to investigate the effect of AA on plant tissues. Different concentrations of AA were used to test the effect on tomato leaves (Figure 1A). Characteristic symptoms of toxicity by AA are veinal necrosis (Figure 1A, b, c) and intercostal necrosis (Figure 1A, a) in leaves of tomato, and a broad chlorosis, which subsequently turns necrotic. Biological assay shows that the severity of necrosis caused by AA in tomato leaves is concentration-dependent (Figure 1A). Veinal necrosis became visible with 0.25 µM AA on the leaf within 24h of application (Fig. 1A. c). Severe veinal necrosis of tomato leaf blade was observed with 25 µM AA as compared to the other concentrations (Figure 1A. a). In the present study, 0.1 µM AA also caused yellowing of the treated site on the leaf (Figure 1A. d). In contrast, as shown in figure 1B, A. solani caused symptoms in tomato leaf 2 days after inoculation, and the necrosis spread further during the next 5 days after the infection. These symptoms (Figure 1B) are different from the veinal necrosis caused by AA treatment (Figure 1A).

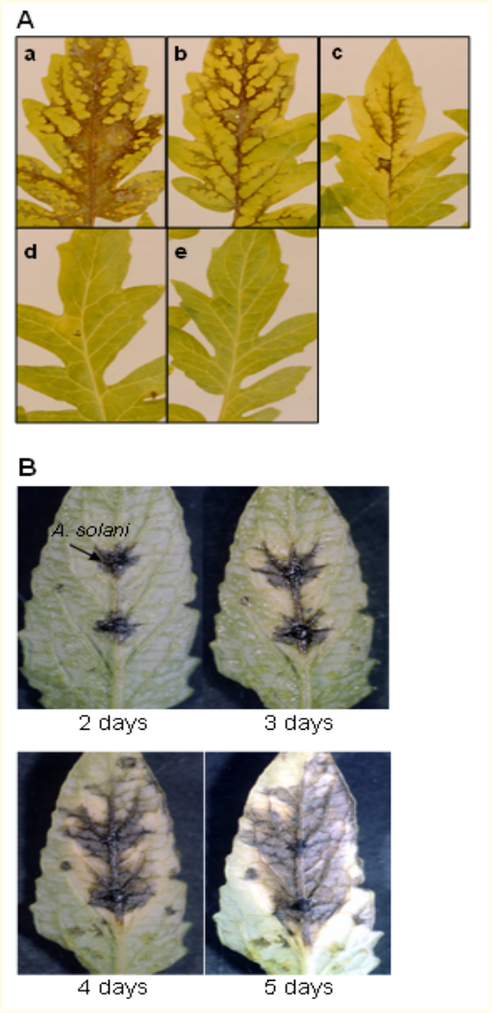

Figure 1: *Bioassay of tomato leaf treated with different concentrations of alternaric acid (AA) produced by Alternaria solani.*

A: The leaf surface was gently punctured with a needle and treated with micromolar concentrations of AA. The observations were made 24h after AA treatment. Treated leaves were incubated under aseptic moist conditions at 23oC for 14h light period. AA concentrations: a, 25 µM; b, 2.5 µM; c, 0.25 µM; d, 0.1 µM; and e, control (water). B: Alternaria solani infection on tomato leaves. Observations were made after 2, 3, 4 and 5 days of the inoculation. A. solani produced AA in the infected plant tissue and neighboring cells causing necrosis.

HR in tomato cells treated with AA was not observed 24h after the treatment under microscopic observation in the present conditions. The necrotic symptoms in this case were the result of AA treatment. This necrotic response of the tomato tissue to AA clearly differs from the early and localized reaction to the infection caused by an incompatible race of *P. infestans* that leads to HR of the host cells.

His-CPK2 contains Ca^{2+} and Mg^{2+} for the activation

His-CPK2 was expressed in *E. coli* cells as fusion with 6xHis tags [33] as described in Materials and Methods. The His-CPK2 was recovered mainly in the insoluble protein fraction though a small amount of the protein was recovered in the soluble fraction as well. His-tagged CPK2 was purified ~90% as judged by visual inspection of SDS-PAGE. To determine if his-CPK2 exhibits Ca^{2+} and Mg^{2+} dependent phosphorylation, the phosphorylation experiments were conducted in the absence or presence of Ca^{2+} and Mg^{2+}. His-CPK2 was phosphorylated only in the presence of Ca^{2+} and Mg^{2+} or Ca^{2+} alone (Figure 2B).

Effect of AA on His-CPK2 with Ca^{2+}

We investigated the effect of AA on purified His-CPK2. His-CPK2 phosphorylation was immediately stimulated ~47% in the presence of AA as compared to the phosphorylation of His-CPK2 without AA (Figure 2A, t=5). The stimulation of phosphorylation increased till 40 minutes in contrast to that of the CPK2 without AA addition. The effect of AA on His-CPK2 phosphorylation was then measured in the absence or presence of either Ca^{2+} alone or Ca^{2+} and Mg^{2+} together. AA showed different effect on the phosphorylation of CPK2 in the presence of Ca^{2+} only (Figure 2C) and Ca^{2+} and Mg^{2+} together (Figure 2D), AA initially inhibited phosphorylation by ~53% with Ca^{2+} alone (Figure 2C, t=0). The inhibition of CPK2 phosphorylation by AA in the presence of Ca^{2+} decreased over time, and the activity was almost the same with and without AA after 30 minutes and was slightly stimulated at 40 minutes (Figure 2C). When the CPK2 containing Ca^{2+} was added with Mg^{2+}, 46% stimulation in the phosphorylation of the RiCPK2 was observed after the initiation of the reaction (Figure 2B, t=5). In the presence of both Ca^{2+} and Mg^{2+} AA stimulated RiCPK2 phosphorylation ~12% (Figure 2D). This stimulation of CPK2 phosphorylation in the presence of Ca^{2+} and Mg^{2+} was 22% higher (Figure 2D) than in the absence of these cations (Figure 2A) as a result of AA addition. This indicated that the presence of Ca^{2+} and Mg^{2+} in the assay played a role in the stimulation of RiCPK2 phosphorylation. In a similar experiment, suppressor of HR from P. infestans also stimulated the phosphorylation of CPK2 with Ca^{2+} and Mg^{2+} just after addition under the same conditions (data not shown).

The present results indicate that the difference between stimulation of phosphorylation of His-CPK2 in the presence and absence of Ca^{2+} and Mg^{2+} together is stronger (Figure 2D) than the difference between stimulation of phosphorylation of His-CPK2 in the presence and absence of Ca^{2+} alone as a result of AA addition (Figure 2C). This demonstrates that these cations together play important role in the stimulation of CPK2 phosphorylation by AA. AA inhibited the phosphorylation of CPK2 in the presence of Ca^{2+} (Figure 2C) and stimulated in the presence of Ca^{2+} and Mg^{2+} together (Figure 2D). These results showed that Ca^{2+} and Mg^{2+} both were required for AA and CPK2 interaction.

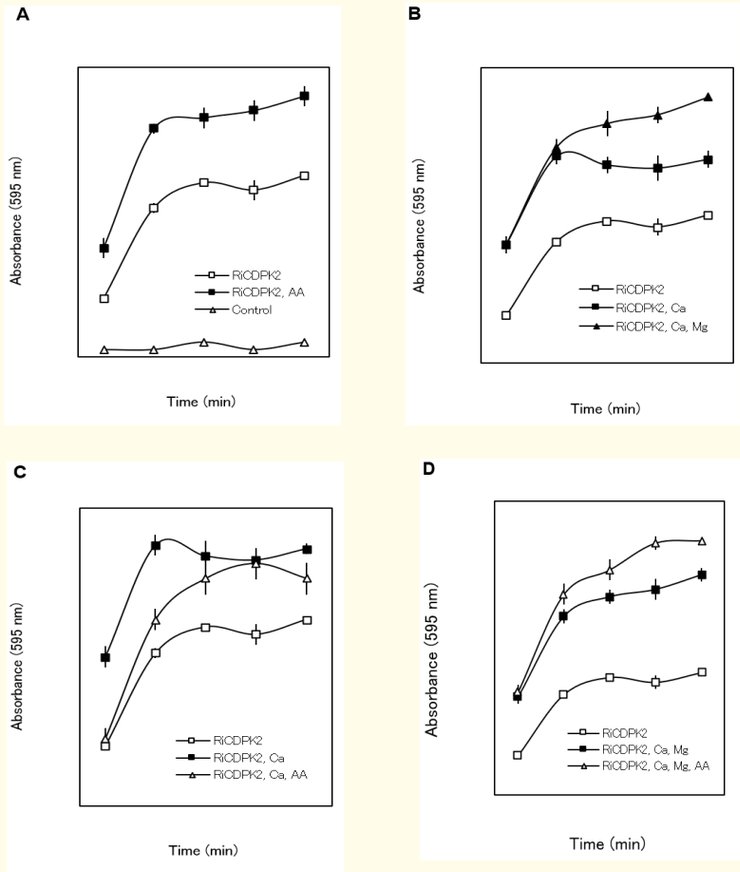

Figure 2: *Effect of alternaric acid (AA) on the phosphorylation of His-CPK2 in the presence of Ca2+ and/or Mg2+.*

A: Effect of AA; B: Effect of Ca2+ or Mg2+; C: Effect of AA with Ca2+; D: Effect of AA with Ca2+ and Mg2+. The final concentrations were: CPK2 (1.5 µg), AA (25 µM). The control treatment (absence of His-CPK2) was identical for all the experiments in panels A-D. Data represent the mean of two independent experiments ± SD.

Discussion

The key role of HSTs in pathogenesis is assumed to be similar to that of a suppressor of host resistance mechanisms [7,12,16,18,28]. Previous report of the analysis of CPK1, and 2 signaling in potato cell and tomato cell in the infected cells, we have reported that the suppressor stimulated CPK signaling in HR, resulting the occurrence and inhibition of cell death of potato cells. These findings indicate that by using these CPK activation signaling pathway, potato cell had caused the inhibition of AOS generation in the infected cells.

These results suggested that the suppressor had inhibited the AOS production by CPK activation in inside cell.

We have presented a hypothesis that alternaric acid of *Alternaria solani* can inhibit the AOS production by the activation of CPK-1, and -2 signaling pathway in host cell. To test this, we used alternaric acid of *A. solani* and the suppressor of *P. infestans* in the same experiments.

AA has the role as a HST during disease development, showing early effect on the plasma membrane of the host cell [12,34] similar to MAP kinase and the receptor protein in host cells as reported [26]. As reported previously, AA significantly delayed the occurrence of HR in potato cells caused by infection with an incompatible race of *P. infestans* but the treatment had no effect on the hyphal growth of *P. infestans* in the infected cells [3,30,35-37]. The present study indicated that AA directly affected the activity of His-CPK2. From these lines of evidence, it was presumed that HST causes a significant delay of HR and when AA stimulates CPK2 in potato, the occurrence of HR in the host cell is inhibited. In potato, early inhibition of HR seems to be important for successful infection and establishment of disease [2,18]. In case of *A. solani* infection to tomato leaves (Figure 1B), the symptoms are not restricted to the veinal necrosis (Figure 1A) but are extended beyond that to the rest of the leaf. It is likely that some other toxins like solanapyrone A are also produced by *A. solani* at a later stage during the infection contributing to the necrosis (Figure 1B, 5 days). Production of AA per spore in germination fluid is very low and low concentration (less than 0.1 μM) of AA does not cause necrosis in host leaf (Figure 1A, d; Figure 1B, before 2 days) while in contrast, low amount of AA (0.25 μM), can delay HR [18]. In the present leaf bioassay more the concentration of AA greater was the damage (Figure 1A, a). The present results show that at the early period of infection *A. solani* produced less than 0.1 μM AA and caused negligible damage (Figure 1B before 2 days) and the production of AA increased with time causing more damage to the leaf surface at later period (Figure 1B, 5 days) different from HR which is localized and early process.

We observed that CPK activity was stimulated by elicitor and suppressor glucan from *P. infestans* in the potato membrane fraction [17] and that a plasma-membrane-bound CPK of 70 kDa was activated just after the addition of elicitor and suppressor in potato membrane fraction [15]. A membrane bound CPK of 68 kDa, activated in Cf-9 tomato cells after elicitation with Avr9 of *Cladosporium fulvum* has also been reported [38]. Biochemical purification and immunochemical studies of potato [2,15] and rice [39] have suggested the association of CPK to the plasma membrane. Since CPK2 stimulation by AA may be related to the mode of action of the toxin as a primary determinant of pathogenesis in the disease, CPK2 may be a key kinase to regulate the inhibition of HR in the host cells. Other reports suggest that AA acts as HST in disease development [24,40]. Several other HSTs like ACT-, AF- and AK-toxins (produced by tangerine, strawberry and Japanese pear pathotypes of *A. alternata*, respectively) show an early effect on plasma membrane of the host cell [7,12] causing electrolyte loss from the host tissue [6]. Electrophysiological analyses of host cell membranes treated with HSTs have been reported. PC-toxin from *Periconia circinata* deprived susceptible tissue of the activity of proton pump [41]. From the present results, we suggest that Ca^{2+} pumps in potato plasma membrane might regulate RiCPK2. Some of these plasma membrane pumps have been reported previously [42]. HSTs cause ion efflux in infected host cells, accumulating Ca^{2+} and Mg^{2+} at the site of infection in the host. Since AA stimulates CPK2 autophosphorylation in the presence of Ca^{2+} and Mg^{2+} (Figure 2D) *in vitro*, AA may stimulate CPK2 *in situ* during *A. solani* infection in potato cells.

We suggest that AA may target a specific plant protein kinase, as shown in the present *in vitro* assay [17] leading to the inhibition of HR.

Our results show that AA and the suppressor of *P. infestans* may regulate CPK2 kinase during the infection process in a compatible interaction between the host and *A. solani*, leading to the inhibition of HR.

We have presented a mechanism that the HST of *A. solan*i and the suppressor of *P. infestans* may inhibit the generation of AOS in host cells by activating CPK 1 and 2 signaling pathways in host plasma membrane and cytosol. (Figure 3, cited from Furuichi., *et al.* (2015), which is different from the FLS2 model) [4,5,26,43-45].

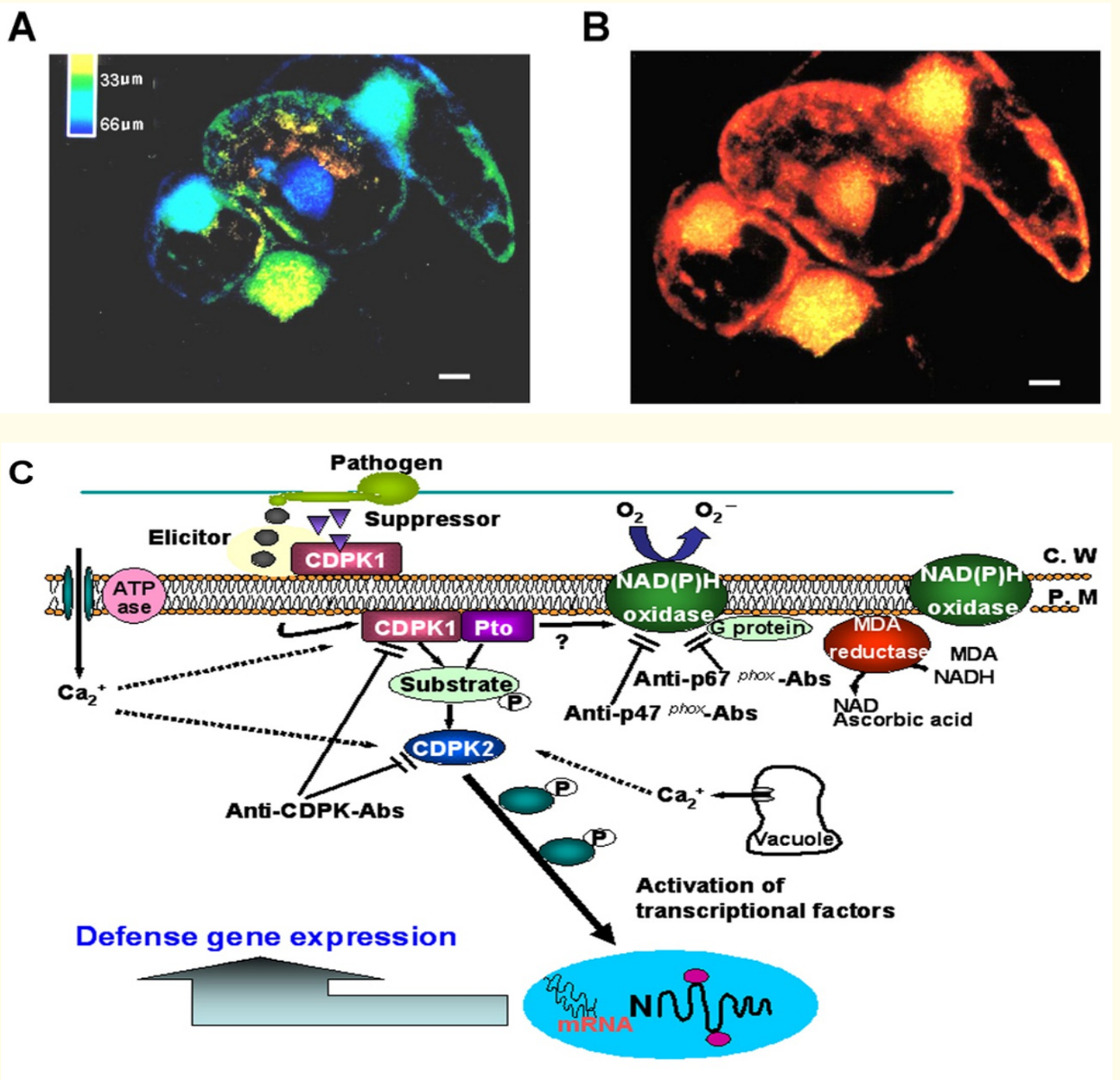

Figure 3

A: The imaging of potato cultured cells and the depth from the cell wall to nucleus.

B: CPK localization in the host cells by the immuno staining of CDPK antibodies.

C: CPK 1 and 2 signal pathway in potato cell, showing the inhibition of AOS (Active Oxygen Species) generation induction and/or inhibition by the suppressor of Phytophthora infestans.

(Cited from Furuichi., et al. 2015, J. Pl Pathol. Microbiology).

Conclusion

We have reported in these experimental data that the suppressor and the host-selective-toxin, Alternaric acid of *A. solani,* have inhibited the generation of AOS in host cells, and inhibit the HR in plant host cells [1,12,40]. The AA was produced in the infection process of *A. solani*, and in the germination fluids of *A. solani* [37]. The HST, AA, have been reported the inhibition of PCD (Programed Cell Death), HR

in potato cells [1,15]. As reported by the suppressor of *P. infestans*, AA have the host selective effector activity and the binding site was the CPK2 of potato cultivars [19].

So far, HST has the host selectivity, the pathogenicity inducing factor, and PCD inhibiting factor as a pathogenicity determinate. As a results of these findings, the AA has the primary pathogenicity effector and the character of the potential HST.

From these experimental data, we report the new Host-Selective-Toxin, Alternaric acid, as a primary determinant of pathogenicity [7,10,28,34].

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