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A cDNA-AFLP based strategy to identify transcripts associated with avirulence in *Phytophthora infestans* ☆

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Abstract

Expression profiling using cDNA-AFLP is commonly used to display the transcriptome of a specific tissue or developmental stage. Here, cDNA-AFLP was used to identify transcripts in a segregating F1 population of *Phytophthora infestans*, the oomycete pathogen that causes late blight. To find transcripts derived from putative avirulence (*Avr*) genes germinated cyst cDNA from F1 progeny with defined avirulence phenotypes was pooled and used in a bulked segregant analysis (BSA). Over 30,000 transcript derived fragments (TDFs) were screened resulting in 99 *Avr*-associated TDFs as well as TDFs with opposite pattern. With 142 TDF sequences homology searches and database mining was carried out. cDNA-AFLP analysis on individual F1 progeny revealed 100% co-segregation of four TDFs with particular AVR phenotypes and this was confirmed by RT-PCR. Two match the same *P. infestans* EST with unknown sequence and this is a likely candidate for *Avr4*. The other two are associated with the *Avr3b-Avr10-Avr11* locus. This combined cDNA-AFLP/BSA strategy is an efficient approach to identify *Avr*-associated transcriptome markers that can complement positional cloning.
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1. Introduction

Many plant–pathogen interactions are governed by specific interactions between pathogen avirulence (*Avr*) genes and corresponding plant resistance (*R*) genes. An interaction where a corresponding pair of *R* gene and *Avr* gene is present and expressed, results in incompatibility and the plant is resistant. When one of the two is inactive or absent, the interaction is compatible and the plant susceptible. This cross talk between host and pathogen was assembled in the gene-for-gene model by Flor (1942), who extracted the concept from his work on the interactions between flax and flax rust. Since, the early nineties numerous *R* genes from model plant or crop species have been identified and cloned (Young, 2000; Dangl and Jones, 2001) and, in parallel, many *Avr* genes mainly from fungi and bacteria (White et al., 2000; Luderer and Joosten, 2001; van't Slot and Knogge, 2002). The availability of both a cloned *R* gene and its corresponding cloned *Avr* gene offers exciting opportunities to elucidate the gene-for-gene interaction at the molecular and cellular level. In recent years, the guard model has won ground particularly by studies on a few model pathosystems, such as the interactions between Arabidopsis or tomato and the bacterial speck pathogen *Pseudomonas syringae*, and tomato and the leaf mold fungus *Cladosporium fulvum* (Innes, 2004;

Rooney et al., 2005). In this model R proteins and pathogen effectors (i.e., AVR proteins) are part of a larger dynamic complex. The pathogen effectors target host cell proteins in order to suppress defense responses or elicit susceptible responses. R proteins evolved as a counter-defense and function to monitor the effector targets.

The subject of our studies is *Phytophthora infestans*, the notorious Irish potato famine pathogen and the causal agent of late blight (Govers and Latijnhouwers, 2004). *Phytophthora* species resemble fungi morphologically but in the tree of life they are classified as oomycetes, a unique group of eukaryotes that evolved independently from fungi. Oomycetes include significant pathogens of insects and animals and they are responsible for a wide variety of destructive plant diseases. All *Phytophthora* species (more than 65), and the majority of the *Pythium* species are plant pathogens, and also all downy mildew diseases and white rusts are caused by oomycetes (Agrios, 1997). Oomycetes not only look like fungi, they also behave like fungi and use the same weaponry to attack plants (Latijnhouwers et al., 2003). Similarly, the R proteins that plants use to defeat oomycetes have the same architecture as R proteins that stop fungal invasions (Ballvora et al., 2002; van der Vossen et al., 2003; Gao et al., 2005; Huang et al., 2005) and many oomycete–plant interactions follow the gene-for-gene model. Genetic analyses on host and pathogen have demonstrated that this model also suits the potato-*P. infestans* pathosystem (van der Lee et al., 2001).

Unlike R proteins, the pathogens' AVR proteins or effectors are highly divergent (Luderer and Joosten, 2001; van't Slot and Knogge, 2002). Many of the fungal *Avr* genes were cloned by reverse-genetics using purified elicitor preparations as starting material. For genetically more tractable fungi, like, for example, *Magnaporthe grisea*, positional cloning appeared to be a suitable approach, and for cloning bacterial *Avr* genes classical bacterial genetics, such as genetic complementation proved to be very efficient (van den Ackerveken and Bonas, 1997; Collmer, 1998). In the case of *Phytophthora*, however, *Avr* gene cloning has lagged behind (Tyler, 2001, 2002). Because of the (hemi-)biotrophic nature of many oomycete–plant interactions purifying elicitors is difficult and, in our hands attempts to identify race specific elicitors from *P. infestans* were unsuccessful (Alfonso and Govers, 1995). Therefore, reverse genetics is not an option. Moreover, low DNA transformation efficiencies and relatively large genome sizes hamper complementation or gene tagging approaches. A more suitable approach is positional cloning and recently three oomycete *Avr* genes have been identified starting off with this approach: *Avr1b*-1 from *Phytophthora sojae* (Shan et al., 2004), and *ATR13* and *ATR1^{NDWsB}* from the Arabidopsis downy mildew pathogen *Hyaloperonospora parasitica* (Allen et al., 2004; Rehmany et al., 2005). These two species are homothallic and the number of inbred progeny that was generated was sufficient to obtain recombinants in the *Avr* regions and to identify closely linked markers.

For cloning *Avr* genes in *P. infestans* we also adopted a positional cloning approach and generated high-density maps of chromosomal regions carrying *Avr* genes (van der Lee et al., 2001). In addition, a BAC library of a strain carrying six dominant *Avr* genes and suitable for marker landing, is available (Whisson et al., 2001). However, *P. infestans* is heterothallic and the problem we face is the inability to generate large segregating mapping populations. Also the relatively large genome size (245 Mb) reduces the marker density and even with high-density linkage maps (van der Lee et al., 2004) we were not able to generate enough markers for efficient landing. To complement the positional cloning strategy we aimed at generating transcriptome markers. In this study, we combined a cDNA-AFLP based strategy with bulked segregant analysis (BSA) to identify *Avr*-associated transcripts. cDNA-AFLP is a relatively simple method to obtain a genome-wide display of differentially expressed genes and it has already been successfully used for gene discovery in *P. infestans* (Avrova et al., 2003; Dong et al., 2004). Many of the known *Avr* genes show a relatively high expression or a stage specific expression in pre-infection stages and therefore, we used germinating cysts as starting material for RNA isolation. cDNA-AFLP patterns obtained from pools of strains with identical AVR phenotypes revealed a high number of putative *Avr*-associated transcript derived fragments (TDFs) for each of the four *Avr* loci that were targeted. Subsequently, segregation of the *Avr*-associated TDFs in an F1 mapping population was analyzed resulting in transcriptome markers for two *Avr* loci.

2. Materials and methods

2.1. P. infestans strains and mapping population

The *P. infestans* strains used in this study are two Dutch field isolates of opposite mating type (80029; A1 and 88133; A2) and 18 F1-progeny (designated as cross 71). The cross 71 mapping population was previously described and characterized by Drenth et al. (1995) and van der Lee et al. (1997). The nomenclature of genes, gene clusters, and phenotypes is according to van der Lee et al. (2001) with one exception; *Avr3* now has the suffix '*b*' to indicate that this avirulence gene elicits resistance on plants carrying resistance gene R3b and not R3a (Huang et al., 2004). Consequently, an avirulent and virulent phenotype on R3b plants is indicated by AVR3b and avr3b, respectively.

2.2. P. infestans culture conditions

Phytophthra infestans strains were routinely grown at 18 °C in the dark on rye agar medium supplemented with 2% sucrose (RSA) (Caten and Jinks, 1968). To obtain germinating cysts for RNA isolation, sporulating mycelium grown on RSA was flooded with ice-cold water and incubated at 4 °C. At this temperature, sporangia release the zoospores into the water. After 4 h incubation, the