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Determination of virulence contribution from *Phytophthora infestans* effector IPI-O4 in a resistant potato host containing the *RB* gene

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ABSTRACT

Late blight of potato, caused by the oomycete pathogen *Phytophthora infestans*, is persistent and costly disease of this important crop plant. Breeders have continually struggled with the evolution and introduction of new pathogen genotypes that threaten to overcome major resistance genes incorporated into new varieties. Wild species relatives of potato have been a valuable resource for the identification of novel resistance genes including the *RB* gene, from the diploid wild potato species *Solanum bulbocastanum*. The *RB* gene confers partial resistance to most *P. infestans* genotypes through its recognition of members of the corresponding pathogen effector protein family IPI-O. Multiple alleles are present at the IPI-O locus and while some alleles are recognized by RB to elicit host resistance (e.g. IPI-O1, IPI-O2), others are able to elude detection (e.g. IPI-O4). In our previous research, we found that in planta expression of *P. infestans* effector IPI-O4 is able to suppress the HR elicited by IPI-O1 in the presence of RB. Additionally, we have observed that *P. infestans* lineages containing IPI-O4 are able to cause more disease on RB plants compared to those without IPI-O4. This led to a hypothesis that the presence of IPI-O4 results in suppression of RB. In this study, we provide evidence that in planta over-expression of IPI-O4 is able to suppress RB-mediated resistance, causing enlarged lesions in RB containing K41 potato lines. The results of this study indicate that even subtle effects from host or pathogen factors during the early stages of infection can heavily influence the ultimate outcome of the interaction.

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1. Introduction

In nature, microbes interact with plants on an enormous scale. The majority of microbes, however, are unable to cause disease on plants because they lack the proper tools to avoid or overcome host defenses. The host's basal defenses, such as cell wall thickening, production of reactive oxygen species and induced synthesis of antimicrobial compounds, are mediated by extracellular pattern recognition receptors (PRRs) that recognize and respond to microbe/pathogen associated molecular patterns [1]. This type of host defense is also called PAMP triggered immunity [PTI; 2]. Plant pathogens can therefore be described as specialized microbes that have evolved the ability to overcome and/or suppress PTI. Phytopathogens accomplish this task using molecular “weapons” termed effectors [3,4]. Effectors function outside pathogens and are highly variable in metabolic function and molecular structure. So far, protein, RNA molecules, and secondary metabolites have been found to serve as effectors [5,6]. The fitness advantage to the pathogen contributed by individual effectors varies. Some effectors are dispensable, either because they may have a minor virulence contribution to the pathogen or they are functionally redundant. Others are widely conserved or have an obvious and significant impact on virulence [7]. A major function of pathogen effectors is to overcome PTI, leading to effector triggered susceptibility (ETS). However, the same set of effectors or others can be recognized by host resistance (R) proteins and elicit effector triggered immunity [ETI; 2]. Like PTI, ETI can also be inhibited by effectors, resulting in another level of ETS. ETI and ETS demonstrate a co-evolutionary arms race between plants and pathogens. PTI and ETI cannot be differentiated based solely on the suite of induced host responses. However, ETI is typically stronger and faster than PTI, and is often associated with the hypersensitive response (HR), a rapid localized programmed cell death, which has the effect of limiting the development of a pathogen at the site of attack [8,9]. Recently a new pathogen-host interaction model, termed the “Invasion Model”, was proposed to integrate the PTI-ETI dichotomy, and view

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plant innate immunity as a system that evolves to detect invasion [10].

Potato (*Solanum tuberosum* L.), belonging to the *Solanaceae* family of flowering plants, is the most important vegetable crop in the world with nearly 400 million metric tons produced worldwide every year [11]. Late blight of potato and tomato is caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary and has historically been one of the most serious and persistent of all crop diseases. In the U.S. and Canada, *P. infestans* is spread as clonal lineages, asexual descendants of a single genotype [12]. In the U.S., a major outbreak of potato and tomato late blight started in 2009, which was caused by a massive distribution of *P.infestans* clonal lineage US22, as well as US23 and US24. Since then, US23 has become increasingly dominant [12].

Currently, most commercially grown potato cultivars are quite susceptible to late blight, and fungicides are used intensively to protect the crops, which is expensive and negatively impacts the environment. Therefore, the best way to control the disease is through natural host resistance. However, most *R* genes introduced from wild potato relatives have already been overcome by specific *P. infestans* genotypes due to mutation or deletion of corresponding effectors, although some still provide moderate resistance in certain regions [13]. Among the most promising genes against *P. infestans* is the *RB* gene, which confers broad-spectrum resistance to late blight. Somatic fusions and transgenic potato plants containing the *RB* gene are highly resistant to late blight even under intense disease pressure [14–17]. The resistance phenotype conferred by RB is distinctive in that it confers partial resistance rather than immunity [18]. The partial resistance phenotype is more pronounced in RB transgenic potato plants, which exhibit a low level of susceptibility and the pathogen is able to sporulate to a small degree [14]. Our previous research demonstrated that RB is able to mediate an HR, but other responses such as callose deposition and defense gene transcription is reduced compared to plants containing late blight immunity [18]. This suggests that responses elicited by RB are either not fully activated or are partially suppressed, allowing *P. infestans* to outgrow the HR lesion.

With the accomplishment of genome sequencing of multiple *Phytophthora* species, such as *P. infestans*, *P. sojae*, and *P. ramorum*, the highly conserved RXLR and dEER motifs have been used as a powerful tool to study effector genomics in oomycete pathogens [19,20]. After the initial screening of putative effectors, a number of RXLR effectors have been exploited to study the mechanisms by which *P.infestans* manipulates host immunity. Multiple host components are targeted by *P. infestans* effectors, including CMPG1 E3 ligase [21], protease C14 [22], transcription factors [23], mitogen-activated protein kinase [24], vesicles [25], autophagy cargo receptor Joka2 [26], and plant PP1c isoforms [27].

The focus of the present study is the *P.infestans* RXLR effector IPI-O (also known as Avrblb1). IPI-O is a multigene family of effectors. This family shares a cell attachment motif Arg-Gly-Asp (RGD), and it has been reported that IPI-O functions in the apoplast to modulate host immunity by disrupting host cell wall and plasma membrane adhesions [28,29]. It was previously determined that this effector family consisted of at least two closely related members, IPI-O1 and IPI-O2 [30], which are induced in planta during the early stage of *P. infestans* infection, supporting a likely role of this gene family in pathogenesis [31]. Sequencing of *IPI-O* from other *P. infestans* genotypes revealed that the IPI-O locus could be extremely variable [32,33]. IPI-O variants have been divided into three classes based on diversity of their deduced amino acid sequences. Class I and Class II variants (e.g. IPI-O1) are found in the majority of *P. infestans* genotypes, and are recognized by the RB protein to elicit ETI. In contrast, class III variants (e.g. IPI-O4) are more rare, and are able to elude recognition by RB [32,33]. In addition, we have previously shown that in planta expression of IPI-O4 is able to suppress the HR elicited by IPI-O1 in the presence of RB in *N. benthamiana* [32,34]. We also found that two IPI-O4 containing Guatemala *P. infestans* isolates were able to overcome resistance in RB transgenic potato plants [32]. Vleeshouwers et al. [35] proposed that the absence of IPI-O1 and IPI-O2 was responsible for the ability of *P. infestans* to overcome RB-mediated resistance based on the performance of two Mexican *S. bulbocastanum* compatible isolates. However, one of them also contains IPI-O4, leaving the possibility that the presence of IPI-O4 is critical. In this study, we have provided evidence that in planta overexpression of *P. infestans* effector IPI-O4 suppresses, but does not eliminate, the RB resistance response. The results of this study will further our understanding on the outcome of interaction between RB containing hosts and IPI-O4 containing *P. infestans*.

2. Materials and methods

2.1. Potato transformation and identification of positive transformants

The effect of in planta expression of the IPI-O4 gene in an *RB* containing resistant background was accomplished through stable transformation of IPI-O4 into resistant potato line K41, derived from a potato-*S. bulbocastanum* hybrid backcrossed with 'Katahdin' or 'Atlantic' three times. *Agrobacterium tumefaciens* strain GV3101 containing N-terminal Myc-tagged IPI-O4 in Gateway®-compatible binary vector pGWB18 [34] was used for transformation. Internode explants from in vitro K41 plantlets were transformed following a standard *A. tumefaciens*-mediated potato transformation procedure [36].

Both wild-type and transgenic K41 plants were propagated from cuttings and maintained in the greenhouse, which was set for 14 h of daylight, a daytime temperature of 23 °C, and a nighttime temperature of 15 °C. Genomic DNA was extracted from leaf tissue using the Qiagen (Hilden, Germany) DNeasy Plant Mini Kit. The oligonucleotides MycIPI-O1F3 (5′-AGAAGACTTGAACGGACTCG-3′) and MycIPI-O4R1 (5′-CCGTGTTGGGCTCTTTTGTGAC-3′) were used to detect the presence of the *IPI-O4* gene in transgenic K41 candidates using polymerase chain reaction (PCR). The PCR conditions were: 1 min at 94 °C followed by 35 cycles of 15 s at 94 °C, 30 s at 55 °C, 30 s at 68 °C, and 7 min at 68 °C.

Seven IPI-O4 positive transgenic K41 candidates A, D, E, F, G, L, R were selected for protein blotting to verify the expression of IPI-O4. To prepare total protein, four-week-old wild-type or IPI-O4 transgenic K41 leaves were ground in liquid nitrogen. One hundred milligrams of leaf powder was thawed and boiled in 200 μl of 4X SDS-PAGE loading buffer (200 mM Tris-Cl (pH 6.8), 400 mM DTT, 8% SDS, 40% glycerol) for 5 min. The suspension was centrifuged for 5 min at room temperature at 13,200 rpm. The supernatant was added to an equal volume of 2X SDS-PAGE loading buffer (100 mM Tris-Cl (pH 6.8), 200 mM DTT, 4% SDS, 20% glycerol), and boiled for 5 min. After a 5 min centrifugation at room temperature at 13,200 rpm, 30 μl of the supernatant was resolved on a 12% SDS-PAGE gel and transferred to PVDF (GE Healthcare, Little Chalfont, UK) according to the manufacturers recommendations. Protein was detected using a 1:5000 dilution of an anti-Myc antibody (Roche, Basel, Switzerland) in an ECL Western blotting detection system (Thermo Fisher Scientific, Waltham, MA) according the manufacturer's instructions.

2.2. P. infestans genotypes and host inoculation

In order to test the effect of IPI-O4 on the ability of *P. infestans* to cause disease symptoms in potato, two *P. infestans* clonal lineages