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Review Article

Effector Signaling in Hypersensitive Response of Plant Microbe Interaction: Single-Molecule-Signaling of Suppressor from *Phytophthora Infestans* and Host Selective Toxin of *Alternaria Solani* on Ca^{2+}-Dependent Protein-Kinase (CDPK)

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Abstract

We have focused on the Hypersensitive Reaction (HR) and cell death in the plant defense between *Phytophthora infestans*, potato late blight pathogen, and host cultivars. We studied the Host-Selective-Toxin (HST), alternric acid and Non-host-selective-toxin, Solanapyron A, as a suppressor of *Alternaria solani* with the receptor candidate, Ca^{2+}-Dependent Protein Kinase (CDPK) by using binding assay and autophosphorylation assay. For the inhibition of HR in host cells, alternaric acid bind to the host membrane CDPK receptor, resulting in the inhibition of HR and cell death of host cells. By using the FCC (Fluorescent Cross Correlation) LSM system, we have investigated the pathogen PAMPS elicitor and suppressor, and the HST from *A. solani*, tomato early blight pathogen, and host membrane receptor signaling during state I - state II (Non-Active to Active transition states) of HR in potato and host cells. In conclusion, the CDPK-1 and CDPK-2 kinase of potato and tomato recognized the suppressor and HST of pathogens, resulting in the inhibition of NADPH oxidase and in the inhibition of occurrence of HR cell death.

Keywords: Hypersensitive cell death; *P. infestans*; Host-Selective-Toxin; Single molecule signaling analysis; CDPK

Introduction

Plants are exposed to pathogen attach in their environment and have developed mechanisms to respond with biotic elicitor and the suppressor or host specific toxin from the pathogens. Among the earliest cellular responses to such attach and stimuli are the production of active oxygen species generation (AOS) [1], the Hypersensitive Response HR [2] and phytoalexin production in host cells, and a specific calcium signature is often reported [3-6]. Suppressor, a glucan, for HR and phytoalexin production was well documented [3,4,7,8].

Suppressor inhibited the AOS generation at an early period of infection with Pi [1,3,9]. Yet, the receptor site for the inhibition of HR in plant cells has not been reported. The suppressor from Pi well control the production of phytoalexin and host cell death in compatible interaction between Pi and the potato cultivar [8,10].

Ca^{2+}-Dependent Protein Kinases (CDPKs) may function as a potential sensor that decodes and translates the elevation of calcium concentration into enhanced CDPK activity and subsequent downstream signaling events [9,11-15]. CDPKs are Ca^{2+}-binding Ser/Thr protein kinases [12].

Upon elicitor stimulation of host cells, the NADPH oxidase produces AOS in the defense response [1,16,17]. The generation of AOS was reported in incompatible interactions between potato and the oomycete pathogen, Pi, and has been considered that production of AOS in the cell was the earliest events in the plant defense response and a signal for induction of HR cell death [18-20].

CDPKs have not been identified in yeast and animal cells. Calmodulin-dependent protein kinases are well characterized as major mammalian calcium dependent signaling molecules [12,15]. CDPKs comprise a large gene family (34 members in *Arabidopsis* sp.). This suggests that individual isoforms have different functions and participate in multiple distinct signaling pathways.

In the present chapter, we would like to describe the recent discovered aspects of HR recognition in plant cells and suppression of the HR of host cells in the potato- *P. infestans* system and to discuss the importance of the switching-on or -off of recognition events in the determination of cultivar- *P. infestans* race specificity, and also, the role of Host-Selective-Toxin (HST), Alternaric acid, of *A. solani*, as the suppressor.

For measuring such random diffusional motion of an individual CDPKs and the host receptor sites, fluorescence correlation laser microscopy (FCCS) is, at present, the only practical method [21,22]. FCCS detects fluctuation of the fluorescence intensity in a confocally defined volume with a sharply focused laser, 0.25 fl. This method has been developed as a unique technique to measure rotational diffusion coefficients of molecules in a solution and in living cells [23,24]. Application of this technique to biological systems has brought to light

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novel aspects of various molecular dynamics, such as the status of the pathogen suppressor, toxin and the cell receptor in plants [25,26].

Here we report whether downstream CDPK-regulated processes control the AOS production in HR response. The challenge that currently faces the CDPK field is not only to defined biological functions to specific CDPK isoforms, but also to integrate CDPK signals into the AOS generation [2,6], HR response [9,27] and phytoalexin, PA, accumulation [28].

Mechanism of Race-Cultivar Specificity in HR

Experiments to determine the basis of race-cv. specificity should be focused on the recognition sites for PiP (38 KD glycoprotein) [29,30] and HWC, the HR-eliciting effector [31], and for the suppressor (the HR inhibiting effector) and Host-Selective-Toxin, Alternaric acid, isolated from *A. solani*, early blight pathogen of tomato [29,32-34], as well as the mechanism of interaction between them.

The starting-on or -off of the HR may possibly determine whether potato tissues react compatibly or incompatibly to infection. Not only is HR nonspecifically elicited by PiP elicitor from any race, but the physiological and immunochemical systems necessary, for HR and the pathway of PA synthesis seems to be present in every potato cv. regardless of their R-genes to *P. infestans* [35,36].

Therefore, experiments to determine the basis of race-cv. specificity should be focused on the recognition sites for elicitor, PiP elicitor (the HR-eliciting PAMPS) [37] and for the suppressor (the HR inhibiting PAMPS) and HST, Alternaric Acid (AA), isolated from *A. solani*, early blight of tomato, as well as the mechanism of interaction between the potato and tomato with the pathogens [29,32].

PAMPs (Pathogen Associated Molecular Patterns) of *P. infestans*, and Host Selective Toxin (HST) of *A. solani*

Pathogens produce either glucans or peptide-oligosaccharides that suppress HR and PA production [3,7,8,38,39]. The receptor protein of the Suppressor from potato plants that mediates the inhibition of HR in plant cells has been reported [40-43]. The Pi suppressor controls the production of PA and HR cell death in compatible interactions between Pi and potato cultivars [3,10,39].

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 HR cell death of plant cells was investigated [44]. The effect of peptide PiP and suppressor from *P. infestans* on the kinase activity of Membrane Protein (MP) from potato (R1 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 of potato cell. 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 from potato cells initiating the signal, which, in the case of PiP, leads to the occurrence and/or inhibition of HR [42,44].

We have reported the role of CDPK-1 and 2 in potato HR cell death after the infection of *A. solani*, tomato early blight pathogen, and *P. infestans*, late blight pathogen, in the HOST-SPECIFIC-TOXIN (HST) of Daisen-International-Congress, Tottori, Japan, in 1997 [26], in which UK scientists, UC San Diego, Washington SU, Utah SU., Toronto U., DA groups of Canada, Wageningen U. and so on, were discussed. This report from us was the first of the role of CDPK kinase for the induction of HR cell death in plant science field (Figures 1-3) [44].

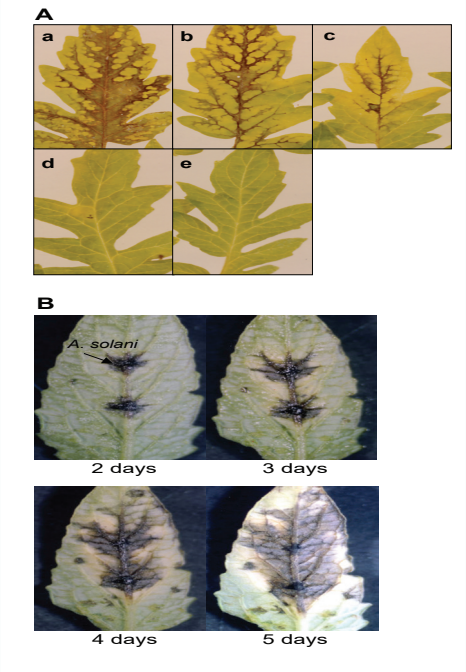

Figure 1: Leaf symptoms of tomato treated with Alternaric Acid (AA) produced by *A. solani*. A: The leaf surface was gently punctured and treated with each concentration of AA. The observations were made 24 h after AA treatment. The toxin concentrations: a: 25; b: 2.5; c: 0.25; d: 0.1 µM; and e: water. B: *A. solani* infection on tomato leaves. Microscopic observations were made after 2, 3, 4, and 5 days of the inoculation. *A. solani* produced AA in the infected plant tissue.

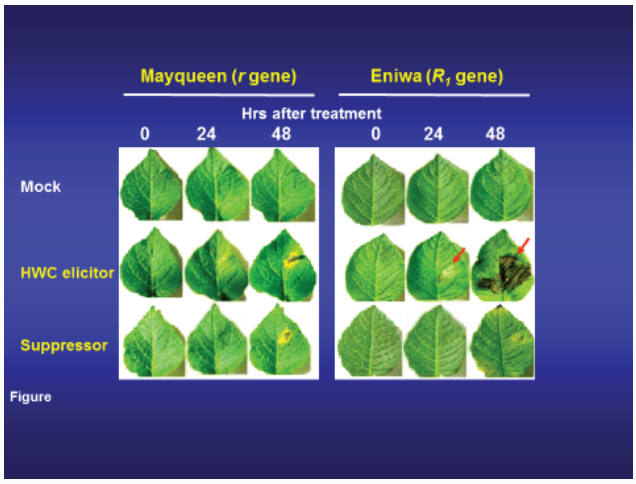

Figure 2: Potato leaf cultivars, Eniwa (R1-gene) and Mayqueen (r-gene) were treated with HWC effector or suppressor of *P. infestans*. PiPE elicitor and suppressor were prepared from *P. infestans* isolate DN101 (race 0), then were treated with 200 ug/ml. Distilled water was treated as a mock treatment.

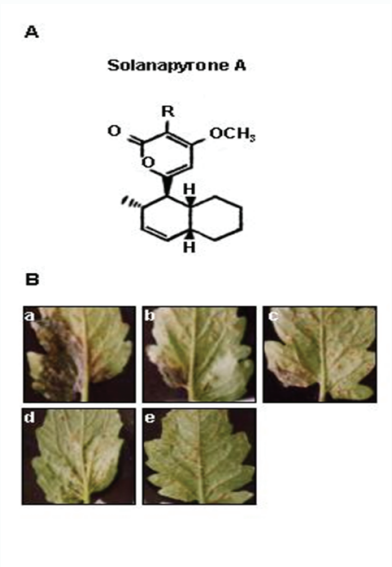

Figure 3: Structure of *Solanapyron A*. The toxin induced tomato leaf necrosis after the treatment on the center vein. a): 2 uMol, the toxin in 20 ul, b): 0.2 uMol, in 20 ul, c): 0.2 uMol treatment, after 24-30 hr, d): water, 20 ul, e): water, 20 ul, 1day after the treatment.

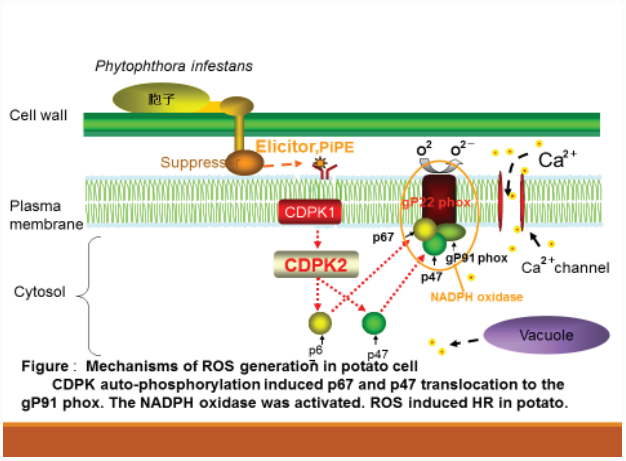

Figure 4: CDPK-1 and -2 signal pathway in host cell, showing the inhibition of AOS generation induction and inhibition by the suppressor of *P. infestans*.

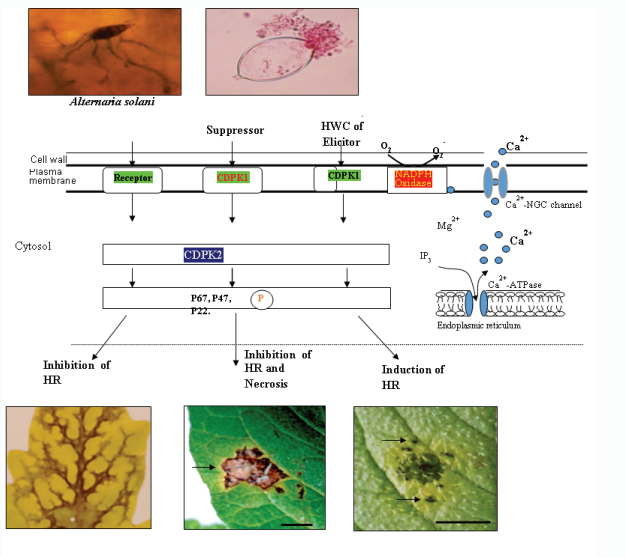

Figure 5: A model of compatible and incompatible host-pathogen interaction between *A. solani*, early blight pathogen, and *P. infestans*, potato late blight pathogen. The inhibition of HR had been occurred by the effect of HST, Alternaric Acid (AA), and the suppressor of *P. infestans*.

The interaction between plants, a suppressor and a HST producing pathogen leads often to suppression of rapid and localized cell death in the context of a HR [45]. The suppressor for HR was reported from *P. infestans*. The hyphal cell wall elicitor, HWC, from hyphal wall of *P. infestans* was also reported from Tomiyama group, Nagoya U [26,36,46].

Recently, we have reported the isolation of PiP effector gene from *P. infestans*, of which peptide induce the generation of AOS and HR cell death in potato and tomato [29,37,47]. In these reported physiological events for the induction of HR and AOS generation, and the inhibition of these resistance responses, CDPK kinase was involved in for the signal cascades in host cell [41,44,48]. We have proposed that CDPK molecule is a switch for the induction or the inhibition of HR response in host cell [26,30,41,42,49].

The mechanisms of these molecular infection events are presumed to be as followings: (1) Initial recognition of the PAMPS (Pathogen Associated Molecular Patterns) and the suppressor of the pathogen by host plasma membrane in the infection process [26,33,50,51,52]; (2) Increase in Ca^{2+} influx and the kinase activation in the cells [53,54]; and induction of biochemical defense in the host cells [35,44,45,46,55].

In our attempt to explore the HR suppressor and the antigenic potential of *P. infestans* derived surface structure to elicit non-specific defense response in potato, we have previously identified PiP (38 KD glycoprotein), an elicitor peptide for HR and the generation of AOS [37,42]. The PiP was shown to serve as recognition PAMP for the activation of HR. Recently, Furuichi et al. [43,49] and other groups, reporting the gp22phox [43,48,56,57], have been reported that CDPK regulated the AOS generation in resistant and compatible interaction of potato- *Phytophthora* by using the single molecule signal analysis [48]. CDPK-1, plasma membrane binding kinase, and CDPK-2, a cytosol localizing one, played a role in AOS regulation in host cell (Figures 4 and 5).

In the FCCS analysis of the binding GFP-CDPK1 and the suppressor of Pi, we have reported that for the analysis of the binding between GFP-CDPK1 as a receptor and the ligand suppressor from Pi in potato cells in vivo, we have used the fluorescence cross correlation spectroscopy, FCCS, laser microscopy [48]. GFP-CDPK1 was constructed for the assay, transformation was done into the potato cells by using particle gun, and the suppressor was incubated with the potato cell under the FCCS system at time intervals. Then the potato cells were treated with the Alexa-anti-suppressor-monoclonal Abs [3,48,58].

In this study we first tested whether the diffusion or binding of GFP-CDPK1 with the suppressor of Pi *in situ* is measurable. We then studied how the dynamics change in the localization of GFP-CDPK1 in the potato cells and the binding between them in the cells after suppressor of Pi addition in the host cells at various levels of micro dynamics. HST and a suppressor of HR are low molecular weight, secondary metabolites belonging to various classes of chemical compounds [57,59-63]. HSTs have been reported as primary determinants of pathogenesis in the recognition of host

cells and in disease development, similar to the suppressor of *P. infestans*. Toxins cause physiological change in host cells altering cell membrane permeability resulting in the rapid increase of electrolyte loss [59,60,64,65] and decrease the membrane potential of potato cell [26,37,40,49,61].

Alternaric Acid (AA) was reported to play a role to determine host specificity and to contribute to disease development caused by *A. solani* [33,66-68]. 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 [34,67]. Tabuchi and Ichihara [62] reported complete stereochemistry and full synthesis of AA, and that biological Diels-Alder reaction is involved in the polyketide pathway for the production of AA [69,70].

The suppressor from compatible pathogens causes inhibition of HR [56,57,71,72]. Suppressors isolated from *P. infestans*, are soluble glucans containing units bonded *via* β-1, 3 and β-1, 6 linkages [40,49,73,74]. Ca^{2+}-Dependent Phosphorylation of various proteins of potato was reported after treatment with the PiP elicitor, H_2O_2, salicylic acid and suppressor from *P. infestans*, showing the role of CDPKs in response to infecting stimuli [67,75].

CDPKs are multifunctional with several isoforms providing specific pathways to control transcription, membrane transport and cell structure [12,56,76-79]. It was reported that the effect of AA on the phosphorylation activity of the purified CDPK-2 (Accession number AB051809), a new isoform of CDPK gene family from potato cv. Rishiri, a highly resistant cultivar to *P. infestans* [40,47,80-82].

To ascertain the role of AA as an HST and its effect on HR in potato and tomato, and to compare the effect of HST with suppressor of HR in the host- *P. infestans* interaction in the CDPK signaling of AOS generation of NADPH oxidase regulation in the infection process at the AOS generating stage are the key points in plant-parasite interactions.

The Interaction of RXLR-Host Receptor

From the reported RXLR-genes of *Phytophthora* species, what is the real product is not yet known. Receptor binding of PiP evokes a PAMP-specific cytoplasmic streaming, and the Brownian movement and gelation in the cytosol, production of AOS as well as translational activation of CDPK kinases, all of which are important elements for the transmission of the PiP signal.

From these evidences, we proposed that PiP elicitor and a HST (Host-Selective-Toxin) from *A. solani*, can regulate HR by the binding with CDPK on the plasma membrane of potato [41,44,30,48]. The RXLR genes from *Phytophthora spp.* interacted with the resistance genes in gene-for-gene level in the host cell. How a secreted protein does from Avr4/6 with an RXLR-dEER protein translocation motif work with the receptors in host cells, is not yet resolved.

AOS (Active Oxygen Species) Production in HR

Upon PiP elicitor stimulation of host cells, NADPH oxidase produces AOS as a defensive response [1,16,17,57,79,83]. AOS is generated during incompatible interactions between potato and Pi, and the production of cellular AOS may constitute the earliest event of the plant defense response and a signal for the induction of HR cell death (Figures 4 and 5) [18,19,48,57,77,84,85,86]. We have report that downstream CDPK-regulated processes control AOS production during HR. Glucan from the oomycete pathogen, *P. infestans* (Pi), represent the suppressor for HR cell death in plants and has been reported to inhibit the accumulation of PA [30,48,88,89].

To evaluate the activation of plant CDPK after the binding of this Pi suppressor, we constructed a chimeric CDPK tandemly fused to green fluorescent protein (smGFP)-CDPK and the suppressor with Alexa-labeled antibodies. Dual-color cross-correlation spectroscopy yielded spectral information on the coincidence of the two fluorescent molecules at the single-molecule level. Furuichi et al. [43,48,49] have reported that the suppressor binds CDPK, which phosphorylates NADPH oxidase, thereby inhibiting its ability to generate AOS (Figure 2).

These results show that HR inhibits the action of plant pathogen toxins to control AOS formation by signal transduction *via.* CDPK-mediated phosphorylation [42,48,49]. The data suggest that CDPK-1 phosphorylated the NADPH oxidase in plasma membrane of potato cells to downstream the suppressor signal for the inhibition of HR and of HR in host cell (Figure 5) [3,7,8,90,91].

Perspectives

In the next step of the future works, we need the single molecule detection and observation *in situ* in host cells. For the new frontier of plant infection mechanisms of the host-pathogens interaction, we will explain the infection process by using the single molecule interaction *in situ* analysis in host cell with regard to the effector-receptor interaction and the signal transduction in a specific host-parasite interaction for the explanation of HR in host cells (Figure 4 and 5) [48].

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