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Induction of putative pathogenicity-related genes in *Verticillium dahliae* in response to elicitation with potato root extracts

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ABSTRACT

Verticillium dahliae is the main pathogen causing Verticillium wilt in potato. Management of this vascular disease is very challenging due to the soilborne nature of the pathogen. A better understanding of the molecular host–pathogen interactions is important for the development of novel strategies to control Verticillium wilt. In this pathosystem, the disease cycle starts with stimulation and germination of the *V. dahliae* microsclerotia through host root exudates. The present study reports on the use of potato root extracts derived from a susceptible (Kennebec) and a moderately resistant (Ranger Russet) cultivar to elicit pathogenicity-related genes in highly and weakly aggressive isolates of *V. dahliae*. Using a combined approach of subtractive hybridization and cDNA-AFLP, 573 transcripts differentially accumulated in one or the other isolate in response to root extracts were detected. Sixteen primer combinations representing *Eco*RI/*Mse*I AFLP primers + A, T, C, or G were used to provide a complete coverage of the subtractive hybridization products. The detected differentially expressed transcripts in the highly and weakly aggressive isolates were 301 and 272, respectively. Among the amplified transcripts, 185 were recovered from the PAGE gel then re-amplified by PCR and further sequenced. BLAST search against the NCBI, the Broad Institute *V. dahliae* genome, and *V. dahliae* ESTs collection COGEME databases showed that some of the differentially expressed transcripts matched with known sequences, with assigned functions in *V. dahliae* such as polygalacturonases or with conserved hypothetical proteins. The remaining sequences had no match in these databases. The results are discussed based on the potential involvement of these genes in *V. dahliae*'s pathogenesis.

1. Introduction

Verticillium wilt is mainly caused by the soilborne pathogen *Verticillium dahliae* Kleb. The disease affects a wide range of plant hosts including herbaceous annuals, perennials, and woody plants (Pegg and Brady, 2002; Fradin and Thomma, 2006) in form of chlorosis and necrosis of the foliage, discoloration of the vascular system, and often wilting and stunting (Alkher et al., 2009). In potato, besides a lower tuber quality, yield losses due to early dying may reach 30–50% in severely affected fields (Powelson and Rowe, 1993).

V. dahliae typically survives in the soil for many years as microsclerotia. Upon stimulation by plant root exudates, the microsclerotia germinate and develop hyphae that penetrate and colonize the root cortex. Once the hyphae reach the xylem, asexual reproduction starts, leading to the formation of conidiospores that germinate, and penetrate new vessels. At the end of the disease cycle, the fungus forms microsclerotia in the dead parts of the plant (Fradin and Thomma, 2006; Klosterman et al., 2009).

Current integrated disease management strategies to control Verticillium wilt in potato include fumigation (Powelson and Rowe, 1993), soil solarization (Katan, 1981), crop rotation (Stevens et al., 2003), biological control (Tjamos et al., 2004; Lopez-Escudero et al., 2007; Ochiai et al., 2007; Uppal et al., 2008), and the use of tolerant varieties, in the absence of completely resistant ones. Deployment of tolerant/resistant varieties will require a better understanding of the host × pathogen interactions. Little is known about potato defense mechanisms against *V. dahliae*, and most information is available in other pathosystems involving *V. dahliae* (Daayf et al., 1997; Fradin and Thomma, 2006). Pathogenicity factors such as extracellularly secreted cell-wall degrading enzymes (CWDEs) and phytotoxic peptides have been characterized using either *V. dahliae* or *V. albo-atrum* isolates. A 29.5 kDa endo-polygalacturonase has been isolated and partially purified from *V. dahliae* culture filtrates (James and Dubery, 2001) while another one from *V. albo-atrum*

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filtrate was of a higher molecular mass (37 kDa) (Huang and Mahoney, 1999). These enzymes are thought to play a role during pathogenesis of vascular wilt and other phytopathogenic fungi (Di Pietro and Roncero, 1998; ten Have et al., 1998; Isshiki et al., 2001) and to work together with xylanases, cellulases, pectinases, β-1,3-glucanases and proteases during the root penetration process (Bidochka et al., 1999; Sattarova, 2001). Jointly with this enzymatic cocktail, activities of cellulases and β-1,4-glucosidases seemed to correlate with the level of aggressiveness of isolates (Novo et al., 2006). In addition, some authors have suggested that *V. dahliae* produces toxins in culture filtrates that might contribute to pathogenicity (Buchner et al., 1982; Pu et al., 2007). *VdNEP*, a gene from the necrosis and ethylene-producing gene family, was suggested to be responsible for wilting in cotton (Wang et al., 2004) while other genes such as *VMK1* and *VDH1*, encoding for a MAP kinase and a hydrophobin, respectively, have been ascribed a role in growth, development and pathogenicity of *V. dahliae* or microsclerotia development and persistence in the soil (Rauyaree et al., 2005; Klimes and Dobinson, 2006; Klimes et al., 2006).

Knowledge about the pathogen's functional genomics and understanding the molecular basis of its pathogenesis will greatly contribute to the adjustment of Verticillium wilt management practices. Screening of genes that are expressed during pathogenic growth and microsclerotia formation of *V. dahliae* has been reported (Neumann and Dobinson, 2003). A comparative proteomics analysis of *V. dahliae* has shown differential expression of several proteins in two isolates with contrasting levels of aggressiveness (El-Bebany et al., 2010). However, the pathogenesis of *V. dahliae* has not been fully understood. So far, pulse field gel electrophoresis revealed that the genome of *V. dahliae* is six to seven chromosomes with an estimated total genomic size ranging from 26.6 to 29.1 Mb, and an average of 28.4 Mb (Pantou and Typas, 2005; Usami et al., 2008). Genetic and physical mapping of the entire genome is ongoing and a series of transcript annotations are on their way to increase the coverage of the existing draft sequence of a reference isolate from *V. dahliae* along with another one from *V. albo-atrum*. Updated annotated genes and transcripts are available from the Broad institute (http://www.broad.mit.edu/annotation/genome/verticillium_dahliae/Info.html). The present study is a contribution to the body of knowledge about *V. dahliae* genomics through the identification of pathogenicity-related candidate genes. Our approach consisted of using a differential model involving two, weakly and highly aggressive, *V. dahliae* isolates, and two potato cultivars, one highly susceptible and the other moderately resistant to the disease. Because interactions in this pathosystem are initiated before any physical contact between potato and *V. dahliae*, through root exudates, the first phase in this interaction is the stimulation of the pathogen by the root exudates. Therefore, we used plant root extracts from a highly susceptible and a moderately resistant potato cultivars to elicit the expression of pathogenicity-related genes in the selected isolates *in vitro*. This approach has the advantage of eliminating interference with accumulated transcripts from the host.

The objective of this investigation was to identify *V. dahliae* genes involved in the earliest stages of its interaction with potato. We used a combined approach of subtractive hybridization and cDNA-AFLP (Henriquez and Daayf, 2010) to selectively amplify only the putative pathogenicity-related genes differentially expressed in one or the other *V. dahliae* isolate, and only after exposure to root extracts from either a susceptible or a moderately resistant potato cultivar. The use of 16 combinations of *Eco*RI/*Mse*I AFLP primers pairs + A/T/C/G allowed a full coverage of the differentially accumulated transcripts. The identified transcripts were further amplified, sequenced and examined against publicly available databases using BLAST search for putative identification.

2. Materials and methods

2.1. Plant material

Two potato cultivars, Kennebec and Ranger Russet, highly susceptible and moderately resistant to Verticillium wilt, respectively, were used in this study. Plants were produced out of *Verticillium*-free potato tuber pieces and planted in plastic trays containing a pasteurized mixture of sand and soil (1:1, v/v). Fifty grams of NPK fertilizer granules (16:20:0) were mixed with the soil. The trays were watered and transferred into a growth room set at 20/16 ± 2 °C day/night, 16 h photoperiod, and 350 μmol m^{-2} s^{-1} light intensity, until used for inoculation after four weeks of growth.

2.2. Fungal isolates and Inoculation procedure

Four *V. dahliae* isolates (Vs06-13, Vs06-14, Vs04-28, and Vd1396-9) were tested in this study. They were selected among more than 60 pre-screened *V. dahliae* isolates, from our laboratory collection (Uppal et al., 2007; Alkher et al., 2009). Each isolate was derived from a single-spore culture and maintained on Potato Dextrose Agar (PDA) at 22 ± 2 °C (Fisher scientific incubator Model 146E) until used.

Conidiospores were scraped off plate surface of 2-week-old cultures of each *V. dahliae* isolate using 10 mL sterilized distilled water (SDW), the inoculum suspension was adjusted to a final concentration of 10^6 conidia/mL and inoculation was performed by root dipping (Alkher et al., 2009). The inoculated plants, along with healthy controls dipped in SDW, were transplanted into 16-cm diameter clay pots filled with a pasteurized mixture of soil, sand, peat and perlite (4:4:4:1, v/v/v/v) containing 5 g L^{-1} NPK (20:20:20). All pots were transferred into a growth room set to the above-mentioned conditions.

2.3. Disease severity

Disease assessment was conducted weekly during the time frame spanning from two to seven weeks after inoculation (w.a.i.), using a pre-established 0 to 5 rating scale (Alkher et al., 2009), with 0 for healthy plants and 5 for 75–100% diseased plants. A disease severity index was calculated based on the disease progress over time. At the end of the experiment, *V. dahliae* was successfully re-isolated on PDA from different parts of the inoculated plants.

2.4. Differential elicitation of V. dahliae transcripts using potato root extracts

Two of the four *V. dahliae* isolates used in the inoculation study were selected to identify the genes differentially elicited by root-extracts. In addition to their contrasting levels of aggressiveness, these two isolates, Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive), were chosen because of their known proteomic profiles (El-Bebany et al., 2010). Fungal 0.9-mm diameter plugs were taken from the edge of cultures actively growing on PDA and transferred into 50 mL Czapek-Dox Broth (CDB) liquid media (Difco Laboratories, MD, USA). The liquid cultures were incubated for two weeks in the dark at 22.5 ± 1 °C on a shaker set at 120 r.p.m. (C2 Platform Shaker, Edison, NJ, USA).

2.4.1. Potato root extracts preparation and elicitation treatments

Five grams of roots from healthy potato plants (cultivars Kennebec and Ranger Russet) were reduced to powder using a mortar and a pestle pre-cooled in liquid nitrogen, then suspended in 25 mL of SDW and agitated for 4 h. After centrifugation at 2000 × *g* for 5 min, the supernatants were filter-sterilized through 0.2 μm