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Transcriptional profiling of canola (*Brassica napus* L.) responses to the fungal pathogen *Sclerotinia sclerotiorum*

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Abstract

To investigate molecular changes in canola (*Brassica napus* L.) accompanying its interaction with the fungal pathogen *Sclerotinia sclerotiorum*, we investigated pathogen-induced changes in gene expression using microarrays. This study led to the identification of a number of interesting canola genes that were responsive to the pathogen-challenge at three different time points. More than 300 transcripts were detected by our studies as being increased or decreased in abundance at least two-fold as compared to uninoculated controls. Pathogen-responsive transcripts included those putatively associated with JA biosynthesis and signaling, reactive oxygen species metabolism, and cell wall structure and function. These genes may play important roles in mediating plant responses to the pathogen. We selected 11 genes that exhibited an increase, and 4 genes that exhibited a decrease in transcript abundance, for validation by quantitative real-time PCR. Results from qRT-PCR analysis demonstrated a similar trend as observed by microarray analysis and highlighted the potential involvement of these genes in mediating plant responses to the pathogen.

Keywords: *Brassica napus*; Microarray; *Sclerotinia sclerotiorum*; Quantitative real-time reverse-transcription PCR; Transcriptional profiling

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary causes soft rot disease and is considered to be one of the most damaging pathogens, which affects at least 400 plant species including canola [*Brassica napus*; 1, 2]. *Sclerotinia* diseases cause estimated annual losses of ~US$ 200 million in the US alone [2 and references therein]. *B. napus* is an agriculturally and economically significant crop that adds over $11 billion to the Canadian economy (http://www.canola-council.org/industry.html) and *Sclerotinia* has the potential to cause significant reduction in its yield [1].

S. sclerotiorum over-winters as mycelia within plants, or as sclerotia, which are hyphal aggregates and long-term survival structures [3]. The sclerotia germinate and form apothecia, which produce asci and a large number of ascospores in the spring or early summer [2]. Either the ascospores or mycelium can invade plants through the stomata into the substomatal chamber, and from here, the fungus progresses rapidly through the leaf tissue [2]. During the infection of plant tissue, oxalic acid secreted by the pathogen is thought to be crucial in facilitating invasion and, for that reason, is considered a pathogenicity factor [4].

Even though the role of oxalate is not completely clear, it has been proposed to remove calcium ions bound to pectins, exposing host cell walls to catabolic enzymes of fungal origin [4]. In addition, oxalic acid also facilitates plant cell wall degradation by shifting the pH of infected plant tissues closer to the optimum of cell wall-degrading enzymes, such as polygalacturonases [5]. Apart from its role in weakening the plant cell wall and facilitating its degradation, oxalic acid has also been shown to suppress defense-related oxidative burst in soybean and tobacco cells [6] and alter guard cell movement by either increasing osmotic pressure via accumulation of potassium ions and starch breakdown or by inhibiting ABA-induced stomatal closure [4].

The plant cell wall-degrading enzymes secreted by *S. sclerotiorum* during infection of plants include cellulolytic and pectinolytic enzymes, which have been studied for their roles in pathogenicity, including penetration, maceration, nutrient

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acquisition, plant defense induction and symptom expression [7,8]. As mentioned earlier, these enzymes are all optimally active under the acidic conditions provided by oxalic acid [7]. In addition to their roles in reducing the integrity of the plant cell walls, the pectin methyl esterases, acid proteases and an aspartyl proteinase secreted by *S. sclerotiorum* are likely required for the degradation of cell wall proteins and the inactivation or inhibition of plant defense response proteins [8].

Even though a considerable amount of information on oxalic acid as well as the virulence factors secreted by the pathogen is available in the literature, very little is known about the molecular events that occur in the plant cell upon infection with this pathogen. One study reported an expressed sequence tag (EST) analysis of two cDNA libraries constructed using either fungal mycelia grown in pectin medium or tissues from infected *B. napus* stems and were used to identify genes involved in fungal development and pathogenesis [9]. This study revealed a number of fungal genes including virulence factors such as exopolygalacturonases and several transporters; however, very little information on the changes in plant gene expression were reported [9]. Transcriptional profiling using microarrays is a powerful tool that can be used to investigate gene expression in different tissues or organs under normal conditions or under abiotic [10] and biotic stresses [11].

The wide application of microarray technology in *B. napus* is limited by the fact that canola microarrays are not commercially available. However, this limitation may be overcome by the use of readily available *A. thaliana* microarrays, given that protein-coding sequences exhibit >86% homology between *A. thaliana* and *B. napus* [12]. Indeed, *A. thaliana* cDNA arrays representing a small number (6120) of genes were previously used to investigate gene expression in canola following challenge with *S. sclerotiorum* [13].

In this study we have used commercially available oligonucleotide (70 mers) arrays representing 26,090 *A. thaliana* genes to profile gene expression in canola leaves following challenge with *S. sclerotiorum*. Our study represents the first report of a detailed investigation into gene expression during this host–pathogen interaction and has revealed many interesting genes responding to this pathogen.

2. Materials and methods

2.1. Fungal and plant materials

A strain of *S. sclerotiorum* was kindly provided by Dr. Stephen Strelkov, Plant BioSystems Group, Department of Agricultural, Food and Nutritional Science, University of Alberta. Sclerotia were subcultured on solid potato dextrose agar (PDA) media (Becton Dickinson, Sparks, MD, USA) under light (24 h/day). After 3 days, 0.2 cm agar plugs were removed with a sterile cork borer from the leading edge of the mycelia and were subcultured on PDA agar plate, 0.5 cm agar plugs were removed from the leading edge of the second two-day old mycelia and used for inoculation. Wild type *B. napus* (Westar) plants were grown in greenhouse with a photoperiod of 16 h light (combination of natural light and T5 fluorescent tubes with a light intensity of 300 $\mu E\ m^{-2}\ s^{-1}$ or μV)/8 h dark for 18 days and used in our experiments. PDA agar plugs prepared as described earlier were placed on the first and second true leaves which were wounded slightly. Leaves of uninoculated, control plants were treated similarly with PDA agar plugs without the mycelia. Plants, both control and inoculated, were placed in a humidity chamber for 24 h after which they were returned to the greenhouse. Leaves from control and inoculated plants were harvested at 12, 24 and 48 h after inoculation, flash frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Leaves from three independent biological replicates were prepared for analysis.

2.2. RNA isolation, microarray slide preparation and hybridization conditions

Total RNA was isolated from both control and inoculated leaf tissue using the RNeasy Plant Mini kit (Qiagen, Mississauga, ON, Canada) with on-column DNA digestion. RNA was quantified by NanoDrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, USA) and the integrity of the RNA was assessed on a 1% agarose gel. cDNA was synthesized from 5 μg of total RNA using SuperscriptII (Invitrogen, Burlington, ON, CA) with RT polyA capture primers (3D Array 900 kit; Genisphere, Hatfield, PA, USA). In these experiments, oligonucleotide (70 mer) arrays that contained a total of 26,090 probes (Array-Ready Oligo Set for *Arabidopsis* genome Version 1.0, Qiagen Operon, Alameda, CA, USA plus additional probes for quality control) spotted on superamine aminosilane-coated slides (TeleChem International Inc., Sunnyvale, CA, USA) were used. The slides were covered with 24 × 60 mm LiferSlips (Eric Scientific, Portsmouth, NH, USA) for all hybridizations which were performed in a two-step format at 55 °C using the 3D Array 900 kit (Genisphere) as per manufacturer's instructions. Slides were scanned immediately using ArrayWoRx (Precision Scientific, Issaquah, WA, USA) and transformed into Tiff images. Two hybridizations were performed for each of the three independent biological replicates for each time point (total 18 hybridizations) which included dye swap hybridizations in order to avoid bias in the microarray evaluation as a consequence of dye-related differences in labeling efficiency and/or differences in recording fluorescence signals.

2.3. Microarray data analysis

Microarray data were analyzed using TM4 suite (http://www.tm4.org [14]). Briefly, spot intensities were measured using the Spotfinder 3.11, normalized using MIDAS2.19 (LOWESS normalization method) and finally analyzed by TMeV4.0. The low intensity cut-off used was 1000 UNITS. Fold changes of gene expression (inoculated/mock) were calculated based on normalized data in Microsoft Excel 2003 and, significance analysis of microarray [SAM; 15] was applied to find differentially expressed genes with fold changes differing significantly from 0 with a false discovery rate (FDR) of ≤5%. All the normalized, intensity-filtered data