Defining the mechanisms underlying resistance to stem rot disease (*S. sclerotiorum*) in *Brassica napus*

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Abstract

Sclerotinia sclerotiorum is a necrotrophic fungal pathogen capable of infecting a wide range of plants, including *Brassica napus*. Genomics approaches were employed to gain insight into the mechanisms responsible for resistance in the Chinese *B. napus* cultivar, Zhong You 821(ZY821). Doubled haploid (DH) lines were created from crosses between ZY821 and susceptible *B. napus* lines. 800 ESTs were generated from each of two subtractive cDNA libraries that were designed to enrich for genes expressed in infected but not in uninfected ZY821 stems and in resistant but not in susceptible DH lines. A total of 58 and 120 uni-genes were identified among the infected vs uninfected and resistant vs susceptible ESTs, respectively. The genes induced in response to infection in ZY821 included many well-characterized anti-fungal proteins such as a hevein-like protein, three chitinases, β-1,3 glucanase, PR-1 and PR-5 as well as several regulatory proteins. The uni-gene set unique to the resistant DH lines included 36 proteins with no ascribed function (two types were highly prevalent) as well as PR1 and a hevein-like protein. Since the subtractive library procedure eliminates transcripts having even some degree of similarity, members of multi-gene families (such as transcription factors) are often eliminated or under-represented. To overcome this limitation, we used a 16,000 feature *B. napus* oligonucleotide micro-array designed to be gene-specific. A time course study was conducted to monitor gene expression profiles in infected ZY821 stems during the very early (6 h) to later (72 h) stages of the infection. We found that three WRKY-like transcription factors were induced (up to 14 times) and these are being further characterized for their contribution to resistance.

Key words: *Sclerotinia sclerotiorum*, *Brassica napus*, resistance, cDNA subtraction, micro-array analysis

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a ubiquitous necrotrophic fungal pathogen capable of infecting at least 408 plant species among 75 families including many important crops such as canola (oilseed rape), soybean, mustard, alfalfa, field beans, peas, lentils and sunflowers (Boland and Hall, 1994). In western Canada, the disease occurs sporadically on canola and is most severe when moist cool conditions prevail, the same conditions that result in high yields in the absence of disease. Losses may exceed 30% in individual fields, while average losses are around 5% annually. Cultural and chemical practices are currently used to manage *sclerotinia* stem rot, but are not economically beneficial due to the unpredictable nature of the disease. Partial resistance has been reported in *B. napus* cv. Zhong You 821 (Li *et al*., 1999; Buchwaldt *et al*., 2003). Zhong You 821 (ZY821) has been evaluated for *sclerotinia* resistance in China, in Canada at AAFC Saskatoon, as well as in the UK at Rothamsted. It is consistently among the most resistant material to stem rot.

The behaviour of *S. sclerotiorum* on and within the host involves a complex series of highly coordinated events that allow the pathogen to cycle between saprophytic (necrotrophic) and pathogenic lifestyles (Hegedus and Rimmer, 2005). While the molecular mechanisms that govern this phenomenon have been elucidated, only a few studies have attempted to address the interplay between this pathogen and host defences (Li et al., 2002, 2004a, b). Here we describe work to uncover the mechanisms that may be responsible for the resistance observed in *B.* napus ZY821 to *S. sclerotiourm* using genomics methods including cDNA subtraction, expressed sequence tags (ESTs) and micro-arrays.

Materials and Methods

Construction of subtractive cDNA libraries

Stems were inoculated with a 0.5 cm agar plug taken from the growing margin of a fungal culture grown on glucose + minimal salts agar that was affixed to the stem with Parafilm™ (a detailed protocol is available upon request). The top epidermal layer (1 -2 mm in depth) was used for RNA isolation. With infected tissues, stem material 0.5 cm around the lesion margin was harvested at 7 days post-inoculation. A subtractive cDNA library was prepared to enrich for transcripts present in infected but not uninfected *B. napus* ZY821 stems. Approximately, 800 cDNA clones were sequenced from the subtractive library. The expression profile of the genes represented by the cDNAs was verified by reverse northern blot analysis.

Micro-array analysis

Zhong You 821 plants were grown in chambers in a randomized design with three biological replications. The plants were inoculated with *S. sclerotiorum* isolate 321 on the stem at the full-flower stage. Stem tissues were harvested as above at 6, 12, 24, 48, and 72 h post-inoculation, with mock-inoculated plants as a control. Total RNA was isolated using the plant RNAeasy kit (Qiagen) and RNA amplification and labelling were conducted using the Amino Allyl Message Amp II RNA amplification kit (Ambion). Cy3/Cy5 labelled amplified RNA was hybridized to oligomer arrays representing 16,000 *B. napus*

genes (Sharpe et al., 2006). Differentially expressed genes were analyzed using GeneSpring 7.3 software. The expression profile of the genes represented by the cDNAs was verified by quantitative PCR analysis.

Results and Discussion

The resistance in ZY821 appears to be associated only with the stem, since neither petals nor leaves exhibited any enhanced resistance when compared to susceptible varieties. Our focus was therefore to identify the suite of *B. napus* ZY821 genes expressed in the stem in response to *S. sclerotiorum* infection. Our initial experiments involved the construction of a subtractive cDNA library to identify genes expressed in tissues at 7 days post-inoculation, ie. after the infection was well-established. Internal standards and the high degree of redundancy of the EST population indicated that the enrichment had proceeded well. The most abundant ESTs (Table 1) represented a MAP3K protein kinase and 6-phosphogluconolactonase. Several types of pathogenesis-related (PR) defense proteins were induced, including 2 types of chitinases, 4 types of carbohydrate binding lectins. Two proteins with unknown functions were also represented. MAP3K kinases are known to control key steps in the defense response such as defense gene expression and re-organization of the cell cytoskeleton allowing for defense protein deployment to the site of attack. 6-phosphogluconolactonase catalyzes a key biochemical branching point for mobilization of the storage carbohydrate reserves to synthesize amino acids (proteins), nucleosides (DNA/RNA) and essential amino acids that are required to mount a defense response. PR1 is a known anti-fungal protein; however, its mechanism of action remains obscure. Class I and II chitinases, the latter possessing an additional lectin-like chitin binding domain, are capable of degrading fungal cell walls. As defence proteins, lectins are most widely known for insecticidal activity due to their ability to disrupt insect digestive processes; however, they may also possess anti-fungal properties through interaction and sequestration of carbohydrates required for cell wall synthesis.

Table 1. Most abundant ESTs from infected vs uninfected ZY821 cDNA library

Most Prevalent ESTs	Fraction (%)
MAP3K protein kinase	17
6-phosphogluconolactonase	9
Proteins with unknown functions (2 types)	8
PR1 (anti-fungal protein)	3
PR3 & PR4 (chitinases)	6
Other PR proteins	3
Lectins (4 types)	9
Transcription factors	<0.5%

Table 2. Most abundant ESTs from resistant vs susceptible DH lines.

Prevalent ESTs	Fraction (%)
Proteins with unknown functions - 34 types	16
- 2 types	6
RNAase	4
Ferritin	2
PR1	2
PR5	1
Lectin (1 type)	1
Transcription factors	<0.1%

A second subtractive cDNA library designed to detect differences in gene expression among infected pools of the most resistant and most susceptible DH lines derived from the ZY821× Westar cross (Figure 2) was prepared. Over 800 ESTs were generated and again the enrichment proceeded very well as indicated by a reasonably high degree of sequence redundancy (data not shown). A total of 120 unique genes were identified with the most abundant ESTs (Table 2) representing 36 proteins that do not have a known function (two types were very prevalent) indicating that a unique mechanism may confer resistance in *B. napus* ZY821. As was observed in the infected vs non-infected subtractive library, PR1 and one of the lectin types were also prevalent.

Identifying the induced defense genes was an important first step toward understanding *sclerotinia* resistance. However, it is equally likely that the resistance is due to alterations in the timing (earlier) and strength (increased) of these defenses, which would be determined by a small number of regulatory proteins. Members of gene families, such as regulatory proteins, are generally under-represented in subtractive cDNA libraries. To overcome this, we used a gene-specific *B. napus* oligonucleotide micro-array to examine gene expression over the time course of the infection from 6 to 72 hours post-inoculation. The micro-array was designed by AAFC and represents 16, 000 uni-genes; the most comprehensive *B. napus* array available. Our analysis revealed that the expression patterns of a large number of genes were altered at the later stages of the infection (Table 3, Figure 1). The up-regulated genes included most of the classical defense genes as well as genes involved in aspects of primary metabolism that are required to mount a defense response. Of equal significance were the vast number of genes that exhibited a decrease in activity; these may represent biochemical pathways or systems that are specifically targeted by the pathogen to compromise the host. Most importantly, we identified 51 genes that were up-regulated

at the very early stages of the infection, i.e. within 6 hours of inoculation. Among these were genes encoding 5 regulatory proteins (including 3 WRKY factors) as well as several chitinases and lectins, of the type identified using the subtractive library approach above.

The expression patterns of candidate resistance genes were verified using quantitative PCR. At present, the most highly resistant and the most highly susceptible lines from the ZY821 × Westar mapping population are being used in time course studies to identify temporal patterns of gene expression unique to the resistance phenotype.

Table 3. *B. napus* genes that exhibit altered patterns of expression in response to *S. sclerotiorum* infection.

Gene expression	6hpi	12hpi	24hpi	48hpi	72hpi
Up-regulated	51	1151	1825	4839	4828
Down-regulated	10	1080	2305	6169	5106
Total	61	2231	4130	10018	9934

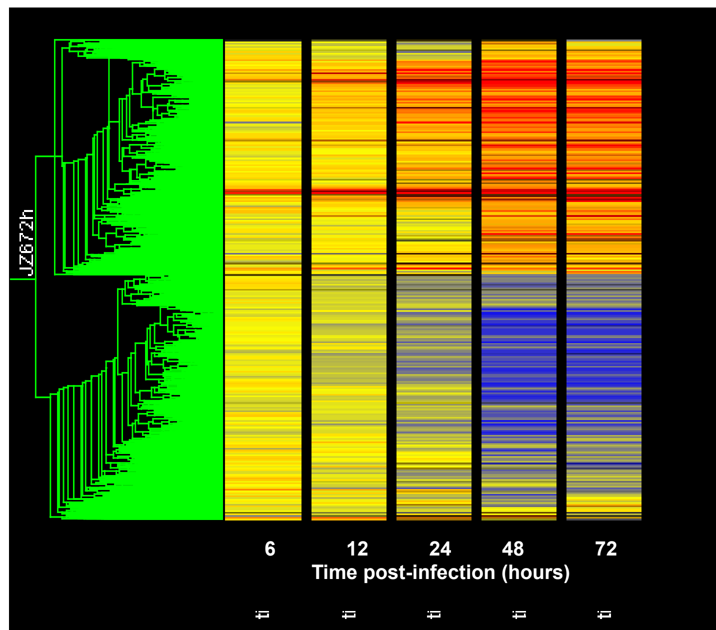

Figure 1. Dendogram showing clusters of *B. napus* ZY821 genes that were up- (red) or down- (blue) regulated at various times post-inoculation with *S. sclerotiorum*. A yellow bar indicates that no alteration in expression was observed at the specific time point. The boxed area (purple) indicates the subset of genes induced at the very early stages of the infection that are being pursued as potential candidates for developing resistance.

We have begun evaluating the candidate resistance genes by expressing them in a susceptible *B. napus* (DH12075) line and the model plant *Arabidopsis thaliana*. The initial set of candidate genes was selected from the subtractive library data and included a highly induced hevein-like class II chitinase and two polygalacturonase inhibitor proteins that inhibit the activity of pathogen polygalacturonases needed to destroy plant tissues. The constructs were designed to express the candidate genes either constitutively or only in the stem in response to *sclerotinia* infection. Homozygous lines have been identified and will be rated for disease resistance. The second set of candidate genes includes the set of WRKY factors identified from the micro-array experiments.

Acknowledgements

The assistance of B. Gjetvaj for EST sequencing and J. Nixon, C. Matsalla and C. Lewis for computational and mathematical support is appreciated. Funding for this work was provided by the Saskatchewan Agriculture Development Fund, the Canola Council of Canada, the Saskatchewan Canola Development Commission and the Federal Matching Investments Initiative.

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