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Potato Early Dying: Molecular Perspectives on Pathogenicity and Host Resistance

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ABSTRACT

Potato early dying is a widespread disease that consistently causes yield losses in almost all potato-growing regions worldwide. Despite this, it does not have a reputation as a devastating disease of potato since symptoms and yield losses are subtle and infection does not lead to complete crop loss. However, the interaction between the vascular fungus *Verticillium dahliae* and the root lesion nematode *Pratylenchus penetrans* to form the early dying complex makes studying this disease very interesting to plant pathologists. The ability of *Verticillium* spores to survive in the soil for many years, and the difficulty in properly treating infested soil makes this one of the most persistent and widespread diseases of potato. The purpose of this short review is to provide readers with an update on recent research developments relating to our understanding of this disease complex and approaches used to control potato *Verticillium* wilt. This review is not meant to be comprehensive, since several excellent review articles with information about the potato early dying complex and *Verticillium* wilts in general are already available. Rather, we have chosen to focus on the impact molecular biology has had on our understanding of this disease and how it has provided opportunities for improvement of resistance in potato cultivars. Although various isolates of *V. dahliae* are important pathogens of multiple plant species, here we will focus mainly on the relationships among *V. dahliae*, *P. penetrans*, and potato.

Keywords: root lesion nematode, vascular wilt resistance, *Verticillium*

CONTENTS

THE POTATO EARLY DYING COMPLEX

Potato early dying (PED), caused primarily by the soilborne imperfect vascular fungi *Verticillium dahliae* Kleb and *V. albo-atrum* Reinke and Berthold, is characterized by unilateral wilting of leaves, chlorosis, and premature senescence (**Fig. 1**; Rowe and Powelson 2002). Yield losses of 50% can occur in sandy soils and 20-30% losses are common. In addition, the disease affects both tuber size and specific gravity. Soil fumigation is the most widely practiced control tactic where potatoes are grown intensively. For example, in the state of Wisconsin, between 60 and 70% of the potato acreage is fumigated each year with metam sodium. At a cost of approximately $400 per hectare to fumigate, the financial impact of disease prevention alone is substantial.

Symptom expression and colonization by *V. dahliae* is enhanced during coinfection with the root lesion nematode *Pratylenchus penetrans* (Martin *et al.* 1982; Riedel and Rowe 1985; Rowe *et al.* 1985; MacGuidwin and Rouse 1990; Rotenberg *et al.* 2004). The synergistic interaction between these two organisms has been well studied. However, many questions related to the role of *P. penetrans* in eliciting increased susceptibility to *V. dahliae* remain to be answered. At population levels too low to induce symptoms using either *P. penetrans* or *V. dahliae* individually,

Fig. 1 Symptoms of potato early dying. (**A**) Leaf exhibiting lateral chlorosis and necrosis. (**B**) Resistant (left) and susceptible (right) potato hybrids in a field plot inoculated with *Verticillium dahliae*.

coinoculation of potato roots with both pathogens leads to impaired photosynthesis as indicated by decreased light use efficiency (Saeed *et al.* 1997). This suggests that the presence of *P. penetrans* enhances *V. dahliae* symptom expression. One possible explanation is that feeding by the nematode compromises structural barriers, leading to increased fungal colonization (Powell 1971; Mai and Abawi 1987). However, several lines of data are inconsistent with this prediction. *V. dahliae* prefers to enter the root cortex via the

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Invited Mini-Review

actively growing tip (Perry and Evert 1983; Bowers *et al.* 1996), whereas *P. penetrans* favors other areas (Zunke 1990). Furthermore, fungal colonies within coinfected roots do not appear at nematode feeding sites or lesions (Malek and MacGuidwin, unpublished data). There also does not appear to be a clear correlation between enhanced nematode root infection and increased fungal infection (MacGuidwin and Rouse 1990; Wheeler *et al.* 1992; Saeed *et al.* 1998). Experiments using stem inoculation of *V. dahliae* suggest that *P. penetrans* infection does not lead to increased susceptibility of roots to fungal entry, but rather leads to physiological changes resulting in increased vascular infection (Rotenberg *et al.* 2004). Unfortunately, the molecular mechanisms involved in eliciting these responses have not been characterized. Molecular biology may provide insights into the regulation of genes involved in this phenomenon. Microarrays to study global gene expression and proteomics to identify differentially accumulated proteins in areas distal to *P. penetrans* infection sites might shed some light on factors involved in increased susceptibility to *V. dahliae*.

Identification of factors involved in pathogenicity

Virtually all molecular data relating to pathogenicity factors involved in eliciting PED symptoms have been derived from experiments with *Verticillium*. Some important factors, such as glycosyl cellulase family genes have been identified in *P. penetrans* (Uehara *et al.* 2001). However, it appears likely that these genes are important in facilitating penetration and migration in roots and are not responsible for inducing increased susceptibility to *V. dahliae* or eliciting PED symptoms in the host.

Individual *V. dahliae* specimens isolated from infected crops throughout the world have not been well differentiated using genetic characterization. However, many strains have been placed into subgroups based on vegetative compatibilities (Leslie 1993). Multiple vegetative compatibility groups (VCG) have been determined for *V. dahliae* based on a specific isolate's ability to anastomose and form heterokaryons, leading to the possibility for genetic exchange of information (Leslie 1993; Leslie and Zeller 1996). Four major VCGs have been defined using diverse *V. dahliae* isolates from multiple hosts and geographic locations (Joaquim and Rowe 1990; Chen 1994; Daayf *et al.* 1995; Elena and Paplomatas 1998; Korolev *et al.* 2000, 2001). VCGs 2 and 4 have been further subdivided based on differential interactions between tester strains (Joaquim and Rowe 1990, 1991). *V. dahliae* isolates associated with PED belong to the VCGs 4A and 4B (Joaquim and Rowe 1991; Strausbaugh 1993; Botseas and Rowe 1994; Dobinson *et al.* 2000). Strains belonging to VCG 4A have the most impact on potato production within North America because they are more virulent than VCG 4B strains and because they exhibit a synergistic interaction with *Pratylenchus penetrans* (Joaquim and Rowe 1991; Strausbaugh 1993; Botseas and Rowe 1994; Omer *et al.* 2000). Recently, DNA analysis of artichoke, cotton, and olive isolates of *V. dahliae* using amplified fragment length polymorphisms (AFLPs) revealed a significant correlation between groupings using VCGs and molecular markers, suggesting that individuals within any given VCG are molecularly similar (Collado-Romero *et al.* 2006). Additionally, members of the same VCG subgroup were found to be genetically more similar to each other than to other isolates collected from the same geographic region or host source (Collado-Romero *et al.* 2006). The ability to use molecular tools to identify and classify *Verticillium* isolates will ultimately speed their characterization and help to identify differences that are important in determining host specificity.

Many plant pathogens produce effector proteins and deliver them to the host cell cytoplasm where they manipulate host defenses to cause disease. The method by which pathogens deliver effectors varies considerably. Gram-negative plant pathogenic bacteria utilize specialized structures such as the type III secretion system to penetrate the host cell wall and plasma membrane in order to translocate effector proteins to the cytoplasm (Cornelis 2006). Effectors produced by oomycetes are delivered to both the apoplast and cytoplasm of plant cells (Kamoun 2007). The latter set of secreted oomycete effectors have an amino acid motif that targets them for import into the host cell cytoplasm (Birch *et al.* 2006). This RXLR amino acid motif, where X is any amino acid, has been identified in hundreds of putative effector proteins from the potato late blight pathogen *Phytophthora infestans* and even more in *P. sojae* and *P. ramorum* (Zody *et al.* 2007). Similar to oomycetes, fungi are capable of delivering effectors to both the inside and the outside of plant cells. AVR2 and AVR4 from the fungal plant pathogen *Cladosporium fulvum* are secreted into the apoplast where they act as inhibitors to tomato cysteine proteases and protect against chitinases, respectively (Rooney *et al.* 2005; van den Burg *et al.* 2006). Other effectors such as AVR-Pita from *Magnaporthe grisea*, AvrL567 from the flax rust fungus *Melampsora lini*, as well as AVRa10 and AVRk1 from the barley powdery mildew fungus *Blumeria graminis* are likely recognized in the host cytoplasm since the corresponding resistance gene encodes a putatively cytoplasmic protein (Jia *et al.* 2000; Dodds *et al.* 2004; Ridout *et al.* 2006). The molecular basis for translocation of fungal effectors into the host cytoplasm remains to be elucidated. While bioinformatics has assisted in the identification of many putative effectors from oomycetes through the recognition of a signal sequence for export from the pathogen and an RXLR motif for import into the host, no discernable amino acid motifs have been identified in effectors of plant pathogenic fungi. However, as more effectors are characterized and as more fungal genomes are sequenced, the probability of finding commonalities among effectors increases.

A draft DNA sequence for *V. dahliae* is now available, which covers all eight chromosomes at 7.5x coverage (http://www.broad.mit.edu/annotation/genome/verticillium_dahliae/MultiHome.html). This assembly of 52 scaffolds covers 33.83 Mb of the genome. The goals of the *Verticillium* genome sequencing project, funded by the USDA-CSREES National Research Initiative, includes the production of a 4x assembly of *V. albo-atrum* and a set of 20,000 expressed sequence tags (ESTs) from *V. dahliae*. One of the major challenges that lies ahead will be to adequately utilize this genomic information to identify effectors and elicitors that are important for pathogenicity.

Several *Verticillium* factors responsible for elicitation of disease symptoms have been identified. The production of cell wall degrading enzymes, toxins, and elicitors have been implicated in pathogenicity and disease symptoms of several plant species (Fradin and Thomma 2006), but these factors have not been well characterized during infection of potato or related hosts. Crude extracts containing phytotoxins from *Verticillium* elicit defense responses, wilting, and cell death (Pegg 1965). Because these extracts contain a mixture of glycoproteins, cell-wall degrading enzymes, and large protein-lipopolysaccharide (PLP) complexes, it has been difficult to determine what components are responsible for elicitation of the disease responses. A moiety of a PLP purified from extracts of a strain of *V. dahliae* pathogenic on potato exhibited phytotoxicity (Buchner *et al.* 1982). This small molecular weight (~3 kDa) peptide was not found in extracts from a nonpathogenic mutant, suggesting a correlation between the presence of this PLP and the ability of the fungus to cause disease symptoms (Buchner *et al.* 1982). Further experiments showed that this toxin accumulates in the cell walls of xylem in stems and tubers of susceptible plants (Nachmias and Krikun 1985). While there appears to be a correlation between the presence of the 3 kDa toxin and disease symptoms in potato, it is likely that there are multiple phytotoxins utilized by *V. dahliae* during infection since many other phytotoxins of differing sizes have been identified (Fradin and Thomma 2006).

More recently, a necrosis and ethylene inducing peptide (NEP) which induces wilting in cotton leaves was identified

in *V. dahliae* (Wang 2004). The ability of this protein, called VdNEP, to induce hypersensitive response-like cell death suggests a role as an elicitor rather than a toxin (Wang 2004). It is tempting to predict that elicitors from *V. dahliae*, such as VdNEP, work similarly to elicitors of other necrotrophic plant pathogens that are required for virulence on specific hosts. For example, the cell death elicitor victorin, a small peptide produced by *Cochliobolus victorae*, is recognized by an *Arabidopsis* protein containing nucleotide binding (NB) and leucine rich repeat (LRR) motifs, common constituents of disease resistance proteins (Lorang *et al.* 2007). In this case, the victorin toxin is secreted into areas not yet colonized by the fungus, where it elicits a resistance response in the host. This resistance response ultimately leads to programmed plant cell death, and provides a suitable growth environment for the necrotroph (Lorang *et al.* 2007). At this point, we can only speculate as to the role of *V. dahliae* phytotoxins in pathogenicity. No relationships between the presence of *V. dahliae* phytotoxins and corresponding host factors have been identified.

The majority of the molecular work done to date on *Verticillium* phytotoxins has relied on the identification of molecules produced during growth in liquid culture. This raises an important question as to whether the toxins that are produced in liquid culture are the same as those secreted *in planta*. Studies of *Verticillium* genes upregulated during xylem colonization and proteins secreted by the fungus during infection will hopefully provide much needed insight into what factors are crucial for pathogenicity and lead to discovery of corresponding host targets (Neumann and Dobinson 2003).

PED resistance breeding in potato

Research efforts focused on the management of *Verticillium* wilt (VW) through irrigation, rotation, fumigation, biological control, and cultural practices have been somewhat effective (Powelson and Rowe 1993). However, the best long-term control strategy remains the development and planting of resistant cultivars. The majority of research has focused on host resistance to *V. dahliae* and *V. albo-atrum*, with less emphasis on resistance to the root lesion nematode. A great deal of effort has been spent on identifying sources of host resistance to *Verticillium* in potato (Akeley *et al.* 1956; Hunter *et al.* 1968; Corsini *et al.* 1990; Hoyos *et al.* 1993; Jansky *et al.* 2004). While immunity has not been reported in *S. tuberosum*, strong, dominant major gene resistance to VW has been reported in wild species germplasm (Lynch *et al.* 1997; Jansky *et al.* 2004). Polygenic resistance has also been identified in the cultivated potato (Simko *et al.* 2004b). In tomato (*Solanum lycopersicon*), resistance to VW is encoded within the *Ve* locus (Schaible *et al.* 1951), which has provided resistance in most cultivated varieties for several decades. The way in which *Ve* confers resistance is not well understood. Some results suggest that *Ve* provides tolerance to the pathogen following its entry into the xylem tissue, and then prevents the pathogen from spreading (Chen *et al.* 2004). In other studies, however, the pathogen was not found in areas other than basal internodes, suggesting a more active immune response to VW in *Ve*-containing tomatoes (Williams *et al.* 2002).

The *Ve* locus in tomato contains two closely linked genes, both of which encode receptor-like proteins (RLPs) with extracellular leucine-rich repeats (LRRs; Kawchuk *et al.* 2001). Both genes, *Ve1* and *Ve2*, confer resistance to VW in potato when stably introduced as transgenes (Kawchuk *et al.* 2001). The LRR domain is common among plant resistance proteins and is a hot-spot for diversifying selection, implying a role in determination of pathogen specificity (Martin *et al.* 2003). The fact that the LRRs of *Ve* putatively lie outside of the plant cell implies recognition of a pathogen elicitor or toxin prior to their entry into the plant cell. The region of the Ve protein located inside the plant cell contains some sequence similarity to mammalian endocytosis signals (Kawchuk *et al.* 2001), suggesting a possible mechanism for capture of extracellular ligands or removal of cell surface receptors.

Homologs of *Ve* have been identified in distantly related Solanaceous species including *S. torvum* (turkeyberry), demonstrating conservation of this gene within the family (Fei *et al.* 2004). In *S. tuberosum*, 11 homologs of *Ve* have been identified at a quantitative trait locus (QTL) for resistance (Simko *et al.* 2004a). However, these homologs have not been shown to confer resistance to VW (Fei *et al.* 2004; Simko *et al.* 2004a). Recently, a marker associated with a potato homolog of *Ve* has been linked to resistance in interspecific wild *Solanum* species hybrids (Bae *et al.* 2008a). A cleaved, amplified polymorphic sequence (CAPS) marker was developed by comparing *Ve*-like sequences from resistant and susceptible progeny of a population segregating for resistance (Bae *et al.* 2008a). This marker was successful in predicting resistance not only in the segregating population, but also in a number of diploid wild species hybrids and some cultivated tetraploid *S. tuberosum* varieties (Bae *et al.* 2008a). Together, these data suggest that the *Ve* locus in some cultivated varieties and wild potato species encodes resistance to VW.

Breeding for VW resistance in potato has been a challenging endeavor. Despite decades of breeding efforts, only a few major cultivars have moderate levels of resistance; all others are susceptible. Limited breeding progress could be due to a lack of genetic resources for resistance genes or the inability to effectively identify resistant clones. It is likely due to the latter, because wild *Solanum* relatives with VW resistance are available and accessible to breeders (Concibido *et al.* 1994; Jansky and Rouse 2000). On the other hand, it has been difficult to identify effective resistance screening methods that can be used on the large scale required by potato breeders. There is typically no clear distinction between VW resistant and susceptible phenotypes, so judgments must be made when categorizing clones. Resistance rankings may vary depending on the screening method used. Finally, the environment is an important component of the VW disease triangle, and the interactions among host, pathogen and environment are not well understood.

Since the 1980's, potato breeders have quantified pathogen populations in host tissue as an alternative or supplement to symptom expression data (Davis *et al.* 1983; Pegg and Street 1984; Davis and Sorensen 1986; Corsini *et al.* 1990; Hoyos *et al.* 1993; Wheeler *et al.* 1994; Jansky and Rouse 2000, 2003; Jansky *et al.* 2004). Stem colonization is typically considered to be a better method of resistance evaluation than symptom expression because it measures actual pathogen levels in plant tissues. Estimates of *V. dahliae* population sizes *in planta* are more effective than visual disease assessment in explaining variability in yield loss due to VW (Frost *et al.* 2007).

Despite being able to provide a picture of pathogen reproduction in host tissue, there are problems associated with stem plating assays, including the time needed to generate data, contamination from other fungi, and a high rate of false negatives. Recently, an effective quantitative PCR (Q-PCR) alternative to stem plating assays has been developed (Atallah *et al.* 2007). The sensitivity of detection of *V. dahliae* is greatly improved using this assay. It can detect the presence of *V. dahliae* even when no colonies are observed on plates (Atallah *et al.* 2007). The Q-PCR procedure reduces the time needed for the detection and quantification of *V. dahliae* from two weeks to one day and improves accuracy compared with plating assays. Additionally, this method provides an unambiguous detection of *V. dahliae*, compared with the less discriminatory detection of multiple fungal species using plating assays. This improved assay was used to study the spatio-temporal colonization by *V. dahliae* of two potato cultivars with varying responses to PED (Bae *et al.* 2007). Colonization of the susceptible cultivar Russet Norkotah consistently increased in both apical and basal samples during the ten weeks of the experiment, while it reached a plateau after week six in the moderately

resistant cultivar 'Ranger Russet'.

Marker assisted selection may provide the best method for identifying clones with VW resistance. The CAPS marker described earlier will be useful for identifying resistant clones in populations segregating for the *Ve* ortholog (Bae *et al.* 2008a). The marker was tested in a broad set of diploid clones and was found to co-segregate with the resistance phenotype. This PCR-based procedure does not rely on expensive molecular methods and is likely to be a valuable tool for potato breeders interested in selecting for VW resistance. The breadth of applicability of this marker is not yet known.

Despite the challenges associated with identifying VW resistant clones, some breeding progress has been made. Resistance breeding using wild *Solanum* species has typically been carried out at the diploid level, where breeding progress is more rapid (Jansky and Rouse 2000). Resistant diploid clones can be brought to the tetraploid level via unilateral sexual polyploidization (4x – 2x hybridization) to create clones that are directly crossable to tetraploid cultivars. In one study, families from 4x × 4x crosses had the highest yield and stem colonization, while 2x × 4x families had the lowest yield (Bae *et al.* 2008b). More importantly, 4x × 2x and 2x × 2x families had high yield and low levels of stem colonization. Consequently, if high yielding diploid VW resistant parents are used in 4x × 2x crosses, then a high proportion of resistant high-yielding offspring can be expected (Bae *et al.* 2008b). Similarly, VW resistance was effectively transferred to the tetraploid level via unilateral sexual polyploidization (Frost *et al.* 2006). In the small families generated by this study, clones with high levels of resistance were produced.

A major focus of breeding for resistance to PED in the future will rely on a better understanding of the molecular mechanisms and interactions resulting in host resistance. Since major and potentially durable resistance has been identified in some wild potato hybrids, future breeding efforts will focus on incorporating this trait into new cultivars.

CONCLUDING REMARKS AND FUTURE PROSPECTS

The majority of cultivated potato varieties used in potato production worldwide are susceptible to PED. Potato growers spend a great deal of time and money on measures to keep pathogen inoculum levels low enough to prevent yield-limiting symptoms. Typically, control of the disease involves soil fumigation, which can have an impact on soil health since it does not differentiate between pathogenic and beneficial microbes. The development of resistant potato cultivars remains a viable alternative to fumigation and other control methods. However, we require a better understanding of the molecular mechanisms mediating resistance and pathogen factors that elicit responses in the host in order to properly breed for durable resistance. Compared to other diseases, our knowledge of resistance mechanisms relating to *Verticillium* and root lesion nematodes in potato is inadequate. A better understanding of the relationship between these two pathogens to cause the early dying complex is also critical to developing durable resistance. The use of molecular tools will be an important part of this future area of research. Advances in our understanding of the molecular interactions between host and pathogen are already being made and are laying the groundwork for future research.

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