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Identification of pathogenesis-related ESTs in the crucifer downy mildew oomycete *Hyaloperonospora parasitica* by high-throughput differential display analysis of distinct phenotypic interactions with *Brassica oleracea*

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Abstract

Crucifer downy mildew is caused by the obligatory biotrophic oomycete *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*). So far, isolates infecting *Arabidopsis thaliana* have proven to be non-pathogenic on other crucifers and, despite its unequivocal merit as a research model, the pathosystem *A. thaliana–H. parasitica* by itself will not provide all the answers onto crucifer downy mildew genetics and biology. In this report, we present the development of a differential display (DD)-based strategy, suitable for high-throughput analysis of expressed sequence tags (ESTs) in plant–pathogen interactions, in this work applied to the analysis of the pathosystem *Brassica oleracea–H. parasitica* interaction transcriptome. Our purpose was the mining for pathogen-specific ESTs that can be used in future research for virulence factors and *Avr* genes. A total of 743 specific cDNAs showing differential expression in *B. oleracea* seedlings infected with *H. parasitica*, as opposed to healthy seedlings, were isolated by DD-PCR. We found 21 exclusively *H. parasitica* cDNAs from 433 sequenced DD clones, 18 encoding for potential new genes. Our results reinforce the abilities of DD-PCR for differential screening of pathosystems transcriptomes, leading to the finding of more new potential genes than the previously used techniques. Both the improved DD-based methodology and the graphical representations based on Venn diagrams from polyominoes are appropriate for large-scale analysis of multiple interaction transcriptomes. The obtained data are also innovative since this is the first approach to study the interaction of *H. parasitica* with its natural host.

Keywords: *Brassica oleracea*; Crucifer downy mildew; Differential display; EST; *Hyaloperonospora parasitica*; Transcriptome differential analysis; Oomycete–plant interaction; Venn diagrams

1. Introduction

Crucifer downy mildew caused by the obligatory biotrophic oomycete *Hyaloperonospora parasitica* (formerly *Peronospora parasitica* (Pers. Ex Fr.) Fr.) is a

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worldwide economically important disease that causes significant damages on Brassica vegetables. Since the disease can kill or affect seedling growth in the nurseries and reduce the productivity and quality of adult plants in the field, the identification and characterization of host resistance genes is a crucial tool to obtain reliable resistant genotypes and assist breeding programmes.

The recognition-dependent disease resistance in plants is ruled by specific interactions between pathogen avirulence (*Avr*) genes and the corresponding plant disease resistance (*R*) genes. As a consequence, when both genes are present the interactions are incompatible but if either one is absent or inactive the interaction is compatible and disease occurs (Flor, 1971).

There are several sources of resistance to downy mildew in *Brassica oleracea* at cotyledon level and subsequent plant development stages (Sousa et al., 1997). The genetic control of the resistance at cotyledon level varies with the source of resistance, e.g. one single gene in cauliflower (Jensen et al., 1999) and two duplicate dominant genes in 'Couve Algarvia' (*B. oleracea* var. Tronchuda Bailey) (Monteiro et al., 2005). Furthermore, different levels of virulence exist in *H. parasitica*, with Portuguese isolates being reported as more virulent than UK and French isolates (Leckie et al., 1999; Agnola et al., 2003). Although resistance in 'Couve Coração-de-Boi' (*B. oleracea* var. Tronchuda Bailey) is race-specific (Bahcevandziev, 2003) and resistance in 'Couve Algarvia' is effective against a wide range of isolates, there is no report on pathogen avirulence genes matching the resistance genes of Portuguese brassicas.

Since the 1990s, the research devoted to the pathosystem *Arabidopsis thaliana–H. parasitica* made enormous progress in cloning and characterizing *R* genes but until recently there has been little advance in characterizing the products or in cloning the *Avr* genes of *H. parasitica* (Rehmany et al., 2005). The isolates infecting *A. thaliana* are non-pathogenic on other crucifers, but there is a clear gene-for-gene relationship within *A. thaliana* ecotypes (Slusarenko and Schlaich, 2003). Despite its unequivocal merit as a research model, the pathosystem *A. thaliana–H. parasitica* by itself will leave many open questions about crucifer downy mildew genetics and biology.

Because *H. parasitica* is an obligatory biotroph, it is difficult to get insight into its genome. The best attempts have been made using techniques that allow the subtraction of host and pathogen transcriptomes. Differential expressed mRNAs in plant systems have been identified by different techniques such as subtractive hybridization (Diatchenko et al., 1996) or cDNA-AFLP display (Van der Biezen et al., 2000). However, the differential display (DD) (Liang and Pardee, 1992) has been a leading technique for cloning differentially expressed genes in major research areas (Matz and Lukyanov, 1998; Sturtevant, 2000; Liang, 2002; Ding and Cantor, 2004). Regarding plant–pathogen interactions, the ability of DD analysis for the detection and isolation of involved genes has already been demonstrated in *Phytophthora capsici* interaction with pepper (Munoz and Bailey, 1998), in cell suspension cultures of soybean infected with *Pseudomonas syringae* pv. *glycinea* (Serhaus and Tenhaken, 1998) and, more recently, after infection of potato with *Phytophthora infestans* (Collinge and Boller, 2001). However, DD analysis was never used to assess entire transcriptomes in plant–pathogen interactions.

DD-PCR-based techniques were designed to be simple, sensitive, systematic and reliable, and for integrating routine molecular biology methods: RT-PCR, DNA sequencing gel electrophoresis and cDNA cloning and sequencing. DD-PCR starts with the synthesis of first-strand cDNAs using total RNA samples and three anchored oligo-dT primers. The resulting three populations of cDNAs are further amplified with the same oligo-dT primer and a short (13-mer) and arbitrary second primer (AP). The differential expressed products are detected by comparing the amplification patterns of two or more samples displayed on a gel and downstream analysis proceeds with cloning and sequencing of differential cDNAs.

Sturtevant (2000) compared subtractive library screening and differential hybridization with DD analysis, and considers that this last methodology is the least labour-intensive and has several important advantages, since no special or expensive equipments are required, there is no need for previous knowledge of gene sequences, more than two RNA samples can be simultaneously compared, the direct detection of signal is obtained through gel display, small amounts of total RNA are enough to cover up to 96% of eukaryotic genome expression and the technique is high sensitive for rare mRNAs. As major drawbacks remain the hands-on time, the susceptibility to variable rates of false positive detection, the short average size of amplified cDNAs representing mainly the 3′-untranslated regions and the number of reactions needed to cover the entire transcriptome.

The need to improve DD technique generated abundant research (Bauer et al., 1993; Vögeli-Lange et al., 1996; Zhang et al., 1996; Bonnet et al., 1998; Jurecic et al., 1998; Lowe, 2000; Cho et al., 2002; Liang, 2002), which solved many of its initial disadvantages or reduced