Elicitor Signal Transduction That Leads to Hypersensitive Reaction in Cultured Tobacco Cells

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Abstract

Plants have an excellent mechanism for responding to pathogen invasion by specific and inducible defense reactions, which is often apparent as the so-called hypersensitive reaction (HR). The molecular dissection of the mechanism of HR is difficult since the biological system involves intricate interactions between plant and pathogen. A simplified system, in which suspension-cultured plant cells are synchronously challenged with a single molecular inducer of HR, elicitor, would greatly facilitate studies of the molecular mechanism of HR. Several components involved in elicitor signaling have recently been identified and characterized using the experimental system.

1. Introduction

Higher plants have the ability to respond to invasion by pathogens, such as fungi, bacteria and viruses, through defense reactions that may provide protection against these pathogens. Although plants do not have specialized cells and tissues, such as cells of immune system in animals, to protect themselves against pathogen infection, they have an excellent mechanism for responding to pathogen invasion by specific and inducible defense reactions, which is often apparent as the so-called hypersensitive reaction (HR). HR resulting from the incompatible interaction between the plant and a corresponding pathogen is a multifaceted defense reaction that include phytoalexin accumulation, ion fluxes, generation of active oxygen species (i.e., oxidative burst), expression of defense-related genes, and hypersensitive cell death (Kombrink and Somssich, 1995).

The molecular dissection of the mechanism of HR is difficult since the biological system involves intricate interactions between plant and pathogen. A simplified system, in which plant cells are synchronously challenged with a single molecular inducer of HR, would greatly facilitate studies of the molecular mechanism of HR.

In the initial step of events during defense reaction, plant cells perceive either plant-derived (endogenous) or pathogen-derived (exogenous) signals. These signal compounds are collectively referred to as elicitors (Ebel and Cosio, 1994, Kombrink and Somssich, 1995). The exogenous elicitors of plant defense responses differ widely in their chemical nature, and include proteins, oligosaccharides, glycoproteins and lipids. Most of the pathogen-derived elicitors identified to date are non-specific, in that they induce various defense responses in a large variety of plant cultivars and species. Inducible defense responses can be activated, not only upon challenge of plant tissues by pathogen, but also upon exposure to elicitors. Therefore, elicitors are now widely used in simple experimental systems to study the molecular mechanisms of defense responses.

The infection of plants by pathogens and the resulting defense responses occur initially at the single-cell level. Suspension-cultured cells have been widely used as a simplified experimental system for investigations of the molecular mechanisms involved in plant-pathogen interactions. Therefore, in combination with elicitors, such systems have provided excellent models for studies of various aspects of defense responses in plant cells. We have been investigating the elicitor signal-transduction pathway of plant cells, using fungal elicitors and suspension-cultured tobacco cells.

In this article, I describe current view of molecular mechanism of induced defense reaction of plant cells based on our own research and recent developments in this field.
2. Defense responses induced by fungal elicitors in tobacco cells.

To investigate an elicitor signal transduction pathway, we have been monitored early changes in morphological, biochemical and molecular events in suspension cultured tobacco cells, line XD6S, which was derived from *Nicotiana tabacum* cv Xanthi (Yamaoka et al., 1969), treated with two different types of fungal elicitors, a crude extract of cell wall of *Phytophthora infestans* (PiE) and a purified xylanase from culture of *Trichoderma viride* (TvX). As shown Figure 1, these two elicitors induce various kinds of defense responses, including activation of a p47 protein kinase, an oxidative burst, alkalinization of culture medium, and expression of a subset of defense-related genes (Fukuda and Shinshi, 1994; Suzuki et al., 1995; Suzuki and Shinshi, 1995; Fukuda, 1996, 1997; Yano et al., 1998; Suzuki et al., 1999; Yano et al., 1999). In addition, TvX induces shrinkage of the cytoplasm, condensation of the nucleus, and finally hypersensitive cell death (Yano et al., 1998; Suzuki et al., 1999; Yano et al., 1999). Therefore, in this experimental system, we can investigate the specific events in hypersensitive cell death as well as general defense responses. Thus, in combination with these elicitors, XD6S cells facilitate further studies on the molecular mechanism of defense responses of plant against pathogen infection.

3. Involvement of protein kinase cascade in elicitor signal transduction.


Molecular genetic studies, based on gene-for-gene complementarity in plant-pathogen interactions, have revealed that several resistance genes encode protein kinases (Martin et al., 1993; Martin GB et al., 1994; Song et al., 1995; Zhou et al., 1995). In addition, recent biochemical studies demonstrated that the hypersensitive cell death in suspension culture of soybean cells (Levine et al., 1994) and of tobacco cells (Suzuki et al., 1999) and in tobacco leaves (He et al., 1994), induced by incompatible bacteria and elicitors can be blocked by protein kinase inhibitors. These results suggest that a protein kinase cascade is involved in the recognition of the elicitor and in the intracellular signal transduction that leads to HR including hypersensitive cell death (Bent, 1996; Suzuki and Shinshi, 1996; Jones, 1997).

4. Elicitor-responsive MAPK-like p47 protein kinase

Correlation of activity of specific protein kinase to defense responses as well as hypersensitive cell
death has rarely been demonstrated. To establish a functional link between protein phosphorylation and transduction of the elicitor signal that leads to defense responses, we have chosen a biochemical approach and attempted to analyze the elicitor-responsive protein kinase in XD6S cells, in which PIE- and TvX-induced defense responses were inhibited by staurosporine (Suzuki et al., 1995; Suzuki et al., 1999) and identified an elicitor-responsive 47-kD protein kinase (Suzuki and Shinshi, 1995) designated as p47 protein kinase (Suzuki et al. 1999). The activity of p47 protein kinase is barely detectable in untreated cells, but the kinase is activated rapidly, transiently and strongly in conjunction with its tyrosine phosphorylation, prior to defense responses, upon treatment of cells with PIE (Suzuki and Shinshi, 1995). By contrast, we found that the p47 protein kinase was slowly and extendedly activated during the TvX-induced hypersensitive cell death (Suzuki et al., 1999). In addition, the activation of p47 protein kinase induced by the fungal elicitors was also inhibited by staurosporine (Suzuki and Shinshi, 1995; Suzuki et al., 1999). These findings are consistent with the model that the p47 protein kinase mediate the phosphorylation-dependent signal transduction pathway in response to fungal elicitors that leads to defense responses and hypersensitive cell death (Fig. 3).

The activity of an upstream protein kinase(s) is required for the tyrosine phosphorylation and activation of the p47 kinase in response to the elicitors (Suzuki and Shinshi, 1995; Suzuki et al., 1999). In addition, anti-ERK1 antibody is bound to the p47 protein kinase (Suzuki et al., 1999). Based on such biochemical features, this kinase has been postulated to be a member of the mitogen-activated protein kinase (MAPK) family (Chasan, 1995; Suzuki and Shinshi, 1995; Hirt, 1997; Mizoguchi et al., 1997; Suzuki et al., 1999). It has also been demonstrated that fungal and bacterial elicitors activate MAPKs and MAPK-like kinases in tobacco cells (Adam et al., 1997; Lebrun-Garcia et al., 1998; Zhang et al., 1998; Romeis, et al., 1999). In parsley, an elicitor-responsive MAPK is probably involved in the activation of transcription of genes since it is translocated to the nucleus upon treatment of cells with elicitor (Ligterink et al., 1997). Thus, an increasing body of evidence indicates that MAPK are involved in the signaling pathway that is triggered by elicitors (Suzuki and Shinshi, 1996; Ebel and Mithöfer, 1998).

Our previous results showed that different stimuli, such as transfer of cell suspension to a new plastic dish, and treatment with PIE and TvX, activate the p47 protein kinase with different kinetics followed by different responses, whereas the magnitude of activation of the p47 protein kinase was similar for these stimuli (Suzuki and Shinshi, 1995; Suzuki et al., 1999). These results suggest that the regulation of the actual duration of activation of p47 protein kinase might be crucial in the determination of subsequent responses of tobacco cell (Fig. 2). Transfer of cell suspension induced very short-term and limited increase of mRNAs for genes, which were also induced by wounding in leaf tissue (Suzuki, K., Yano, A., Nishiuchi, T., and Shinshi, H., unpublished results). Therefore, it is reasonable to postulate that very short-term and limited activation of p47 protein kinase, for example by the transfer (probably mechanical) stress, is insufficient for sustained induction of downstream events in the elicitor-initiated signal transduction cascade (Fig. 2A). By contrast, the elicitor-induced rapid and
transient activation of the p47 protein kinase might be sufficient for initiation of defense responses such as the oxidative burst and expression of defense genes (Fig. 2B). Furthermore, it is plausible that the elicitor-induced slow and prolonged activation of the p47 protein kinase might be a prerequisite for hypersensitive cell death (Fig. 2C).

A similar paradigm has been suggested for the mammalian MAPK pathway. Several studies have revealed the importance of the duration of activation of c-Jun N-terminal kinase (JNK)/stress-activated kinase (SAPK) and/or p38 MAPK in determination of cell fate (Xia et al., 1995; Chen et al., 1996a and b; Goillot et al., 1997). In Jurkat T-cells, T-cell activation signal induced the rapid and transient activation of JNK and the proliferation of T-cells, by contrast a lethal dose of γ radiation or UV-C induced the delayed and persistent activation of JNK and apoptotic cell death (Chen et al., 1996a and b). Thus, the p47 protein kinase in tobacco cells appears to be functionally similar to SAPK/JNK and/or p38 MAPK in mammalian cells and the p47 protein kinase may play a role as a component of the elicitor signal transduction (Suzuki and Shinshi, 1996).

In contrast to TvX-induced slow and prolonged activation of p47 protein kinase (Suzuki et al., 1999), the immediate and transient nature of the activation that occurs in response to fungal elicitors appears to be a common feature of various myelin basic protein kinases including MAPKs (Suzuki and Shinshi, 1995; Ádám et al., 1997; Ligerink et al., 1997; Zhang et al., 1998). Both synthesis of protein de novo and protein phosphatase activity might be required for the attenuation of p47 kinase activity and other MAPKs (Suzuki and Shinshi, 1995; Meskiene et al., 1998). The process, which induces the delayed and prolonged activation of the p47 protein kinase by TvX, could involve either prolonged activation of upstream kinases or inhibition and/or down-regulation of specific protein phosphatases (Fig. 3). The observation that calyculin A induced the prolonged activation of p47 protein kinase (Suzuki and Shinshi, 1995; Suzuki et al., 1999) suggests possible posttranslational negative modulation of the constitutive activity of a p47 protein kinase and its upstream kinases via dephosphorylation-mediated down-regulation.

At 1 μM, staurosporine enhanced TvX-induced cell death and the activation of p47 protein kinase (Suzuki et al., 1999), whereas it inhibited the TvX-induced defense responses (Suzuki et al., 1999), the PiE-induced activation of the p47 protein kinase (Suzuki and Shinshi, 1995) and the expression of defense genes (Suzuki et al., 1995). By contrast, the staurosporine at 10 μM prevented the TvX-induced activation of the p47 protein kinase and cell death as well as the defense responses (Suzuki et al., 1999). Therefore, we postulated that TvX might activate distinct forms of p47 protein kinase, which have varying sensitivities to staurosporine and which are involved in the induction of varying cellular responses (Fig. 3).

5. Involvement of Ca²⁺ in elicitor signal transduction.

An influx of Ca²⁺ ions has been implicated in the activation of early defense responses induced by elicitors (Kombrink and Somssich, 1995; Ebel and Mithöfer, 1998). In addition, an influx of Ca²⁺ ions across the plasma membrane might be involved in the bacterial induction of hypersensitive cell death in plants (Atkinson et al., 1990; Levine et al., 1996) and such an influx of Ca²⁺ ions has often been implicated in the induction of apoptosis in animal cells (Martin JS et al., 1994). In human B lymphocytes, apoptosis and sustained activation of SAPK and p38 MAPK, which were induced by cross-linking of membrane IgM, were inhibited by a calcium-channel blocker (Graves et al., 1996). We have also shown that a calcium-channel blocker prevented not only sustained activation of the p47 protein kinase but also induction of hypersensitive cell death in XD6S cells treated with TvX (Yano et al., 1998; Suzuki et al., 1999). Our results have shown that the entry of extracellular Ca²⁺ ions might be required for the induction of a diverse array of...
early events in tobacco cells in response to PiE and TvX (Suzuki et al., 1995; Suzuki and Shinshi, 1995; Fukuda, 1996; Yano et al., 1998; Suzuki et al., 1999). Therefore, it is likely that Ca²⁺ ions might function at immediate downstream of the initial elicitor–receptor interaction (Fig. 3).

6. Involvement of reactive oxygen and proteases in elicitor signal transduction.

The rapid production of reactive oxygen intermediates (ROIs), which include superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂), by plant cells that have been challenged by pathogens is one of the most striking events during the early phase of the HR. The oxidative burst has been shown to be induced via a protein kinase cascade. ROIs have been implicated in multiple phenomena that occur during plant defense responses (for review see Doke et al., 1996; Low and Merida, 1996; Wojtaszek, 1997). H₂O₂ has been postulated to be a mediator of defense gene expression and cell death in soybean culture (Levine et al., 1994). Recent report suggests that ROI may be also rapidly induced at distal uninfected leaves following a local oxidative burst in infected leaves and function as a second messenger for systemic acquired resistance (Alvarez et al., 1996). Superoxide is also a key regulator of induction of the spreading cell death associated with the lsd1 mutant of Arabidopsis (Jabs et al., 1996). Furthermore, nitric oxide (NO) has recently been identified as a second messenger during HR (Delledonne et al., 1998; Durner et al., 1998).

However, it has been also reported that a normal level of ROIs production by the oxidative burst is not sufficient to trigger cell death (Glazener et al., 1996; Jabs et al., 1997). PiE did not induce cell death in XD6S cells, even though the level of ROIs from the oxidative burst after treatment was similar to that observed after cells had been treated with TvX (Suzuki, K and Shinshi, H., unpublished result). This result is consistent with the results reported by Glazener et al. (1996) and Jabs et al. (1997). In addition, co-treatment of XD6S cells with xanthine and xanthine oxidase, which were used as an extracellular superoxide–generating system (Jabs et al., 1996), did not induce cell death (Yano et al., 1999). The degradation of H₂O₂ by catalase, the scavenging of ROIs by N-acetyl-L-cysteine and the prevention of production of O₂⁻ by inhibition of NADPH oxidase by diphenyleneiodonium, which completely inhibited the TvX–induced accumulation of H₂O₂ in the culture medium, did not affect the TvX–induced cell death (Yano et al., 1999). We have also found that the TvX induces activation of p47 protein kinase even in the presence of such inhibitors (A. Yano, K. Suzuki, and H. Shinshi, unpublished results). These results suggest that accumulation of ROIs is not necessary for the induction of TvX–mediated activation of p47 protein kinase and cell death and it is possible that TvX induces cell death in tobacco cells via a ROS–independent signaling pathway which is distinct from the ROI–mediated pathway (Yano et al., 1999). However, ROI (and probably NO) produced by the TvX–induced oxidative burst might act to potentiate defense responses and hypersensitive cell death, since a variety of experiments strongly support a model whereby ROI and NO potentiate the overall HR in plant (for review see Richberg et al., 1998).

In animal cells, members of a family of cysteine proteases, designated caspases, have been shown to play important roles in the regulation of the development of programmed cell death (pCD) (Nicholson and Thornberry, 1997). Although no homologs of caspases have been identified in plant cells, it was shown that possible involvement of activity of cysteine protease and/or caspase–like protease but not of serine protease in induction of HR (del Pozo and Lam, 1998; Solomon et al., 1999). On the contrary, a serine protease might be involved in the early signaling of hypersensitive cell death because inhibitors of serine proteases, such as leupeptin, 3,4-dichloroisocoumarin, and 4–(2-aminoethyl)-benzenesulfonyl–flonyl–flouride (AEBSF), but not of caspases prevented the TvX–induced cell death and the inhibitory effect was observed during only early phase of the cell death (Yano et al., 1999).

7. Regulatory mechanism of elicitor–inducible defense gene expression

To elucidate the regulatory mechanism of expression of defense genes, we are investigating the cis– and trans–acting elements that are involved in transcription of genes for class I basic chitinases and class I basic β-1,3-glucanase responsive to fungal elicitors. In tobacco XD6S cells, it has been shown that PiE and/or TvX activates transcription of CHN48, CHN50 and GLN2 (Fukuda and Shinshi, 1994; Suzuki et al., 1995; Yamamoto, S., Suzuki, K., and Shinshi, H., in preparation). Deletion analysis of the promoter of CHN50 in transgenic XD6S cells showed that the region of the gene between positions –788 and –345 from the site of initiation of transcription is sufficient for the PiE–induced activation of transcription (Fukuda and Shinshi,
proteins to the GCC box have been identified and shown to function as an ethylene-responsive element (Shinshi et al., 1995; Ohme-Takagi and Shinshi,1990; Zhou et al., 1997) and has been called as EREBPs (Eulgem et al., 1999). Recently, we identified four tobacco genes encoding proteins that are homologous to WRKY proteins, namely, NtWRKY1, 2, 3, and 4 (Yamamoto, S., Suzuki, K., Yano, A., and Shinshi, H., in preparation). These proteins are conceivable candidates for transcriptional regulators of elicitor-inducible defense genes (Rushton and Somssich, 1998). The presence of elicitor-inducible and sequence specific DNA binding activity that interacts with EIRE in nuclear extracts from elicitor-treated tobacco cells was demonstrated (Fukuda and Shinshi, 1994). In addition, the elicitor-inducible activity of the binding to EIRE was reduced in nuclear extracts prepared from the cells that had been treated with cycloheximide and astaurosporine (Fukuda, 1997). Therefore, it is tempting to speculate that elicitor signal may be transmitted to the DNA binding protein and activate the transcription of the chitinase genes via protein kinase cascade including the p47 protein kinase (Fig. 3).

Three parsley W box-binding proteins, namely, WRKY1, 2, and 3, were identified (Rushton et al., 1996). The mRNAs corresponding to WRKY1 and WRKY3 are rapidly and transiently increased in parsley cells in response to fungal elicitor treatment (Rushton et al., 1996) and WRKY1 may function as a transcriptional activator mediating fungal elicitor-induced gene expression via W box elements (Eulgem et al., 1999). Recently, we identified four tobacco genes encoding proteins that are homologous to WRKY proteins, namely, NiWRKY1, 2, 3, and 4 (Yamamoto, S., Suzuki, K., Yano, A., and Shinshi, H., in preparation). These proteins are conceivable candidates for transcriptional regulators to modulate elicitor-inducible transcription of genes via (T)TGAC(C) motif in functionally defined EIRE (Fig. 3).

A GCC box is found in the promoter regions of a large number of defense genes (Ohme-Takagi and Shinshi, 1990; Zhou et al., 1997) and has been shown to function as an ethylene-responsive element (Shinshi et al., 1995; Ohme-Takagi and Shinshi, 1995; Suzuki et al., 1997). Specific binding proteins to the GCC box have been identified and called as ERFs, formerly designated as EREBP (Ohme-Takagi and Shinshi, 1995). We also found that level of mRNAs for all ERFs were increased by ethylene and wounding (Ohme-Takagi and Shinshi, 1995; Suzuki et al., 1997). Recently, we found that TvX induced gradual increase of ERF2 mRNA in contrast to transient increase of mRNAs for ERF3 and ERF4 and the activation of transcription of gene via the GCC box independently of ethylene production in tobacco XD6S cells (Fig. 3, Yamamoto, S., Suzuki, K., and Shinshi, H., submitted). In addition, it was suggested that both protein phosphorylation and dephosphorylation might be required for the TvX-induced expression of gene for ERF2 and for the TvX-activated GCC box-mediated transcription of gene (Fig. 3).

8. Perspectives

As described here, an availability of the model system should facilitate the biochemical and molecular biological analysis of the elicitor-inducible processes that lead to HR, and the system should also provide a tool for the isolation and identification of the specific components that are responsible for perception of elicitor, elicitor signal transduction, transcriptional regulation of defense genes, hypersensitive cell death and also survival. For example, identification of the AEBSF-sensitive serine protease and cloning of the corresponding gene will advance our understanding of the role of this protease in not only hypersensitive cell death but also programmed cell death in plant. The isolation of binding proteins for TvX will provide important information on the molecular identification of receptor protein for TvX, since the xylanase activity of TvX is probably not required for the elicitation process (Sharon et al., 1993), and the high-affinity binding site for TvX has been identified on plasma membrane in tobacco cells (Hanania and Avni, 1997). The molecular identification of the p47 protein kinase will help us to elucidate the role of this kinase in the elicitor signal transduction and the possible relationships between this kinase and known tobacco MAPKs, which seem to function as signal mediators responsive to various stimuli (Wilson et al., 1993; Wilson et al., 1995; Seo et al., 1995; Zhang and Klessig, 1997). The future analyses of function of the components identified in this system will also contribute to our understanding of the common mechanism of signal transduction in response to environmental stimuli in plant.

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