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Exploring the interaction between small RNAs and R genes during *Brachypodium* response to *Fusarium culmorum* infection

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ABSTRACT

The present study aims to investigate small RNA interactions with putative disease response genes in the model grass species *Brachypodium distachyon.* The fungal pathogen *Fusarium culmorum* (*Fusarium* herein) and phytohormone salicylic acid treatment were used to induce the disease response in *Brachypodium*. Initially, 121 different putative disease response genes were identified using bioinformatic and homology based approaches. Computational prediction was used to identify 33 candidate new miRNA coding sequences, of which 9 were verified by analysis of small RNA sequence libraries. Putative *Brachypodium* miRNA target sites were identified in the disease response genes, and a subset of which were screened for expression and possible miRNA interactions in 5 different *Brachypodium* lines infected with *Fusarium*. An NBS-LRR family gene, *1g34430,* was polymorphic among the lines, forming two major genotypes, one of which has its miRNA target sites deleted, resulting in altered gene expression during infection. There were siRNAs putatively involved in regulation of this gene, indicating a role of small RNAs in the *B. distachyon* disease response.

1. Introduction

Brachypodium distachyon, a member of the Pooideae subfamily, is a wild annual grass endemic to the Mediterranean and Middle East whose genome sequence has recently been completed (International *Brachypodium* Initiative, 2010). It has morphological and genetic characteristics making it attractive to plant biologists as a model system for grass species, as well as being a close phylogenetic relative of the Triticeae, which includes the major crop species wheat and barley.

Fusarium culmorum is a fungal plant pathogen causing a destructive disease called Fusarium head blight (FHB) on wheat as well as other cereals. It infects several plant tissues resulting in symptoms such as seedling blight, crown rot, ear blight and stalk rot. In addition to reduced plant growth, FHB-infected crops are useless because some secondary metabolites, primarily mycotoxins such as trichothecenes, produced by Fusarium species within infected grain are toxic to human and animal consumers (Peraldi et al., 2011). Due to its high genome complexity, genetic studies of *Fusarium* infection were initially performed on surrogate species, one of which is *Arabidopsis thaliana*. However, as an evolutionarily distant and dicotyledonous species, *Arabidopsis*-related findings are less applicable to wheat. Additionally, rice, widely accepted as a model species for monocotyledonous plants, is susceptible to *Fusarium* species. Yet, wheat and rice have been shown to differ significantly in response to *Fusarium* mycotoxins (Llorens et al., 2004). Host–microbe interactions have also been modelled in barley, a close relative of wheat. The application of such studies to wheat suffers from the inherent resistance of barley to disease spread (Foroud and Eudes, 2009). Recently, *Brachypodium distachyon* has been proposed as a model system to study FHB of wheat due to striking similarities observed on *Fusarium* infected *Brachypodium* leaves and spikes. Although the genetic mechanisms controlling the progression of infection and host responses are yet to be elucidated, phenotypic symptoms of infection including bleaching patterns and accumulation of phenolic compounds are suggestive of the resemblance in underlying molecular mechanisms between two species (Peraldi et al., 2011).

Fusarium infection elicits plant defense mechanisms through the induction of several pathogenesis-related proteins (Scherm et al., 2013). Secretion of effector proteins and suppression of host defenses are required for proliferation in the host cell (Dangl et al., 1996). The

Abbreviations: ABA, Abscisic Acid; ANOVA, Analysis of Variance; bp, base pairs; BLAST, Basic Local Alignment Search Tool; cDNA, DNA complementary to RNA; CDS, Coding Sequence; DCL, Dicer-Like (nuclease); DNA, deoxyribonucleic acid; DNase, Deoxyribonuclease; ds, double-stranded; EST, Expressed sequence Tag; ET, Ethylene; FHB, Fusarium head blight; gDNA, genomic DNA; GSS, Genomic Survey Sequence; HR, Hypersensitive Response; IPTG, isopropyl β-D-thiogalactopyranoside; JA, Jasmonic Acid; L-Amp, Luria-Bertani Agar supplemented with Ampicillin; LRR, Leucine Rich Repeat; miRCB, miRNA Candidate Brachypodium; miRNA, microRNA; miRNA*, mature miRNA complementary sequence; mRNA, messenger RNA; NBS, Nucleotide Binding Site; nt, nucleotides; PCR, Polymerase Chain Reaction; pre-miRNA, precursor miRNA; pri-miRNA, primary microRNA; R genes, Resistance genes; RISC, RNA-induced silencing combplex; RNA, Ribonucleic Acid; RNase, Ribonuclease; rRNA, ribosomal RNA; RT, reverse transcriptase; RT-qPCR, Reverse Transcriptase-quantitative PCR; SA, Salicylic Acid; siRNA, small interfering RNA; Tm, melting temperature; UTR, untranslated region; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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pathogen–host interactions lead to the activation of systemic signal transduction pathways by phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) (Bari and Jones, 2009; Fujita et al., 2006). These induce the hypersensitive response (HR), which includes the activation of various protein kinase cascades, an oxidative burst, and increased expression of numerous defense related genes. The plant proteins required for the detection of pathogen effectors are known as R (resistance) genes. So far several R genes have been cloned from various plant species, containing common sequence motifs encoding nucleotide binding site (NBS) and leucine-rich repeat (LRR) domains and indicating roles in signal transduction (e.g. Brueggeman et al., 2008; Feuillet et al., 2003; Johal and Briggs, 1992; Liu et al., 2010). Recently, it has been suggested that microRNAs can play a role in the R gene response to invading pathogens (Gupta et al., 2012).

MicroRNAs (miRNAs) are a class of 19-25 nt small non-coding RNAs that can play critical roles in stress responses, including disease resistance, by negatively regulating gene expression at the post-transcriptional level (Bartel, 2004). First discovered in *C. elegans*, miRNAs were later found to be abundant and ubiquitous in eukaryotes, with the first plant miRNA discovered in 2002 (Llave et al., 2002). Plant miRNA genes are transcribed from non-protein coding loci by RNA Polymerase II and capped and polyadenylated in the nucleus (Xie et al., 2005). This primary miRNA (pri-miRNA) forms a hairpin-like secondary structure which is further processed by the RNaseIII endonuclease, cleaving at sites either side of the primary stem loop. The resulting precursor miRNA (pre-miRNA) is further processed by DICER-like (DCL) nucleases to produce mature miRNA duplexes, which are characterized by 2 nt 3′ overhangs (Meyers et al., 2008). The duplexes are then methylated by HEN1 and exported to the cytoplasm by HASTY. Here the duplex is unwound and a single strand loaded into the RNA-induced silencing complex (RISC). Until recently, it was thought that only one strand of each miRNA duplex is functional, this being the mature miRNA, while the other strand (referred to as miRNA*) is rapidly degraded. However, there is now considerable evidence that many miRNA* species are also functionally active (e.g. Yang et al., 2011). Although the miRNA produced from one arm of the pre-miRNA hairpin is usually much more abundant than the other, the dominant strand may be swapped in different organisms or even for a single miRNA in different tissues. Therefore a more neutral nomenclature is now recommended, referring to the 2 mature products of any miRNA duplex as 'miRXXX-5p' and 'miRXXX-3p,' with the suffix indicating whether it originates from the 5′ or 3′ arm of the pre-miRNA hairpin. Whichever strand is loaded on to the RISC directs this complex to bind its complementary target messenger RNA (mRNA), to down-regulate gene expression by either mRNA cleavage or translational repression (Bonnet et al., 2006).

Plant miRNAs are endogenously expressed and well conserved across evolution (Unver and Budak, 2009; Unver et al., 2009). The majority of plant miRNA targets are developmentally important transcription factors and stress related genes (Budak and Akpinar, 2011; Kantar et al., 2010, 2011; Sunkar and Zhu, 2004; Unver et al., 2010). While mature plant miRNAs are shorter on average (21 nt) than animal miRNAs, they have longer precursors ranging from 50 to 350 nt compared to 70–80 nt in animals.

MicroRNAs have been discovered using three basic approaches: direct cloning, forward genetics, and bioinformatic prediction followed by experimental validation (Jones-Rhoades et al., 2006). Cloning efforts are unable to detect some miRNAs if they are only expressed in restricted cell types or specific environmental conditions, so computational approaches have been developed to complement experimental approaches to miRNA gene identification. Those approaches based on homology searches have revealed orthologs and paralogs of known miRNA genes. Another simple approach has been to search the vicinity of known miRNA genes for other stem loops that might represent additional genes of a genomic cluster. This is effective because some miRNAs are present tandem arrays within operon-like clusters. In addition, unlike animal miRNAs, plant miRNAs are highly complementary (3 or fewer mismatches) to multiple conserved target mRNAs, which allows fast and confident bioinformatic identification of plant miRNA targets (Jones-Rhoades and Bartel, 2004).

In addition to miRNAs, the small RNA population of most plant cells also contains large numbers of small interfering RNAs (siRNAs). These are similar to miRNAs in that they are cleaved from larger precursors by DCL nucleases, are loaded onto the RISC complex, and repress transcription of their target sequences (Bonnet et al., 2006). However, they differ in their biogenesis; instead of single-stranded hairpin structures, siRNAs are produced from longer dsRNA duplexes that may derive from transcribed inverted repeat sequences, antisense overlapping gene pairs, or dsRNA synthesized by RNA-dependent RNA polymerases (e.g. from aberrant mRNAs; Voinnet, 2009). Many siRNAs do not target endogenous genes, but are thought to help protect the integrity of the genome from transposable elements and exogenous RNA, e.g. from viral infection. Plant siRNAs are typically more diverse and comprise a greater proportion of the total small RNA population than miRNAs (Lu et al., 2005). Unlike miRNAs they are derived from both strands of perfectly base-paired dsRNA and do not have a 3′ overhang (Khraiwesh et al., 2011). Their length is determined by the DCL nuclease(s) involved in their biogenesis; for example, in *A. thaliana* miRNAs are produced by DCL1 with lengths of 18–21 nt, but siRNAs may also be generated by DCL2, 3 or 4 which give lengths of 22, 24 and 21 nt respectively (Voinnet, 2009).

R gene expression must be tightly regulated to enable the plant to mount an effective disease response, but avoid the damaging effects of continuing activation of the HR. The possible role of miRNAs in controlling R gene expression has not been studied extensively. In this report, we aim to test the hypothesis that miRNAs regulate R genes in *Brachypodium* in response to *Fusarium culmorum*. To achieve this goal, we determined putative resistance genes and miRNAs that may regulate them by a computational approach. Relative expression of those genes and miRNAs was examined by RT-qPCR in a panel of 15 different *Brachypodium* lines.

2. Materials and methods

2.1. Data sources and bioinformatics tools

The complete unmasked *B. distachyon* v1.0 genome sequence (International Brachypodium Initiative, 2010), along with annotated proteins, coding sequences (CDS), and 1000 bp upstream and downstream of each predicted open reading frame were downloaded from the *Brachypodium distachyon* project (Munich Information Center for Protein Sequences; http://mips.helmholtz-muenchen.de/plant/brachypodium/download/index.jsp, last accessed 31.05.13), genome annotation v.1.2. All mature miRNA sequences from miRBase (Griffiths-Jones et al., 2006; Kozomara and Griffiths-Jones, 2011) were downloaded from http://www.mirbase.org/ftp.shtml (last accessed 31.05.13). Sequences from 4 *B. distachyon* small RNA libraries, sequenced by Illumina, were downloaded as processed data tables from the Gene Expression Omnibus (Barrett et al., 2009; http://www.ncbi.nlm.nih.gov/geo/, last accessed 30.05.13). The accessions used were GSM406302, GSM406303 (Wei et al., 2009), GSM506620 & GSM506621 (International Brachypodium Initiative, 2010). The first two of these libraries were produced from the reproductive tissues (young spikes) and vegetative tissues (roots, shoots and leaves) respectively of inbred line Bd21-3, while the latter two were both derived from panicles of Bd21, but produced in two different laboratories. Protein domain annotations were verified using the batch web CD-Search tool (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi; Marchler-Bauer and Bryant, 2004) searching against the Conserved Domain Database (Marchler-Bauer et al., 2011) with an e-value threshold of 0.1. Other sequence similarity searches were carried out with BLAST