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Priming with a double-stranded DNA virus alters *Brassica rapa* seed architecture and facilitates a defense response

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ABSTRACT

Background: Abiotic and biotic stresses alter genome stability and physiology of plants. Under some stressful situations, a state of stress tolerance can be passed on to the offspring rendering them more suitable to stressful events than their parents. In plants, the exploration of transgenerational response has remained exclusive to model species, such as *Arabidopsis thaliana*. Here, we expand transgenerational research to include *Brassica rapa*, a close relative to economically important plant canola (*Brassica napus*), as it is exposed to the biotic stress of a double-stranded DNA virus *Cauliflower mosaic virus* (CaMV).
Results: Parent plants exposed to a low dose of 50 ng purified CaMV virions just prior to the bolting stage produced significantly larger seeds than mock inoculated and healthy treatments. The progeny from these large seeds displayed resistance to the pathogen stress applied in the parental generation. Differences in defense pathways involving fatty acids, and primary and secondary metabolites were detected by *de novo* transcriptome sequencing of CaMV challenged progeny exhibiting different levels of resistance.
Conclusions: Our study highlights biological and cellular processes that may be linked to the growth and yield of economically important *B. rapa*, in a transgenerational manner. Although much remains unknown as to the mechanisms behind transgenerational inheritance, our work shows a disease resistance response that persists for several weeks and is associated with an increase in seed size. Evidence suggests that a number of changes involved in the persistent stress adaption are reflected in the transcriptome. The results from this study demonstrate that treating *B. rapa* with dsDNA virus within a critical time frame and with a specified amount of infectious pathogen produces economically important agricultural plants with superior coping strategies for growing in unfavorable conditions.

1. Background

Plants have developed protection and defense strategies for dealing with adverse environmental conditions and biological stresses. Induced resistance is one of the strategies that plants use to combat pathogens and it involves pre-treating a plant with a stress to obtain reduced losses associated with subsequent stressful events (Conrath, 2011). There have been several examples of induced resistance being carried over to the next generation, thus giving rise to a transgenerational response (for reviews on transgenerational response in plants see Holeski et al., 2012; Hauser et al., 2011). To some extent, primed plants, whether in the same or next generation, have an elevated level of basal resistance and this prepared state allows for the plant to defend itself from subsequent stress and possibly offering a broad-spectrum resistance (Conrath, 2011; Kathiria et al., 2010).

Several pathogens such as single-stranded positive-sense (ss(+)) RNA viruses, Gram negative bacteria or synthetic chemicals resembling a pathogen elicitor have demonstrated an ability to generate resistance in a transgenerational manner (Kathiria et al., 2010; Slaughter et al., 2012; Luna et al., 2012). *Nicotiana tabacum* was primed by tobacco mosaic virus (TMV), a ss(+)RNA virus and the progeny of the treated plant had lower TMV titer, up-regulation of salicylic acid pathway marker pathogenesis related 1 (PR1) and more abundant callose deposition than the mock treated control group (Kathiria et al., 2010). In a second study, transgenerational pathogen resistance to virulent *Pseudomonas syringae DC3000* pv *tomato* (Pst) and up-regulation of pathogen defense genes were observed in the progeny of *Arabidopsis thaliana* primed with β-aminobutyric acid or an avirulent isolate of Pst. (Slaughter et al., 2012). In a third study, *A. thaliana* was primed with Pst and transgenerational pathogen resistance was measured as fewer colonies

Abbreviations: CaMV, cauliflower mosaic virus; Pst, *Pseudomonas syringae* DC3000 pv *tomato*; SAR, systemic acquired resistance; GST1, glutathione S-transferase; ROS, reactive oxygen species; PR1, pathogenesis-related protein 1; FPKM, fragments per kilobase of transcript per million mapped reads

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of bioluminescent Pst and altered regulation of pathogen defense genes in primed plants in comparison to the non-treated control plants (Luna et al., 2012). These studies clearly demonstrate that these pathogens trigger a limited transgenerational effect; however, to explore transgenerational diversity and specificity, more types of plant pathogen groups need to be used as stressors in the parent generation. One of the broad-based plant pathogen types that remains to be explored includes dsDNA viruses.

Cauliflower mosaic virus (CaMV) is a dsDNA virus that uses reverse transcriptase and a RNA intermediate during replication (Scholthof et al., 2011). CaMV infects a host plant, which most often belongs to family *Brassicaceae*, following transmission in a non-circulative, semi-persistent manner by an aphid vector such as *Myzus persicae* (Haas et al., 2002). The virus systemically infects young host plants and produces severe symptoms including leaf mottling and mosaic, reduced growth, developmental abnormalities and stunting.

Transgenerational effects have been mainly demonstrated to occur in model laboratory plants (i.e. tobacco and *A. thaliana*) (Kovalchuk et al., 2003; Boyko et al., 2007, 2010). To characterize transgenerational effects in economically important plant species, we evaluated disease responses in *Brassica rapa* as the next step in exploring economically important members of the *Brassicaceae* family. *B. ra*pa (AA, n = 10) is a diploid species and hybridizes with *Brassica oleracea* (CC, n = 9) to give rise to the allotetraploid *Brassica napus* (AACC, n = 19), also known as canola. Together, *B. napus* and *B. rapa* are major crops in Canada and they are grown for the production of seed oil, high grade animal feed and biofuel (Rempel et al., 2014). Our study examines the transgenerational response of *B. rapa* following exposure to CaMV, producing a compatible pathogen interaction that elicits a disease response. Since host response to a pathogen is often dosage-dependent and influenced by the developmental stage of the host plant (Gutiérrez et al., 2012), these variables were examined experimentally for the onset of a transgenerational response in the form of physiological attributes and pathogen resistance. In addition, RNA transcriptome sequencing was used to identify candidate genes in biochemical pathways or signaling transduction influencing the transgenerational responses. Evidence is presented that transgenerational disease resistance is inducible in economically important plant species, resistance persists for extended periods and critical physical and biochemical characteristics of the plant can be improved.

2. Material and methods

2.1. Plant material and experimental design

To evaluate transgenerational inheritance, seed was collected from one *B. rapa cv* R018 parent plant and used to generate the first self-fertilized generation (S1). All plants were grown at 20 °C in controlled greenhouse conditions with 16 h photoperiod and with light levels of 100 $\mu E.m^2s^{-1}$. The parental generation was exposed to either 50, 100 or 200 ng of purified CaMV at host plant age of two, three or four weeks following germination. Purification of CaMV virions was carried out according to Hull and Shepherd (1976) and the concentration of particles was determined using spectrophotometry using an $OD_{260} = 7$ equivalent to 1 mg mL^{-1} while adjusting for light scattering. Individual plants were inoculated with a 10 μl suspension containing either 50, 100 or 200 ng of virus and abrasive 250–400 mesh carborundom (Sigma). Leaves containing the inoculation sites were removed from the plants within 24 h following pathogen exposure to explore signaling rather than pathogen movement throughout the plant. Plants were grown to set seed and the resulting self-fertilized progeny treated with the pathogen were called P0pS1. Control plants consisted of healthy (P0cS1) or plants that were treated with the inoculation buffer consisting of 0.01 M sodium phosphate, pH 7.2 (P0bS1).

2.2. Examination of stable complex traits and virus resistance

Seed size was estimated using image analysis software and a transmitted light flatbed scanner as described by Herridge et al. (2011). Briefly, 50–300 seeds per plant were spread onto the scanner bed ensuring that no seeds were touching one another. An image was taken for each plant at a resolution of 1200 dpi with transmitted light. ImageJ particle analysis software was used to measure seed area using the threshold feature (Abramoff et al., 2004). The greyscale value was 162 and the lower limit of particle analysis was 30,000 μm^2. Other stable complex traits that were measured in the progeny were rate of germination, number of days until first flower, number of days until first 10 flowers, foliage dry weight, total height, root collar diameter, total number of leaves and average crown radius with the latter four measurements being completed at four and eight weeks following germination.

To examine CaMV resistance, progeny were challenged with CaMV and virus titer was measured at 14 days post-inoculation (dpi) using a double anitibody enzyme linked immunosorbent assay (DAS-ELISA). Polyclonal and alkaline phosphatase conjugated goat anti-rabbit (Sigma) were used as the primary and the secondary antibody, respectively. CaMV titer was measured for three to nine progeny for each treatment during three separate experiments. To remove the influence of wounding, the measured variables for pathogen treated plants (P0pS1) were normalized by the average of buffer treated plants (P0bS1) and the pathogen treatments were compared to the healthy treatments. Data were compiled in Microsoft Excel and statistics completed using SAS version 9 (SAS Institute).

2.3. cDNA library preparation and sequencing for transcriptome analysis

Fresh leaf tissue was homogenized in liquid nitrogen and total RNA extracted using a Plant/Fungi purification kit (Norgen Biotek Corp., Canada). The quality of RNA was assessed with agarose gel electrophoresis and spectrophotometrically before generating the mRNA-Seq library and sequences previously described by Kalischuk et al. (2013).

The mRNA-Seq library was generated following Illumina's sample preparation recommendations. Briefly, the poly[A] + RNA was enriched from 20 μg of total RNA using Oligo(dT) magnetic beads. This RNA was fragmented into small (200–400 bp) fragments and the short fragments were used as templates for random-hexamers to prime first strand followed by second strand cDNA synthesis. Short fragments were purified with a QiaQuick PCR Extraction Kit (Qiagen) and used in cluster generation on Illumina's Cluster Station. Sequencing was performed as paired-end of 101 nt read length on Illumina HiSeqTM 2000. Raw sequencing intensities were extracted and the bases were called using Illumina's real-time analysis software, followed by sequence quality filtering.

2.4. Sequence analysis

All raw reads generated from the sequencer were *de novo* assembled into contigs using the Trinity program (Haas et al., 2013). Assembled contigs were aligned to sequences of 2,487 proteins of *B. rapa* from the NCBI database (http://www.ncbi.nlm.nih.gov/protein/?term=txid3711[Organism:exp]) using BLASTx and homologous genes with the e-value $<10^{-5}$ were identified. The Blast2GO program was used to obtain alignments to the Gene Ontology (GO) database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.blast2go.org). Trancript abundance evaluated as Fragments per kilobase of transcript per million mapped reads (FPKM) was determined by mapping raw reads back to the assembled contigs using the Tophat and Cufflinks suite (Trapnell et al., 2012).