Genome-wide identification and characterization of miRNAs resonsive to *Verticillium longisporum* infection in *Brassica napus* by deep sequencing

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A number of pathogens affect rapeseed crop, leading to severe and widespread yield losses . *Verticillium* wilt is one important rapeseed disease caused by *Verticillium longisporum*, a soil-borne fungal pathogen with host specificity for the genus Brassica. However, the molecular mechanism of the systemic development of *V. longisporum* in rapeseed and other Brassica species is poorly understood. Recent research in our laboratory demonstrated that elimination of several compatibility factors can result in a decline of susceptibility in *V. longisporum*-infected Arabidopsis plants. For a more comprehensive understanding of the underlying molecular mechanism(s) during *V. longisporum* infection, the post-transcriptional gene silencing (PTGS) mechanism was investigated in this study. Since miRNAs act as important regulators in PTGS, we assumed that miRNAs function as critical plant-derived factors controlling the plant-fungus interaction, either by activation or suppression of plant defense responses, or as developmental cues for the completion of the fungal lifecycle in plants.

To explore the role of miRNAs in the *Verticillium* response system, two comparable small RNA libraries were constructed from mock- and *V. longisporum*-infected rapeseed roots in this study. Analysis of sequencing data provided two major findings: i) about the origin and evolution of miRNAs in the rapeseed genome and ii) an overview of potential miRNAs involved in the *V. longisporum*-rapeseed interaction, which are described below.

i. Whole genomic sequences of *Brassica rapa* (AA) and *Brassica oleracea* (CC), the progenitor species of rapeseed (AACC), were used for deep sequencing data analysis, overcoming the plight of miRNA annotation caused by unavailable genome information of rapeseed. In total, 360 conserved and 533 novel rapeseed miRNAs were identified, which for a first time allow a new insight into the origin and evolution of miRNAs during the polyploidization of rapeseed. Microsyntenic analysis of conserved miRNAs revealed 137 syntenic miRNA pairs between the *B. rapa* and *B. oleracea* genomes. Nucleotide substitution rates and frequency analysis of syntenic miRNA pairs indicated that - besides of the ancient γ whole genome duplication (WGD) predating the divergence of dicot plants - another WGD might have occurred after divergence between Brassica species and *Arabidopsis*. These genome-wide and gene-level duplications contributed to the emergence and intensive expansion of rapeseed miRNAs and were illustrated in this study.

ii. By comparison of the transcriptional profile of the miRNAs between the two libraries, 138 conserved and 82 novel miRNAs were identified as *V. longisproum*-responsive miRNAs in rapeseed. Among them, 119 conserved and 49 novel miRNAs were down-regulated, while 19 conserved and 33 novel miRNAs were upregulated upon *V. longisporum* infection. Target prediction and validation of *V. longisproum*-responsive miRNAs suggest a multi-function of miRNAs in the regulation of physiological processes in rapeseed. Further, quantitative RT-PCR confirmed the majority of the data detected by miRNAseq. Notably, a larger fraction of miRNAs was down-regulated during *V. longisporum* infection, including several highly-conserved miRNA families, such as miR168, miR160, miR167, miR164, miR390, miR396, miR403 and miR5654. This observation suggests that most of the miRNAs identified in this study probably act as positive regulators in plant resistance mechanisms. miR1885, highly induced upon *V.*

longisporum infection, was validated to target TIR-NBS-LRR disease resistance proteins in this study, indicating its role in regulating innate immunity receptors.

Based on deep sequencing results, miR168, which was highly suppressed in *V. longisporum*-infected roots (~ -2.5 fold), was selected for further functional characterization. miR168a,c-f shows a down-regulated expression at 6 and 12 days post-inoculation (dpi) in rapeseed roots after *V. longisporum* infection. In *Arabidopsis*, a miR168b T-DNA insertion mutant (*mir168b*) showed increased susceptibility, and correspondingly, its target gene AGO1 mutant (*ago1-t*) was less susceptible. Fungal biomass and the expression level of *PEN3* in *mir168b* and *ago1-t* mutants also reflected these results. This provided some evidence that miR168 negatively regulates AGO1 to suppress *V. longisporum* development in plants. Moreover, vigorous variation in the *mir168b* mutant of two upstream genes of the SA signaling pathway (EDS1 and NPR1), suggests that SA-dependent regulation plays a role in the plant-*V. longisporum* interaction.

Taken together, this study allows for the first time a whole genome-wide exploration of miRNAs in *B. napus*. The results obtained in the present work proved the importance of the PTGS mechanism in the regulation of the plant-*Verticillium* interaction. A set of *V. longisporum*-responsive miRNAs revealed from this study, laid the foundation for further understanding of miRNA function in the regulation of *Verticillium* defense responses in rapeseed.