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Molecular Mechanisms Involved in Bacterial Speck Disease Resistance of Tomato

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An important recent advance in the field of plant-microbe interactions has been the cloning of genes that confer resistance to specific viruses, bacteria, fungi or insects. Disease resistance (R) genes encode proteins with predicted structural motifs consistent with them having roles in signal recognition and transduction. Plant disease resistance is the result of an innate host defense mechanism, which relies on the ability of plant to recognize pathogen invasion and efficiently mount defense responses. In tomato, resistance to the pathogen *Pseudomonas syringae* pv. *tomato* is mediated by the specific recognition between the tomato serine/threonine kinase Pto and bacterial protein AvrPto or AvrPtoB. This recognition event initiates signaling events that lead to defense responses including an oxidative burst, the hypersensitive response (HR), and expression of pathogenesis-related genes.

Keywords : Pto kinase, AvrPto, AvrPtoB, disease resistance; signal transduction, recognition specificity, PR genes

Plants have evolved complex mechanisms to recognize, and defend themselves against many potential pathogens (Lamb et al., 1989; Lamb 1994). These mechanisms include a rapid, localized cell death at the site of infection (Hypersensitive response, HR), increased expression of defense-related genes, and the oxidative burst (Bogdanove, 2002; Cutt & Klessig 1992; Goodman & Novacky 1994; Levine et al., 1994; Mehdy 1994). In many plant-pathogen interactions, recognition of pathogen is mediated by a plant disease resistance (R) gene that responds to the presence of corresponding avirulence (*avr*) gene in the pathogen. In interactions where the specific *R* gene in the plant or the corresponding *avr* gene in the pathogen is lacking, there is no concerted defense response and disease ensues. Gene-for-gene interactions can be envisaged to involve four steps including: (i) delivery of a pathogen-produced elicitor molecule to the plant cell; (ii) recognition of this signal molecule by the plant cell; (iii) signal transduction, which may involve several pathways; and (iv) the activation of a variety of defense responses. Elucidation of the molecular mechanisms by which plant defense systems are activated after specific recognition of a pathogen offers great potential for increasing the effectiveness of natural plant resistance by genetic engineering (Keen et al., 1993; Staskawicz et al., 1995).

Over the past ten years, many R genes have been isolated that confer resistance to various pathogens including virues, bacteria, fungi or nematodes (Martin et al., 2003). With a few exceptions (e.g. *Hm1, mlo, Hspro-*1), these R genes condition disease resistance in a gene-for-gene manner (Dangl 2001; Staskawicz et al., 1995; Martin et al., 2003).Based on predicted protein sequences, these R gene products can be divided into five classes: (i) intracellular protein kinases (e. g. Pto); (ii) proteins with an extracellular leucine-rich repeat (LRR) domain and a cytoplasmic protein kinase region (e.g. Xa21); (iii) intracellular proteins containing a region of a LRRs and a nucleotide binding site (NBS; e.g. RPS2, RPM1); (iv) intracellular proteins containing a region of homology to the Toll/IL-1R proteins in addition to LRRs and a nucleotide binding site (e.g. N, L6, RPP5); and (v) proteins with LRRs that appear to encode membrane-bound extracellular proteins (e.g. Cf-4, Cf-9). Proteins with these motifs are known to have important roles in signal recognition and transduction in mammals. For example, LRRs have been implicated in protein-protein interactions and the binding of peptide hormones by transmembrane receptors, the NBS may play a role in activation of kinases or G-proteins by binding to the nucleotide triphosphate ATP or GTP, and protein kinase participate in phosphorylation

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cascades that are central to many signal transduction pathways.

Bacterial Speck disease resistance and fenthion sensitivity

Pseudomonas syringae pv. *tomato* is a common bacterial pathogen that causes leaf speck disease when infecting susceptible tomato plants. An R gene to the pathogen, Pto, was originally discovered in a wild-type species of tomato *Lycopersicon pimpinellifolium*, and has subsequently been introgressed into many cultivated tomato (*L. esculentum*) cultivars by backcrossing. The *Pto* locus confers resistance specifically to *P. s.* pv. *tomato* strains that express the avirulence gene *avrPto* (Ronald et al., 1992; Martin et al., 1993). Interestingly, most tomato cultivars that carry bacterial speck resistance rapidly develop small necrotic lesions when exposed to the organophosphorous insecticide fenthion, and the gene controlling sensitivity, *Fen*, is located very close to the *Pto* locus on chromosome 5 (Laterrot 1985; Martin et al., 1993). Mutagensis of a bacterial speck-resistant tomato line identified gene near *Pto*, named *Prf*, that is required for both *Pto*-mediated resistance and fenthion sensitivity (Salmeron et al., 1994). This finding, along with the observation that overexpression of a *Pto* transgene in tomato confers mild sensitivity to fenthion in addition to resistance to *P. s.* pv. *tomato* strains expressing *avrPto* (Martin et al., 1994), suggests that Pto and Fen share some components in a common signal transduction pathway.

Pto and *Fen* were isolated by map-based cloning (Martin et al., 1993, 1994) and it was found that they belong to a small clustered gene family consisting of five members. Recently orthologues of *Pto* and *Fen* have been isolated and characterized from tomato lines that are susceptible to bacterial speck resistance and are insensitive to fenthion sensitivity (Jia et al., 1997). Introduction of a *Pto* or *Fen* transgene into a susceptible or fenthion insensitive tomato cultivar results in a marked increase in resistance to *avrPto*-expressing strains of *P. s.* pv. *tomato* and sensitivity to fenthion, respectively (Martin et al., 1993, 1994). *Pto* and *Fen* encode serine-threonine protein kinases that share 80% identity. Expression of *Pto* and *Fen* in E. coli and subsequent in vitro kinase assays indicated that they are both functional kinases with autophosphoryla-tion activity on serine and threonine residues (Loh & Martin 1995). Therefore, we have hypothesized that a protein phosphorylation cascade is involved in Pto-mediated disease resistance and Fen-mediated fenthion sensitivity.

Unlike other R gene products, the Pto protein contains no obvious membrane-spanning or extracellular domain, suggesting an intracellular localization. A potential myristylation site was found at the N-terminus of Pto. Myristylation is often involved in recruiting proteins to the plasma membrane and frequently plays a role in signal transduction pathways in mammalian cells. However, mutation of the invariant glycine residuce in the mristylation motif does not impair Pto-mediated resistance to bacterial speck disease (Loh et al., 1998).

Isolation of the *Prf* gene revealed that it encodes a large protein with a leucine-zipper, nucleotide-binding site, and a region of LRRs similar to those found in other plant R gene products (Salmeron et al., 1996). Interestingly, the rice *Xa21* gene product which confers resistance to bacterial blight contains an N-terminal region with similarity to the LRR-type proteins and a C-terminal protein kinase domain with sequence similarity to Pto (Song et al., 1995). This interesting structure of Xa21 protein suggests that LRR-type R gene products (e.g. Prf) may physically interact with protein kinase such as Pto to transmit the recognition of a pathogen signal to the plant cell(Oldroyd and Staskawicz, 1998; Rommens et al., 1995; Tang et al., 1999). However, there is no evidence to support this and the role of Prf in Pto- or Fen-mediated recognition or signaling remains unclear.

Molecular basis of gene-for-gene specificity in the bacterial speck disease resistance

Pto recognition specificity. Evidence for the direct interaction of an R and Avr protein has come from work with Pto-avrPto system. A domain swaping analysis between Pto and closely related Fen kinase has identified a region in the Pto kinase activation domain which is determinant for Pto-AvrPto interaction and specificity (Frederick et al., 1998; Kim et al., 2002). Within this region, Thr-204 is required for the specific recognition of AvrPto and AvrPtoB and for the triggering the HR. Moreover, introduction of a Thr to Fen at the amino acid location corresponding to Pto Thr-204 confer the ability to interact with AvrPto and AvrPtoB. It seems that a substitution at this position of Pto alters the structure of protein or hinder the phosphorylation status of Pto by another host protein kinase. However, this residue has not been found to be phosphorylated *in vitro* (Sessa et al., 2000). It is interesting to note that the Pelle and IRAK kinases also have a Thr conserved at the position corresponding to Pto Thr-204 (Cao et al., 1996).

Role of AvrPto in specificity and disease. Since the early 1980s it had been proposed that the molecular basis of gene-for-gene interactions might be the direct physical interaction of an elicitor molecule produced by the pathogen and a receptor encoded by the plant R gene. In support of this receptor-ligand model, a physical interaction was detected

between the Pto kinase and AvrPto protein, by using yeast two-hybrid system (Tang et al., 1996; Scofield et al., 1996). The Pto-AvrPto interaction detected in yeast was strictly correlated to the expression of disease resistance in plants. Deletion of AvrPto sequence required for Pto interaction in yeast impaired the ability of AvrPto to elicit Pto-mediated defense responses. Conversely, removal of AvrPto portions dispensible for Pto interaction did not affect resistance (Tang et al., 1996; Scofield et al., 1996).

During pathogen attack, the bacterial *Hrp* secretion system delivered AvrPto into the plant cell where it interacts with the Pto kinase. The question remains as to how the physical interaction of AvrPto to Pto activates the disease resistance response. First, it is possible that binding of AvrPto to Pto may effect a conformational change in the Pto kinase thus enhancing its phosphorylation activity directed at downstream components. In support of this model, it was found that the mutant Pto (Y207D), which has a substitution in a residue of the kinase activation domain, produced an HR-like cell death in the absence of AvrPto (Rathjen et al., 1999). Interestingly, Pto (Y207D) is able to induce an HR-like response only in the presence of an additional gene, *Prf*, which is required for bacteria speck resistance and fenthion sensitivity (Salmeron et al., 1996). Alternatively, it is plausible that AvrPto mediates the interaction between Pto and other plant proteins in a receptor complex, perhaps including Prf, and this interaction serves to activate Pto (Fig. 1). Based on dual requirement of Prf and Pto for AvrPto-triggered resistance, it was designated as the "guard" hypothesis (Van der Biezen and Jones, 1998).

In tomato plants lacking Pto, *Pseudomonas* strains with avrPto grow to higher levels and induce more severe disease symptoms than strains without avrPto (Chang et al., 2000; Shan et al., 2000a). Extensive mutagenesis study of AvrPto revealed residues required for its avirulence fuction

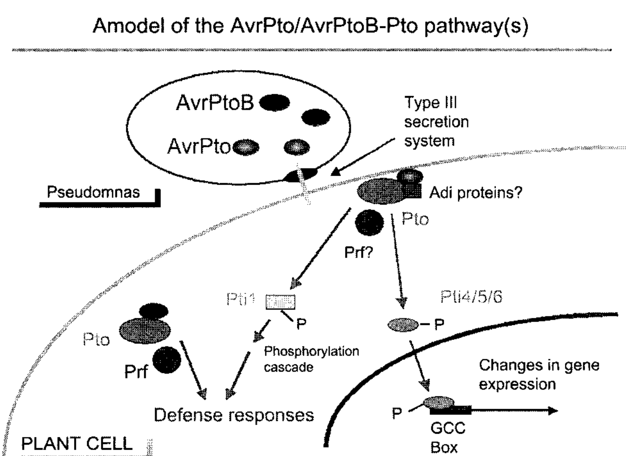

Fig. 1. A model of AvrPto/AvrPtoB-Pto pathways.

but dispensable for this role in virulence. Thus AvrPto provides an example in which structureal adaptations to evade recognition by an R protein could be made without loss of virulence function. This example supports the notion important to plant breeding that R gene durability will depend not only on the tendency of the Avr protein to be conserved due to an virulence function but also will require specifically that the structure the R protein recognizes is indispensable for that virulence function (Shan et al., 2000a).

In highly saturated screens in two different laboratory, 19 residue of AvrPto possibly required for the interaction with Pto (Chang et al., 2000; Shan et al., 2000a). Further mutagenesis of AvrPto revealed that GINP motif in the center of AvrPto required for recognition in tomato expressing Pto, also residues in the C-terminus are involved in recognition of tobacco Pto-like protein (Shan et al., 2000b).

Pto recognition of a second avirulence protein, AvrPtoB. When avrPto was originally cloned, it was used to develop gene-replacement mutants in *Pseudomonas* strain DC3000, which a single *avrPto*-hybridizing fragment is observed (Martin et al., 1993; Ronald et al., 1992). Surprisingly gene-replacement mutant was found to be avirulent on *Pto*-expressing tomato lines. This indicated that some *P. s.* pv. *tomato* strain might have a second *avr* gene recognized by Pto. This second avr gene, now refered to as *avrPtoB*, was isolated from DC3000 LexA fusion genomic libraries by using a "cross-kingdom" yeast two-hybrid screen using Pto as a bait protein (Kim et al., 2002). AvrPtoB is much larger than AvrPto and lacks an myristylation motif as seen in AvrPto. However, AvrPtoB interacts with an identical spectrum of Pto variant forms and mutant alleles as does AvrPto and elicits a Pto- and Prf-dependent HR in tomato (Kim et al., 2002).

AvrPtoB-like sequences are present in a wide array of phytopathogenic bacteria, including *P. syringae* pathovars, *Xanthomonas vesicatoria* and even *Erwinia carotovora* (Kim et al., 2002).This wide distribution of AvrPtoB-like sequence suggests that expression of avrPtoB-like sequence provides some advantage to the pathogen. In fact, AvrPtoB has been shown to have virulence activity in a bean pod assay and to promote growth of *P. s. pv. phaseolicola* in bean leaves (Jackson et al., 2002). Also AvrPtoB was shown to promote bacterial virulence by acting as a general inhibitor of programmed cell death in the plant cell as well as yeast (Abramovitch et al., 2003).

Although AvrPtoB and AvrPto are very different proteins, possible clues to their shared Pto-specific avirulence activity are present in nine subregions of both proteins where they have identical amino acids with similar spacing between them. The functional significance of most of these

regions in unknown; however, mutations of subdomain V(called GINP motif) abolished their interaction with Pto in yeast two-hybrid system and elicitation of disease reistance by virulence strains expressing mutants of AvrPtoB, but not AvrPto. The GINP motif might play a role as a direct contact point with Pto or, alternatively, it could affect the overall structure of AvrPto and AvrPtoB in a way that facilitates interaction of another region of the effector proteins with Pto. The latter possibility seems more likely for AvrPtoB in light of recent results showing that a form of that protein entirely lacking the GINP region still interacts with Pto (Kim et al., 2002).

Dual recognition specificities of Pto kinase. Unlike animals, plants do not have a circulating immune system, all plant cell are individually capable of recognizing pathogens and turning on the defense system (Baker et al., 1997). This type of defense responses achieved through the "gene-for-gene" interaction that single R proteins recognize single avirulence proteins. However, some R proteins recognize more than one effector proteins from pathogen. RPM1 and Pto each recognize different pairs of structural distinct effectors from pathogens. Pto recognize AvrPto and AvrPtoB, which is three times the mass of AvrPto. Both effectors share very short regions of similar sequences in common and at least one of the regions play a role in interaction with Pto. RPM1 recognize AvrB and AvrRpm1. This recognition mediated by a third protein, RIN4, as no direct interaction of RPM1 observed (Mackey et al., 2002). Mi-1 confers resistance to a nematode and an aphid. Also some loci tightly linked, HRT (recognize a coat protein of turnip crankle virus) and RPP8 (recognize effector of *Peronospora parasitica*) of *Arabidopsis* and Gpa2 and Rx1 (recognize a coat protein of Potato Virus X) of potato, show dual specificity (Cooley et al., 2000; McDowell et al., 1998; Van der Vossen et al., 2000; Vos et al., 1998). Thus dual (or multiple) recognition specificity may turn out to be a common feature of defense response. Also it provide some genomic and physiological economy for plants, which are faced with perhaps thousands of potential pathogens (or uncountable pathogens in co-evolution of plant-microbe interaction history). This notion is consistent with the recent report that Arabidopsis contains only 200 potential members of the five major classes of R proteins (Meyers et al., 2003). The studies of AvrPto and AvrPtoB suggests that common structural motifs embedded within diverse pathogen proteins might account for the limited number of R genes in plant.

Pto-mediated signal transduction and subsequent defense response

The Pto-AvrPto recognition event is postulated to activate the Pto kinase and induce phosphorylation of downstream components in signal pathways leading to defense response (Figure). Downstream effectors, which physically interact with Pto and represent putative targets for its phosphorylation, were isolated by using a yeast two-hybrid screen (Zhou et al., 1995; Zhou et al., 1997). Four of Pto-interacting proteins have been investigated further: Pti1, a protein kinase, and three related transcriptional factors Pti4, Pti5 and Pti6. Other genes with possible roles in Pto-mediated signaling have been isolated by yeast three-hybrid system to identify proteins that interact when Pto bind to AvrPto. Prf was not isolated from this screen. However, five AvrPto-dependent Pto-interacting (Adi) proteins were isolated (Bogdanove and Martin 2000).

Pti1 is a cytoplasmic serine/threonine protein kinase, and its role in Pto-mediated disease resistance was assessed in transgenic tobacco plant (Zhou et al., 1995). In these plants, overexpression of Pti1 enhanced the HR in leaves inoculated with *P. s.* pv. *tabaci* containing avrPto gene. In vitro analysis revealed that Pti1 is able to autophosphorylate via an intramolecular mechanism and it is specifically phosphorylated by Pto kinase (Zhou et al., 1995; Sesse et al., 1998). Taken together, this evidence suggests that Pti1 acts downstream of Pto in a phosphorylation cascade that triggers the HR. However, there are currently no loss-of-function evidence to support the involvement of Pti1 in Pto-mediated resistance. Another screen using yeast three-hybrid system, Pti1 was identified as a group of Adi.

A major target of signal transduction pathways leading to defense responses in plants is the transcriptional activation of defense-related genes (Zhang and Klessig, 2001). In bacterial speck resistance of tomato and tobacco, transcripts of a set of defense genes encoding PR proteins accumulate earlier during resistant interaction than in susceptible interactions (Thilmony et al., 1995; Zhou et al., 1997). The Pti4/5/6 proteins were found to physically interact with Pto in yeast two-hybrid system (Zhou et al., 1997). Sequence analysis revealed the Pti4/5/6 contain a DNA-binding domain, a region of acidic residues possibly involved in transcriptional activation and nuclear localization sequences. Pti4 and Pti6 are induced by the bacterial speck pathogen (Thara et al., 1999). Gu et al. (2000) showed that phosphorylation of Pti4 by Pto enhances binding of transcriptional factor to the GCC box. Transgenic Arabidopsis that constitutively express Pti4, 6, or 6 demonstrate that these transcriptional factors increase the expression of PR-genes containing GCC box in promoter region. Pti4 overexpression *Arabidopsis* plants increased resistance to powdery mildew and tolerance to bacterial pathogen *P. s.* pv. *tomato* DC3000 (Gu et al., 2002).

Perspective

Clearly a great deal remains to be learned about the

mechanisms that contribute to specific recognition of AvrPto and AvrPtoB by the Pto kinase, transduction of this information and subsequenct activation of defense responses. However, much has been revealed by identifying proteins that physically interact with Pto from bacterial and tomato libraries, cDNA microarray, SAGE, GeneCalling system, Virus Induced Gene Silencing Screen (VIGS) and now focusing on their biological significance in the plant cell (Mysore et al., 2002). Further biochemical, molecular, and genetic characterization will be required to elucidate precisely how interaction with Pto activates downstream components, and attempts to reveal the biological relevance of such interactions *in vivo* will be the main challenge in the future (Fig. 1). A better understanding of the molecular strategies that plants utilize to defend themselves against pathogen will allow improving disease resistance in economically important crops.

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