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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).

3. Results

To study if prior exposure to a pararetrovirus produces a transgenerational effect conferring disease resistance and other physiological changes, *B. rapa* cv. 018 was inoculated with cauliflower mosaic virus isolate LRC2010 (CaMV). The parent plants were inoculated with one of three concentrations of CaMV (50, 100 or 200 ng purified virions) and virus exposure for the hosts was either one, two or three weeks old following germination. Control plants included healthy uninoculated or inoculated with virus suspension buffer (i.e. without virus). Parental plants were grown to set seed and the resulting self-fertilized progeny were called P0pS1 for pathogen treated, P0cS1 for healthy and P0bS1 for the buffer treated (Fig. 1).

3.1. Low dose of cauliflower mosaic virus applied just before bolting

To explore if phenotype could be used to describe the primed state of *B. rapa* after exposure to CaMV, the progeny of the CaMV infected plants (P0pS1) were compared with the control plants (P0cS1 and P0bS1) to evaluate differences in physiological attributes, progeny development and pathogen resistance. Variation in agronomical traits of *B. rapa* such as germination rate, flowering rate, foliage dry weight, total height, root collar diameter, number of leaves and crown radius were observed while treatment effects of pathogen dose and timing of host plant pathogen exposure were not detected using one-way or two-way analysis of variance (ANOVA). However, when the parent generation was exposed to 50 ng purified CaMV particles per plant, plants that produced larger seeds generated progeny that were more resistant to CaMV (Fig. 2; Supplementary Fig. 1). The timing of pathogen exposure was also important in this relationship. For example, later exposure to virus (i.e. four versus two weeks) increased the number of plants producing larger seeds and the abundance of CaMV resistant progeny (Supplementary Fig. 1). From these analyses and for further comparisons involving P0pS1, only plants exposed to 50 ng purified CaMV at four weeks following germination were characterized and the pathogen treated plants (P0pS1) were separated into two groups designated as P0pS1R for large seed and CaMV resistant phenotype and P0pS1S for small seed and susceptible to CaMV phenotype.

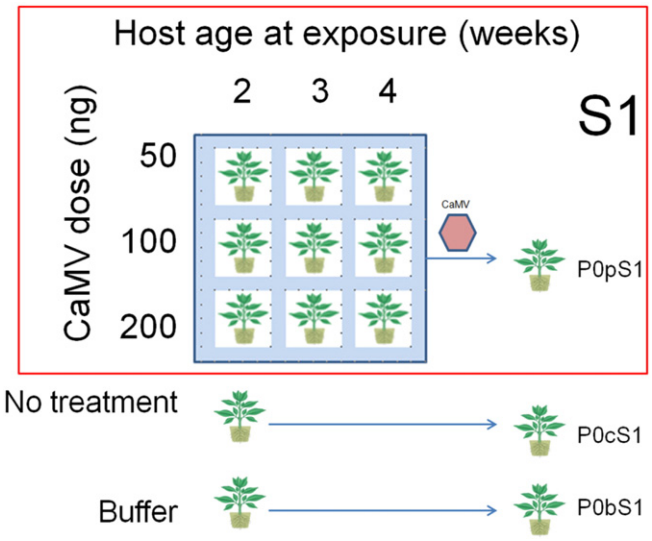

Fig. 1. Study design for evaluating transgenerational response in *Brassica rapa* following exposure to cauliflower mosaic virus (CaMV). Seed collected from one *B. rapa cv* R018 parent plant was used to generate the first self-fertilized generation (S1). The parental generation was exposed to either 50, 100 or 200 ng of purified CaMV at host plant age of two, three or four weeks following germination. Inoculation sites were removed at 24 h following pathogen exposure. Parent 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 which was absent of infectious material (P0bS1). Progeny were screened for stable complex traits and CaMV resistance. Measured variables for pathogen treated plants (P0pS1) were adjusted for by the buffer treated plants (P0bS1).

Significant differences in seed size and CaMV titer were detected for healthy (P0cS1), susceptible (P0pS1S) and resistant phenotypes (P0pS1R) (Fig. 2). The average seed sizes were 2.12 +/− 0.08 mm, 1.99 +/− 0.08 mm and 2.09 +/− 0.09 mm for the resistant, susceptible and healthy treatments, respectively (Fig. 2). Evaluation of CaMV resistance in the progeny involved double-antibody sandwich enzyme linked immunosorbant assay (DAS-ELISA) and showed that virions were significantly lowest in the resistant (P0pS1R) plants, and significantly highest in the susceptible (P0pS1S) plants (Fig. 2; Supplementary Fig. 1). Symptom expression following the CaMV challenge also correlated with CaMV titer measurements obtained by DAS-ELISA. Following CaMV inoculation, plants grown from small seed (P0pS1S) and both control plants (P0cS1 and P0bS1) displayed similar uniform and severe symptoms of CaMV infection including stunting, reduced growth and flowering, leaf deformities, mosaic and mottling within two weeks after being exposed to the virus. Surprisingly, when challenged with the same CaMV pathogen, plants grown from large seeds (P0pS1R) showed a continuum of symptoms ranging from healthy to mild which consisted of a minor intensity rating of stunting and leaf mottling and mosaic. The onset of symptoms that appeared in the P0pS1R plants after being challenged by CaMV were delayed 10 to 14 days in comparison to similarly challenged P0pS1S, P0cS1 or P0bS1.

3.2. RNA sequencing

High throughput RNA transcriptome sequencing was used to identify differentially expressed loci involved in biochemical pathways that showed a relationship to seed size and/or CaMV resistance. Deep sequenced samples included CaMV challenged P0pS1R, CaMV challenged P0bS1 and healthy P0cS1. Sequences were obtained from a pooled sample of tissue 21 days after pathogen challenge for treatments and time of sampling corresponded to 100% of P0bS1, 20% of P0pS1R and 0% of P0cS1 plants showing CaMV symptoms. The RNA sequencing produced 55 551 366, 54 658 348 and 54 233 142 raw reads from P0pS1R, P0bS1S and P0cS1, respectively. *De novo* assembly of all sequences generated 39 183 contigs with a mean size of 724 bp that ranged between 201–16 032 bp.

3.3. Resistant and susceptible phenotypes exposed to CaMV have contrasting profiles of differentially expressed loci

Genes displaying significant changes in expression were identified in CaMV challenged resistant and susceptible phenotypes. A total of 644 (365 up-regulated and 299 down-regulated) and 3193 (1250 up-regulated and 1943 down-regulated) differentially expressed genes (DEGs) were detected after exposure to CaMV in resistant (P0pS1R) and susceptible (P0bS1S) phenotypes, respectively (Supplementary Fig. 2).

3.4. Functional annotation of differentially regulated genes

To understand the functions of DEGs, the transcripts yielding a two-fold increase or decrease relative to the healthy control group were mapped to terms in the Gene Ontology (GO) database (Gene Ontology Consortium, 2004; Figs. 3 & 4). Fisher exact testing was used to determine enrichment of sequences mapping to GO term annotations between the resistant and susceptible phenotypes and was performed using a false discovery rate (FDR) adjusted p-value of <0.01 as the cut-off.

For DEGs up-regulated in resistant and susceptible plants relative to the healthy control, cellular component GO annotations for the up-regulated transcripts that were enriched in the resistant treatment were “cell wall”, “intracellular organelle”, “chloroplast stroma”,

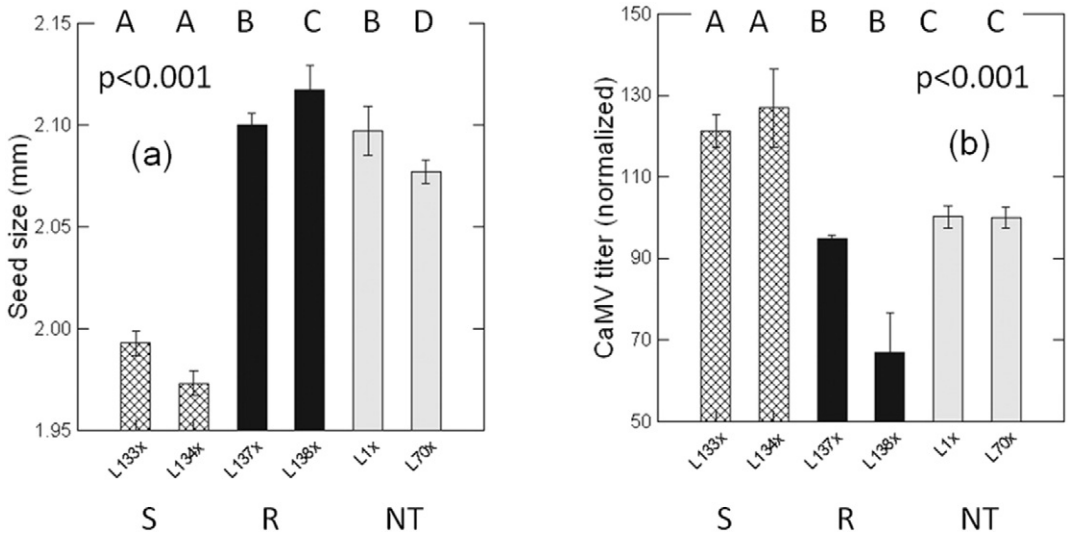

Fig. 2. Seed size (a) and cauliflower mosaic virus (CaMV) resistance (b) of *B. rapa* progeny with the parental generation exposed to 50 ng purified CaMV at four weeks following germination. Hatched bars represent lines of plants with small seeds and rated as susceptible (S) to CaMV. Dark bars represent lines of plants with large seeds and rated as resistant (R) to CaMV. Bars with light shading represent the line of plants that were not challenged during the parent generation (NT). Different letters above the bars indicate significance at $p < 0.001$.

"ribosome" and "intra-cellular non-membrane-bound organelle" and represented 27, 82, 11, 29 and 44% of the DEGs, respectively. "Integral to membrane" was the only cellular component annotation enriched for the susceptible treatment whereby representation was 8% and 1% of the total DEGs for the susceptible and resistant phenotypes, respectively.

According to molecular function for the up-regulated transcripts relative to the healthy control, the DEGs that mapped to "transmembrane transporter activity", "kinase activity", "transferase activity", "metal ion binding" and "catalytic activity" were enriched in the susceptible plants and represented 7–52% of the DEGs. The resistant plants were enriched in different annotations including "RNA binding", "myosin heavy chain kinase activity", "structural constitute of ribosome", "unfolding protein binding" and "structural molecule activity" representing 3–22% of the total DEGs.

Functional annotation using GO terms categorized into biological process for the up-regulated transcripts suggested that the resistant plants were enriched in "RNA methylation", "nucleotide biosynthetic

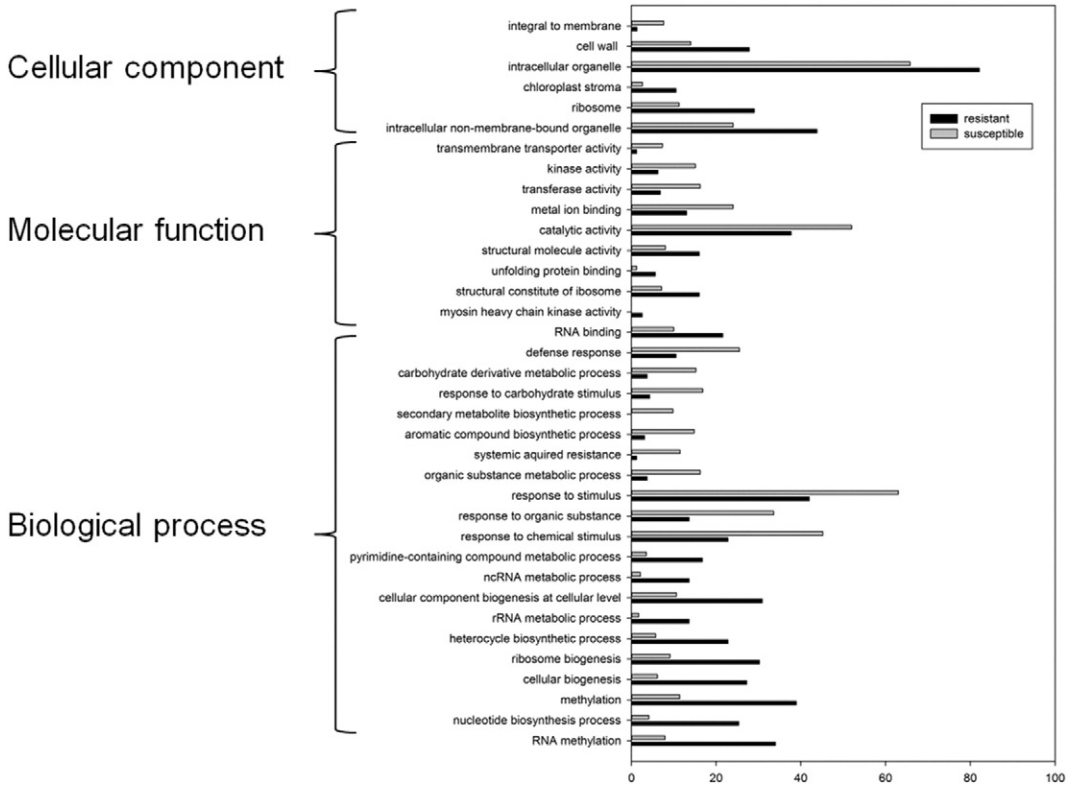

Fig. 3. Go annotations for up-regulated sequences of resistant and susceptible plants relative to healthy plants. Significant differences were detected between resistant and susceptible plants for all annotations using Fisher Exact Testing ($p < 0.01$). Only the top 5–10 over and under represented genes for cellular component, molecular function and biological process are shown.

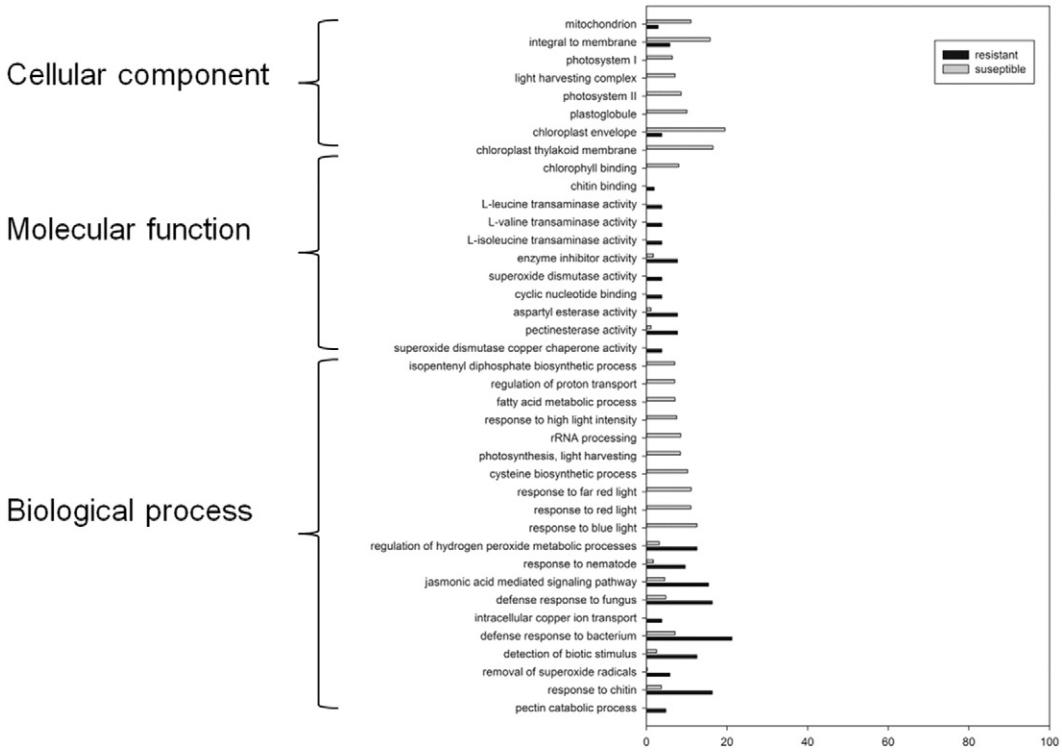

Fig. 4. Go annotations for down-regulated sequences of resistant and susceptible plants relative to healthy plants. Significant differences were detected between resistant and susceptible plants for all annotations using Fisher Exact Testing ($p < 0.01$). Only the top 5–10 over and under represented genes for cellular component, molecular function and biological process are shown.

process", "methylation", "cellular nitrogen compound biosynthetic process", "ribosome biogenesis", "heterocycle biosynthetic process", "rRNA metabolic process", "cellular component biogenesis", "ncRNA metabolic process" and "pyrimidine-containing compound metabolic process". Mapping of these annotations were represented by 13–34% of the total DEGs for the resistant phenotype and 2–11% of the total DEGs for the susceptible phenotype. "Response to chemical stimulus", "response to organic substance", "response to stimulus", "organic substance metabolic process", "systemic acquired resistance", "aromatic compound biosynthetic process", "secondary metabolite biosynthetic process", "response to carbohydrate stimulus", "carbohydrate derivative metabolic process" and "defense response" were enriched in the susceptible treatment, in comparison to the resistant treatment. These annotations were represented by 10–63% of the total DEGs of the susceptible treatment and 0.1–42% of the total DEGs of the resistant treatment.

For DEGs down-regulated in resistant and susceptible plants relative to the healthy control, cellular component GO annotations for the down-regulated transcripts enriched in the susceptible plants were "mitochondrion", "integral to membrane", "photosystem I", "light harvesting complexes", "photosystem II", "plastoglobule", "chloroplast envelope" and "chloroplast thylakoid membrane". These transcripts represented between 6–20% of the total DEGs. Cellular component annotations down-regulated relative to the healthy control but enriched for the resistant treatment were absent.

According to molecular function for the down-regulated transcripts relative to the healthy control the DEGs that mapped to "L-leucine transaminase activity", "L-valine transaminase activity", "L-isoleucine transaminase activity", "enzyme inhibitor activity", "superoxide dimutase activity", "cyclic nucleotide binding", "asartyl esterase activity", "pectinesterase activity" and "superoxide dismutase copper chaperone activity" were enriched in the resistant plants and represented 2–8% of the total DEGs. "Chlorophyll binding" was the only molecular function annotation enriched for in the susceptible treatment and represented 8% of the total DEGs.

Functional annotation using GO terms categorized into biological process for the down-regulated transcripts suggested that the resistant plants were enriched in "pectin catabolic process", "response to chitin", "removal of superoxide radicals", "detection of biotic stimulus", "defense response to bacterium", "intracellular copper ion transport", "defense response to fungus", "jasmonic acid mediated signaling pathway", "response to nematode", and "regulation of hydrogen peroxide metabolic processes". These annotations represented 4–21% of the total DEGs for the resistant phenotype and 0–5% of the total DEGs for the susceptible phenotype. "Response to blue light", "response to red light", "response to far red light", "cysteine biosynthetic process", "photosynthesis and light harvesting", "rRNA processing", "response to high light intensity", "fatty acid metabolic process", "regulation of protein transport" and "isopentenyl diphosphate biosynthetic process" were enriched in the susceptible treatment in comparison to the resistant treatment. These annotations represented 7–13% of the total DEGs of the susceptible treatment and 0–0.1% of the total DEGs of the resistant treatment.

3.5. Mapping differentially expressed genes to KEGG pathways

Mapping the DEGs to KEGG pathways shows that defense pathways, such as flavonoid biosynthesis, fatty acid biosynthesis and glucosinolate biosynthesis were more active in the resistant plants than the susceptible plants (Fig. 5). Pathways associated with growth and development such as starch and sucrose metabolism, carbon fixation and glycolysis were more active in the susceptible plants, and less active in the resistant.

3.6. Common and unique genes among phenotypes

Of the 3857 total number of DEGs analyzed, 222 and 2751 were unique to the resistant and susceptible phenotypes, respectively (Fig. 6). Among the 442 common DEGs, hierarchical clustering was used to detect any differences in the abundance of transcripts between

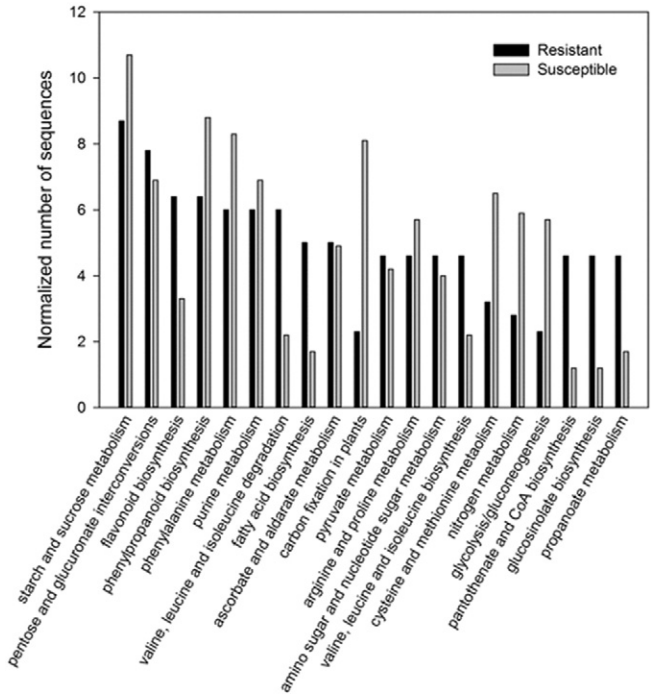

Fig. 5. KEGG pathways enriched in the DEGs between resistant and susceptible phenotypes.

the two treatments (Fig. 6). Most notable, absolute numbers of transcripts of the susceptible and healthy plants responded similarly when compared with the resistant phenotype.

4. Discussion

This study shows the changes in transcriptome associated innate immunity and confirms that in addition to ss(+) RNA viruses, Gram negative bacteria and chemical elicitors (Kathiria et al., 2010; Slaughter et al., 2012; Luna et al., 2012), dsDNA viruses can also generate a transgenerational response in the form of pathogen resistance. Resistant and susceptible plants displayed differences in seed size, pathogen titer and expression profiles. Pathogen response measured as prolonged resistance was exhibited in the progeny and appeared as a 10 to 14 day delay in symptom response and in some lines, symptoms and/or titers of CaMV were absent. The data support temporal differences in defense responses between primed plants showing pathogen resistance and plants that did not receive a priming treatment, similar to that observed in model hosts (Kathiria et al., 2010). Following pathogen exposure at the same developmental stage, the plants that were not primed exhibited elevated levels of infection whereas, symptom development progressed much more slowly in the primed plants. Symptom development, pathogen titer and RNA sequence transcriptome profiling was used to characterize these differences between primed first generation stress resistant and susceptible phenotypes.

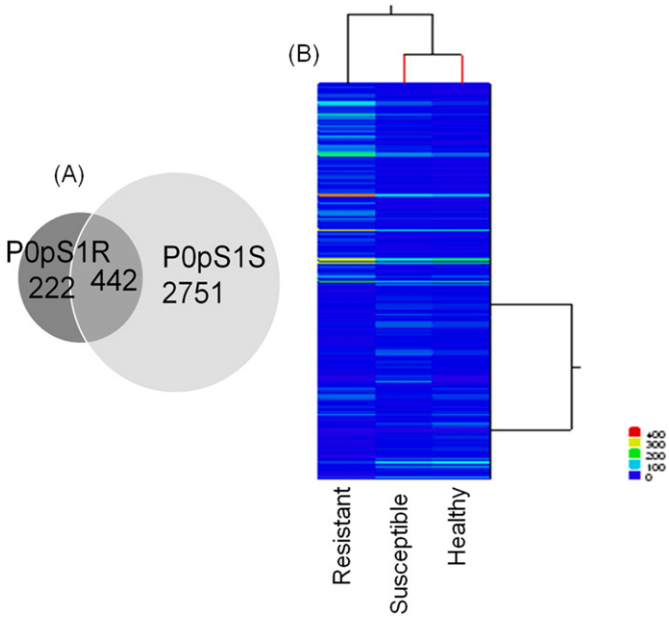

Fig. 6. (A) Venn diagram illustrating the number of differentially expressed genes of resistant and susceptible phenotypes. Dark shade represents resistant phenotype and light shade represents susceptible phenotype. (B) Hierarchical clustering of the 442 common DEGs among the resistant and susceptible phenotype.

We report pathogen resistance as a trangenerational response in *B. rapa* and this finding is similar to many other recent studies with other pathogens and non-agricultural plants (Kathiria et al., 2010; Luna et al., 2012; Slaughter et al., 2012). However, this study differs from the previously mentioned studies in that pathogen titer and symptom development evaluations were observed at 21 days post inoculation (dpi) rather than at early phases (2–48 h) of local pathogen infection. At 21 dpi CaMV is systemic in the host enabling the evaluation of advanced stages in pathogen infection, including quantification of the pathogen using immunological assays (Farzadfar and Pourrahim, 2013). Transgenerational responses in the form of pathogen resistance that have been reported only last 6–48 h (Kathiria et al., 2010). Others have made similar suggestions in that the expression of transgenerational effects may not be confined to the seedling stage, but that they may also be observed in adults (Galloway and Etterson, 2009; Case et al., 1996). In this study, a prolonged period of 10 to 14 days of delayed symptom and titer was obtained by measuring the time-point of systemic rather than local infection.

Susceptible and resistant first generation progeny were challenged with CaMV and during systemic viral infection, RNAs were extracted and sequenced to obtain the transcriptomes. Comparisons among transcriptomes revealed distinct differences between the resistant and susceptible phenotypes. Progeny exhibiting strong resistance to CaMV had an increased abundance of transcripts associated with pathogen defense including glucosinolate biosynthesis, flavonoid biosynthesis and fatty acid biosynthesis, while the susceptible phenotype did not share this profile. The glucosinolate, flavonoid and fatty acid biosynthesis pathways were previously identified as being involved with pathogen defense (Montillet et al., 2013; Ferreyra et al., 2012; Wang et al., 2011). Although the transcriptome profile of the susceptible plants also displayed pathogen defense activity the profile was different from the resistant phenotype. The susceptible phenotype had activity in phenylpropanoid biosynthesis, phenylalanine metabolism and there was an abundance of transcripts involved in systemic acquired resistance (SAR). Love et al. (2005) had previously shown that glutathione S-transferase (GST1), a marker gene for reactive oxygen species (ROS), pathogenesis-related protein 1 (PR1), a marker for salicylic acid pathway, PR2 and PR5 had increased expression at 20 dpi in the compatible reaction between *A. thaliana* and CaMV. Here, we provide additional evidence that SAR appears to be involved in pathogen defense during the later stages of compatible CaMV infected *B. rapa* in a transgenerational mechanism.

Differences in the activity of pathways involved in primary metabolism (i.e. growth and development) between the two phenotypes were also detected. The susceptible plants had an increased abundance of transcripts associated with growth and development processes including photosynthesis and glycolysis, relative to the resistant plants. These findings coupled with observations of early bolting and flowering indicates faster flowering as a developmental response of *B. rapa* infected with CaMV. Alteration in reproductive development after exposure

to pathogens was previously described (Agnew et al., 2000; Peters, 1999). For example, accelerated reproductive development was observed in *Arabidopsis* infected with *Pseudomonas syringae*, *Xanthomonas campestris* or *Personospora parasitica* (Korves and Bergelson, 2003). Transcription factors and phytohormones such as SA, JA, ethylene or auxins link pathogen defense and development responses because they are involved in both processes with cross-talk between gene networks (Liu et al., 2014; Wang et al., 2002). This study further shows strong evidence of differences between pathogen defense and development response supported by an increased abundance of transcripts involved with cellular components associated with structure including the cell wall, cell membrane constituents and intracellular organelles displayed in the resistant plants, compared to the susceptible. Plants coordinate their cellular structures with the production of secondary metabolites to inhibit the movement of viruses and other pathogens during infection (Zavalieu et al., 2011). Additionally some pathogens including CaMV alter the host's cellular structure to facilitate movement throughout the plant (Carluccio et al., 2014). These defense mechanisms alter gene expression of structural host cell machinery and reduce CaMV titer in resistance plants leading to a high level of resistance.

The mechanism behind pathogen resistance as a transgenerational response remains elusive but the meiotic inheritance of epigenetic signatures, such as DNA methylation, acetylation of histone tails, chromatin remodeling and small RNAs have been suggested to give rise to transgenerational responses (Jablonka, 2013; Jablonka and Raz, 2009). Although in this study, the mechanism behind this resistance appears to involve several pathways and possibly mechanisms, enhanced methylation and non-coding RNA metabolic processes were detected in the resistant plants, suggesting an epigenetic mechanism behind the resistance. Further support for an epigenetic mechanism is the nuclear localization of CaMV nucleic acid throughout it's lifecycle. Replication of CaMV requires the viral translational transactivator protein P6 that is present in the nucleus and is essential for CaMV infectivity (Haas et al., 2008). One nuclear function for P6 is to supress RNA silencing, a gene regulation mechanism that provides antiviral capabilities by inactivating the nuclear protein DRB4 that is required by the major plant antiviral silencing factor DCL4. Besides a regulatory role, RNA silencing confers a sequence-specific antiviral immunity to plants through virus-derived short interfering RNA (reviewed in Ding and Voinnet, 2007). Localization of CaMV components such as P6 in the nucleus of the plant cell provides an opportunity for genome and transcriptome modifications. These modifications may provide transgenerational protection to other pathogens (Kathiria et al., 2010) and influence other phenotypic characteristics such as seed size as observed in this study.

We also uncovered a relationship whereby, parent plants that produced larger seeds produced progeny more resistant to CaMV. The phenotypic transgenerational effect of an increase in seed size was previously observed with exposure to low temperature, herbivory or shaded conditions (Galloway and Etterson, 2009; Agrawal, 2001; Case et al., 1996). Although a linkage between these two traits remains to be determined, as single variables, both of these traits were demonstrated to be inherited in a transgenerational manner and have shown to be correlationed to DNA methylation (Amoah et al., 2012; Luna et al., 2012; Kathiria et al., 2010). Although there is uncertainty over stable transgenerational inheritance (Iwasaki and Paszkowski, 2014; Boyko and Kovalchuk, 2011; Pecinka et al., 2009) because a mechanism awaits to be determined, this study provides evidence that under a specified treatment regime, seed size and CaMV resistance may be transferred to first generation progeny in a durable transgenerational fashion. Also notably our results indicate that disease resistance was not acquired equally across all progeny, but that there was variability between progeny produced from the same plant, limiting maternal inheritance as influencing the traits observed.

Further examination of the inheritance of stable epigenetic signatures and metabolic profiling would advance our understanding of the mechanism behind the resistance and determine if there is a linkage between seed size and resistance. This work provides insight into the use a non-transgenic means for introducing stress resistance into *B. rapa* germplasm which is an economically important crop. It would be beneficial to further explore the type of resistance that was demonstrated in this study and continue to investigate if it could be applied to other economically important crops.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.12.016.

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