Comparative transcriptomics revealed differential regulation of defense related genes in *Brassica juncea* leading to successful and unsuccessful infestation by aphid species

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Productivity of Indian mustard (*B. juncea*), a major oil yielding crop in rapeseed-mustard group is heavily inflicted by mustard aphid, *L. erysimi*. Mustard aphid, a specialist aphid species on rapeseed-mustard crops, rapidly multiplies and colonizes the plants leading to successful infestation. In contrary, legume specific cowpea aphid, *A. craccivora* when released on *B. juncea* plants fails to build up population and thus remains unsuccessful in infestation. In the present study, differential host response of *B. juncea* to the two aphid species, one being successful insect-pest and the other being unsuccessful on it has been studied based on transcriptome analysis. Differential feeding efficiency of the two aphid species on mustard plants was evident from the amount of secreted honeydews. Leaf-transcriptomes of healthy and infested plants, treated with the two aphid species, were generated by RNA sequencing on Illumina platform and de novo assembly of the quality reads. A comparative assessment of the differentially expressed genes due to treatments revealed a large extent of overlaps as well as distinctness with respect to the set of genes and their direction of regulation. With respect to host-genes related to transcription factors, oxidative homeostasis, defense hormones and secondary metabolites, *L. erysimi* led to either suppression or limited activation of the transcript levels compared to *A. craccivora*.

Further, a comprehensive view of the DEGs suggested more potential of successful insect-pests towards transcriptional reprogramming of the host. qRT-PCR based validation of randomly selected up- and down-regulated transcripts authenticated the transcriptome data.
many plant species during their life cycle\(^3\). For example, the green peach aphid, *Myzus persicae* has a wide range of hosts while its close relative pea aphid, *Acrithosiphon pisum* has a limited number of hosts within the leguminous plants. The determinants of such differences in host range within the aphid species remain elusive\(^4\). Finding a specific host by a winged aphid involves a series of complex behavioural events between plant and aphids such as host finding, landing, probing etc. before the establishment of uninterrupted feeding. A small number of plant species can kill or repel aphids by toxic compounds secreted by the glandular trichomes\(^5\). Aphids are likely to use a phototactic visual response and phytochemical cues in determining the host\(^6\). For example, soybean aphid, *Aphis glycines* identifies soybean over the other nonhost plants through olfactory chemical signalling and it is evident as interuption by nonhost plants through odours decreases their ability to locate and colonize the host plants\(^7\).

During feeding from the sieve tube elements in phloem, aphids introduce effector molecules into the host cells for suppression of the host defense responses\(^8,9,10\). In the past decade, several effector molecules and their role in aphid virulence have been identified by either over-expression or suppression studies in the host plants. For example, over-expression of *M. persicae* effectors Mp10, Mp42, Mp56, Mp57, and Mp58 in Arabidopsis and tobacco plants reduced aphid virulence\(^10,11\). In contrary, similar over expression of a pea aphid C002 ortholog from *M. persicae* MpC0002 and other *M. persicae* effectors such as Mp1/PlntO1 and PlntO2 were found to be involved in increased virulence\(^7,12\). Further, it has been demonstrated that the effectors’ interaction with host proteins is species-specific. For example, the *M. persicae* Mp1 effector interacts with the host protein VPS52 to promote aphid virulence; whereas over-expression of potato StVPS52 in tobacco plants significantly reduced *M. persicae* fecundity\(^11\). Similarly, host plants over-expressing pea aphid effector C002 did not affect the performance of *M. persicae*. Such impasse did not allow to hypothesize more universal role of any effector which could be targeted for devising resistance strategy against aphids.

Broad-spectrum, nonhost resistance against pathogens involves recognition and activation of the plant immune system. In contrary, the host defense system is either suppressed or evaded by effectors in case of compatible host-pathogen interactions\(^13\). Significant progress has been made on understanding how the plants respond to pathogens in interacting as a host or nonhost. Opposing to that, not much is known in understanding the plant-response as nonhost in case of plant-insect or plant-aphid interactions. Jaouannet et al.\(^16\) through microarray analysis demonstrated set of genes specifically affected during host or nonhost interactions with specific aphid species. However, because of limited evidence, lack of more studies and validation, our knowledge of nonhost resistance mechanisms in plant-aphid interactions remains limited. More recently, it was shown that BAK1 (Brassinosteroid insensitive 1-associated kinase 1), a key regulator of several leucine-rich repeat-containing PRRs (pattern recognition receptors) is involved in nonhost resistance to aphids. The pea aphid, *A. pisum* for which Arabidopsis is normally a nonhost survive better on bak1-5 mutant plants suggesting that BAK1 contributes to nonhost resistance\(^11\). Similarly, bird-cherry oat aphid, *Rhopalosiphum padi* for which Arabidopsis is a nonhost survive longer on vspl and atroboh F-3 mutant plants, indicating that VSP1 and AtRbohF contributes to nonhost resistance against this aphid\(^10\). However, similar role of these genes in case of other species is not known.

In the last decade, other than *M. persicae*-Arabidopsis interaction, a few more studies based on microarrays or RNA-seq analyses have been carried out in different plant species for elucidating plant-responses’ to aphid infestation\(^17,18\). These studies revealed involvement of ROS homeostasis, cell signalling and production of secondary metabolites as a major part of the host defense response against aphids in addition to components of primary metabolism of the host plants. However, in case of *Brassica* sp. which comprise a major group of economically affected crops only limited information is available on host and nonhost-response to aphids\(^19\). For overcoming such bottleneck, in the present study, we have made a comparative assessment of the transcriptional responses of *B. juncea* under compatible and incompatible interaction with aphid species *L. erysimi* and *Aphis craccivora*, respectively. Out of that comparative analysis, the differentially regulated genes involved in pathways related to host resistance have been highlighted.

**Results**

**Feeding performance of aphid species on *B. juncea* as host and nonhost.** The feeding efficiency of an aphid species on a host is directly proportional to the amount of honeydew excretion\(^20\). Indian mustard, *B. juncea* is the natural host and a nonhost for the aphid species *L. erysimi* and *A. craccivora*, respectively. The feeding efficiency of *L. erysimi* and *A. craccivora* released on *B. juncea* plants was measured as a function of the amount of honeydew excreted by them in 24 h of feeding. The excreted honeydews collected on the Whatman paper discs were stained and quantified by Ninhydrin reagent\(^24\). The honeydews developed purple colour spots with different intensities after staining with ninhydrin. The number of purple spots produced in case of *L. erysimi*-feeding was much higher than the *A. craccivora*-feeding (Fig. 1A). Further, the spectrometric quantification of the spots revealed that, *L. erysimi* excreted 4.5 folds higher amount of honeydews compared to *A. craccivora* (Fig. 1B). Fecundity estimates of the aphids during the rearing period revealed that *A. craccivora* released on *B. juncea* out performed *L. erysimi* and *A. craccivora* respectively. Out of that comparative analysis, the differentially regulated genes involved in pathways related to host resistance have been highlighted.

**Transcriptome sequencing, data records and de novo assembly.** Whole transcriptome of *B. juncea* leaves independently infested with *L. erysimi* and *A. craccivora* were sequenced to identify the set of differentially regulated host-genes in case of successful and unsuccessful colonization. Sequencing was carried out on Illumina platform using 2 × 150 paired-end chemistry and the mean sizes of the inserts in the libraries were in the range of 450–675 bp. The raw reads obtained in uninfested (control), *L. erysimi*-infested (LE)
and _A. craccivora_-infested (AC) samples were 5.7, 5.4 and 5.2 Gb, respectively (Table 1). All sequence reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession SAMN12924495-SAMN12924497) under Bioproject PRJNA576081(https://www.ncbi.nlm.nih.gov/sra/PRJNA576081). After removing the adaptor sequences, low quality reads and over-represented sequences, high-quality clean reads were obtained from the above three libraries. These high-quality reads were _de novo_ assembled using CLC Genomics workbench 6.0 on optimized parameters which led to the identification of 48775, 49646 and 42182 transcripts in case of control, LE and AC samples. The minimum and maximum contig size was 500 bp and 16812 bp, respectively with an N50 value greater than 1000. The size distribution of transcript lengths has been shown in Supplementary Fig. S1. Further, the assembled transcript contigs were analysed for identifying coding regions which revealed a total of 47806, 48638 and 41389 CDS with varying length in control, LE and AC infested samples, respectively.

Functional annotation of aphid inducible transcriptome in _B. juncea_. The assembled transcripts were aligned with the non-redundant NCBI plant protein database using blastx with a cut-off E-value of 10^{-6}. The number of CDS showing significant matches were 43485, 44507, 38068 and unmatched CDS were 5290, 5139, 4114 in control, LE and AC infested samples, respectively. The transcriptome data sets were also annotated against TAIR10 protein database. In TAIR blastx 39813, 41184, 37630 coding sequences in case of control, LE and AC infested samples, respectively found significant matches. Species distribution of the best match sequences suggested that majority of the hits were from the Brassicaceae members comprising _Eutrema salsugineum, Arabidopsis thaliana, A. lyrata, Capsella rubella, Thellungiella halophila, Brassica rapa, B. napus, B. oleracea_ and _B. juncea_ (Fig. 2). The transcriptome data sets also shared similarity with species outside the Brassicaceae family viz., _Phaseolus vulgaris_ and _Glycine max_. Further, the annotated CDS were mapped on to the GO database for

![Figure 1](https://www.nature.com/scientificreports/images/figure1.png)

**Figure 1.** Feeding efficiency, survival and reproduction rate of _L. erysimi_ and _A. craccivora_ on _B. juncea_. (A) Ninhydrin staining of aphid honeydew after 24 h of feeding. (B) Quantification of honeydew by ninhydrin staining measured at λ₅₀₀. (C) Fecundity of the two aphid species on mustard plants. (D) Survival rate of the two aphid species after 4 days of release. Bars represent means ± SE. Means with different letters are significantly different (Student’s t test, p < 0.05).

|                         | Control | LE   | AC   |
|-------------------------|---------|------|------|
| Data output (Gb)        | 5.7     | 5.4  | 5.2  |
| High quality reads      | 20606561| 19387846| 19321340|
| Number of transcripts   | 48775   | 49646| 42182|
| Maximum transcript size (bp) | 16812 | 8529 | 9223 |
| N50                     | 1053    | 1207 | 1182 |
| Number of CDS           | 47806   | 48638| 41389|

Table 1. Summary of sequencing and assembly data of control, LE and AC infested _B. juncea_.

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identifying nodes comprising of GO functional groups. CDS associated with similar functions are assigned to the same GO functional group. Based on the sequence homology, 30681 (Control), 31383 (LE) and 26088 (AC) CDS were grouped under Biological Process, 28877 (Control), 29468 (LE) and 24339 (AC) CDS were assigned under Molecular Function and 30929 (Control), 32028 (LE) and 26371 (AC) CDS were categorised under Cellular Component (see Supplementary files 1–3). All the CDS were also compared against the KEGG database (www.kegg.jp/kegg/kegg1.html) using blastx with threshold bit-score value of 60 (default)\(^{25}\). The mapped CDS represented metabolic pathways of major biomolecules such as carbohydrates, lipids, nucleotides, amino acids, glycans, cofactors, vitamins, terpenoids and polyketides etc. Details of the functional pathways and their sub-categories are provided in Table 2.

Table 2. KEGG pathway distribution of *B. juncea* leaf transcriptome.

| Pathway                              | Control | LE  | AC  |
|--------------------------------------|---------|-----|-----|
| **Metabolism**                       |         |     |     |
| Carbon and fatty acid metabolism     | 519     | 570 | 512 |
| Carbohydrate metabolism              | 591     | 631 | 572 |
| Energy metabolism                    | 556     | 561 | 503 |
| Lipid metabolism                     | 358     | 388 | 324 |
| Nucleotide metabolism                | 226     | 247 | 220 |
| Amino acid metabolism                | 483     | 517 | 476 |
| Metabolism of other amino acids      | 195     | 218 | 182 |
| Glycan biosynthesis and metabolism   | 112     | 131 | 112 |
| Metabolism of cofactors and vitamins | 287     | 317 | 299 |
| Metabolism of terpenoids and polyketides | 184 | 187 | 158 |
| Biosynthesis of other secondary metabolites | 156 | 163 | 159 |
| Xenobiotics biodegradation and metabolism | 116 | 123 | 83 |
| **Genetic Information Processing**   |         |     |     |
| Transcription                        | 334     | 345 | 312 |
| Translation                          | 879     | 970 | 806 |
| Folding, sorting and degradation     | 676     | 708 | 621 |
| Replication and repair               | 176     | 196 | 167 |
| **Environmental Information Processing** |     |     |     |
| Membrane transport                   | 36      | 30  | 29  |
| Signal transduction                  | 551     | 616 | 550 |
| Signalling molecules and interaction | 1       | 1   | 4   |
| **Cellular Processes**               |         |     |     |
| Transport and catabolism             | 390     | 392 | 340 |
| Cell motility                        | 58      | 65  | 60  |
| Cell growth and death                | 212     | 230 | 202 |
| Cell communication                   | 64      | 69  | 64  |
| **Organismal Systems**               |         |     |     |
| Environmental adaptation             | 210     | 214 | 207 |

Figure 2. Species distribution of the top blastx matches following nr annotation of unigenes in leaf transcriptome of *Brassica juncea* treated with successful and unsuccessful aphid species.
B. attenuated by LE-infestation (Fig. 4D). The analysis empirically advocated suppression of defense related pathways. AC and LE infested transcriptome showed significant differences in biotic stress and secondary metabolism responses (Fig. 4). Under biotic stress, the transcripts belonging to hormone signalling, cell wall modification, proteolysis, redox state including glutathione S-transferase (GST), signalling, secondary metabolites, transcription factors, and heat shock protein categories were found to be up-regulated in response to AC infestation. In AC-infested samples enriched GO terms represented defense response to bacterium (GO:0042742), hormone transport (GO:0009914) and carbohydrate metabolic process (GO:0006790). Further, in biological processes enriched GO terms of down-regulated transcripts in case of LE-infested sample distinctly represented defense related mechanisms such as secondary metabolic process (GO:0019748), response to oxidative stress (GO:0006979), phenylpropanoid biosynthetic process (GO:0009699), sulfur metabolic process (GO:0006790), and glucosinolates biosynthetic process (GO:0019761), characteristically present in members of Brassicaceae.

Further, to understand the mechanistic differences in AC and LE induced host responses, differentially expressed genes were analyzed by the MapMan software built in biotic stress and secondary metabolism overview under pathways. AC and LE infested transcriptome showed significant differences in biotic stress and secondary metabolism responses (Fig. 4). Under biotic stress, the transcripts belonging to hormone signalling, cell wall modification, proteolysis, redox state including glutathione S-transferase (GST), signalling, secondary metabolites, transcription factors, and heat shock protein categories were found to be up-regulated in response to AC infestation (Fig. 4A,B). However, most of these pathways were suppressed within 24 h of successful host colonization by LE (Fig. 4C). Similarly, the transcripts belonging to biosynthetic pathways of secondary metabolites such as phenylpropanoid, carotenoids, flavonoids, lignin and glucosinolates were induced in response to AC infestation (Fig. 4B). The analysis empirically advocated suppression of defense related pathways in B. juncea in case of successful colonization by LE.

**Differentially expressed transcription factors in response to L. erysimi and A. craccivora.** Transcription factors (TF) are the key regulatory proteins involved in modulating gene expressions. Out of the 1307 differentially expressed genes in LE and AC transcriptomes compared to uninfested controls, 158 genes were identified as encoding transcription factors (TFs). Out of 158 TFs, 91 were from LE and 67 from AC samples (see Supplementary file 6). Further analysis showed that most of the TFs were distinct to LE or AC library whereas, at the most 10% were overlapping (Fig. 5A). Further, a comparative assessment of down-regulated TFs showed that higher number of distinct TFs are suppressed or down-regulated in LE compared to AC samples and only 7% TFs were commonly down-regulated in both the samples (Fig. 5B). In structural grouping, the differentially expressed TFs were spanned over 32 families. Twenty-one family were specific to LE and 4 families (Nin-like, CAMTA, M-type, TCP) were specific to AC. The major families of the differentially expressed TFs in LE sample were bHLH (11), GeBP (9), NAC (8), B3 (5) and CO-like (5). These 5 families of TFs represented almost similar, representing response to abiotic stimulus (GO:0009628), cellular process (GO:0009987), response to stimulus (GO:0050896), developmental process (GO:0032502) and catalytic activity (GO:0003824). However, 143 up- and 34 down-regulated genes in LE and AC samples (Fig. 3). Analysis of gene ontology terms of the differentially expressed transcripts in LE and AC infested samples over the control was represented in Supplementary file 5 and Fig. S2. In case of up-regulated transcripts in both the treatments, significantly enriched GO terms in molecular function and biological processes were almost similar, representing response to biotic stimulus (GO:0009628), cellular process (GO:0009987), response to stimulus (GO:0050896), developmental process (GO:0032502) and catalytic activity (GO:0003824). However, in AC-infested samples enriched GO terms represented defense response to bacterium (GO:0042742), hormone transport (GO:0009914) and carbohydrate metabolic process (GO:0006790). Further, in biological processes enriched GO terms of down-regulated transcripts in case of LE-infested sample distinctly represented defense related mechanisms such as secondary metabolic process (GO:0019748), response to oxidative stress (GO:0006979), phenylpropanoid biosynthetic process (GO:0009699), sulfur metabolic process (GO:0006790), defense response to bacterium (GO:0042742), defense response (GO:0006952) and glucosinolates biosynthetic process (GO:0019761), characteristically present in members of Brassicaceae.

**Analysis of oxidative stress related transcripts in B. juncea as host and nonhost to aphid infestations.** Transient activation of redox related genes constitutes the secondary signalling in plant
defense response. The genes involved in transient generation as well as scavenging of free radicals determine the ROS homeostasis. The ROS homeostasis genes showing differential expression between LE vs uninfested and AC vs uninfested were further compared for identifying the set of genes in common as well as distinct and the direction of their regulation (Table 3). The analysis showed that quantitatively a greater number of distinct transcripts involved in scavenging process are up-regulated in *B. juncea* in response to *L. erysimi* compared to response against *A. craccivora*. In plants, small intracellular thiol molecules, glutathione is considered as a strong non-enzymatic scavenger of reactive oxygen species (ROS). It serves as a substrate to the glutathione peroxidase
and glutathione S-transferase in scavenging process. We observed that several transcripts involved in glutathione metabolism such as glutathione S-transferase, ascorbate peroxidase and L-ascorbate oxidase were induced in case of AC sample. In case of LE-infestation though peroxidases and glutaredoxin family proteins were activated, metabolism such as glutathione S-transferase, ascorbate peroxidase and L-ascorbate oxidase were induced in S and glutathione indicated limited activation of ROS scavenging enzymes leading to elevated levels of free radical production in L. erysimi also showed differential regulation when the response against A. craccivora and L. erysimi compared to its susceptible response to L. erysimi.

### Analysis of phytohormone-related transcripts

Phytohormones are known to play important role in plant defense against various biotic and abiotic stresses. Therefore, we investigated the differential transcript activation of the genes related to phytohormone biosynthetic pathways in *B. juncea* transcriptome in response to *L. erysimi* and *A. craccivora*. The analysis indicated differential expression of genes related to primarily six phytohormones viz., auxin (9 genes), abscisic acid (ABA; 4 genes), ethylene, (ET; 4 genes), jasmonic acid, (JA; 3 genes), salicylic acid (SA; 1 gene) and brassinosteroid (BR; 7 genes) (Table 4). Among the transcripts involved in auxin homeostasis UGT775B, PIN4 and UGT774B1 were down-regulated in LE-infested samples, whereas TIR3 and ARF18 showed up-regulation in both LE and AC infested samples. Interestingly, LAX3, PIN7 and ARF2 were specifically up-regulated in AC-infested sample. Three transcripts AFB4, KOB1, NCED4 which are involved in ABA signalling were up-regulated in the LE transcriptome, while ABA-responsive element binding protein3 (AREB3) transcripts were down-regulated in AC transcriptome. ET signalling pathway related genes such as multigene bridging factor 1C (MBF1C) and cooperatively regulated by ethylene and jasmonate1 (CE1) were down-regulated and DEAD box RNA helicase 20 (RH20) was up-regulated in case of LE. In contrary, EIN3-binding F-box protein2 (EBF2) was up-regulated in case of AC set.

JA and SA-related genes in *B. juncea* also showed differential regulation when the response against *L. erysimi* and *A. craccivora* was compared (Table 4). The expression of NPR3, a paralog of NPR1 involved in SA signalling was down-regulated in LE infestation. In case of JA pathway, the transcripts encoding fatty acid desaturase3 (FAD3) was up-regulated and coronatine-induced protein1 (CO1) was down-regulated in LE infested sample. CO1 is involved in signal transduction process of JA signalling. Thus, the result indicated likely suppression of JA signalling pathways in *B. juncea* in case of *L. erysimi* infestation. A similar suppression was not detected in case of AC sample which rather showed an increase in level of 12-oxophytodienoate reductase2 (OPR2) involved in JA biosynthetic pathway. Transcripts encoding BR-related pathway such as brassinosteroid signalling positive regulator (BZR1) family protein and squalene monooxygenase were down-regulated in AC. Whereas the transcript BRI1 suppressor1 (BRS1), a serine carboxypeptidase and BRI1-associated receptor kinase1 (BAK1) were

| Common hit ID | TAIR ID | Log2 (LE/Control) | TAIR Description |
|---------------|---------|-------------------|------------------|
| BAF00793      | at5g37830 | 4.35              | Oxoprolinase 1 (OXP1) |
| XP_002875944  | at3g95110  | 2.86              | Peroxidase 33 (PER33) |
| AAD17935      | at4g35090  | −2.91             | Catalase 2 (CAT2) |
| XP_006291823  | at3g26600  | −2.33             | Antioxidant/peroxiredoxin |
| AAB69001      | at3g10920  | −2.48             | Manganese superoxide dismutase 1 (MSD1) |
| XP_002870217  | at4g15680  | 2.43              | Glutaredoxin family protein |
| ESQ58351      | at4g33040  | 2.11              | Glutaredoxin family protein |
| XP_002887881  | at1g64500  | −2.10             | Glutaredoxin family protein |
| XP_006294288  | at2g41330  | −3.11             | Glutaredoxin family protein |
| XP_006298621  | at3g15360  | −2.61             | Thioredoxin M-type 4 (TRX-M4) |
| AAP58393      | at2g02930  | −2.84             | Glutathione S-transferase F3 (GSTF3) |
| RAJ33811      | at3g03190  | −2.26             | Glutathione S-transferase F11 (GSTP11) |
| XP_002893466  | at1g26340  | 2.34              | Cytochrome b5 isofrom A (Cb5-A) |
| ESQ39310      | at2g46650  | 2.63              | Cytochrome b5 isofrom C (Cb5-C) |
| ESQ49825      | at3g02870  | 2.86              | L-galactose-1-phosphate phosphatase |
| NP_568360     | at5g18120  | 2.49              | APR-like 7 (APRL7) |
| ABB17025      | at1g77510  | 2.31              | Protein disulfide isomerase-like 1-2 |
| AET14214      | at4g26850  | −2.00             | Vitamin C defective 2 (VTC2) |
| Common hit ID | TAIR ID | Log2 (AC/Control) | TAIR Description |
| AAN60795      | at1g07890  | 2.50              | Ascorbate peroxidase 1 (APX1) |
| ESQ24233      | at3g21105  | 3.10              | L-ascorbate oxidase |
| NP_172147     | at1g08620  | 2.01              | 2-oxoglutarate-dependent dioxygenase |
| NP_177955     | at1g78320  | 2.44              | Glutathione S-transferase tau 23 (GSTU23) |
| ESQ47277      | at5g37830  | 2.25              | Oxoprolinase 1 (OXP1) |
| NP_568360     | at5g18120  | 2.08              | APR-like 7 (APRL7) |
| ABB17025      | at1g77510  | 2.72              | Protein disulfide isomerase-like 1-2 |

Table 3. Differentially expressed transcripts related to oxidative stress in response to successful and unsuccessful *B. juncea* - aphid interactions.
down-regulated and BRASSINOSTEROID INSENSITIVE1 (BRI1), a brassinosteroid receptor was up-regulated in LE-infested samples (Table 4).

### Transcript response related to secondary metabolite biosynthesis.

Plants synthesize secondary metabolites such as phenols, terpenes, flavonoids, sulphur and nitrogen containing compounds to combat against microbial infections and insect herbivory. In the transcriptome data, several transcripts showing differential level were mapped to the genes involved in the synthesis of various secondary metabolites belonging to isoprenoid, flavonoid, carotenoid, lignin and glucosinolates. A total of 36 transcripts involved in secondary metabolite production were differentially expressed in LE and AC infested samples compared to their uninfested controls (Table 5). The transcript responses against the two aphid species with respect to genes involved in secondary metabolism consist mostly of common set of genes and with similar pattern of regulation (Table 5). Nevertheless, the transcripts which were distinct in LE and AC were also identified. Interestingly, the direction of regulation of the differentially expressed genes in case of *A. craccivora* infested sample (AC), was essentially activation type; whereas the majority of differentially expressed genes showed down-regulation in case of LE infested plants.

| Common hit ID | TAIR ID | Log2 (LE/Control) | TAIR Description |
|---------------|---------|-------------------|------------------|
| Auxin         | AAL09350 at1g24100 | −2.44 | UDP-glucosyl transferase 74B1 (UGT74B1) |
|               | BAD93921 at2g04120 | −2.58 | PIN-FORMED4 (PIN4) |
|               | BAJ33712 at1g05560 | −2.15 | UDP-Glucosyltransferase 75B1 (UGT75B1) |
|               | ESQ49882 at3g02260 | 3.06 | Transport inhibitor response3 (TIR3) |
|               | AFD01316 at3g61830 | 2.52 | Auxin response factor18 (ARF18) |
|               | ESQ33837 at1g03330 | 2.02 | Auxin response factor6 (ARF6) |
| ABA           | ESQ47969 at3g19290 | 2.06 | ABRE binding factor4 (ABF4) |
|               | NP_187467 at3g08550 | 2.68 | KOBITO (KOB1) |
|               | BAJ34120 at4g19170 | 2.27 | Nine-cis-epoxycarotenoid dioxygenase4 (NCED4) |
| ET            | XP_002876019 at3g50260 | −2.26 | Cooperatively regulated by ethylene and jasmonate1 (CEJ1) |
|               | ESQ59967 at1g55150 | 2.64 | DEAD box RNA helicase, putative (RH20) |
|               | NP_189093 at3g24500 | −3.60 | Multipeptide bridging factor1C (MBF1C) |
| IA            | AAN51933 at1g19670 | −2.92 | Coronatine-induced protein1 (COR1) |
|               | ADJ50819 at2g29980 | 3.09 | Fatty acid desaturase3 (FAD3) |
| SA            | ESQ59613 at5g45110 | −3.21 | Regulatory protein NPR3 (NPR3) |
|               | ESQ50688 at2g07050 | 2.63 | Cycloartenol synthase1 (CAS1) |
|               | ESQ51435 at4g30610 | −2.72 | BRI1 suppressor1 (BRS1) |
|               | BAJ34430 at4g33430 | −3.90 | BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) |
| BR            | AFU83230 at4g39400 | 2.13 | BRASSINOSTEROID INSENSITIVE1 (BRI1) |

| Common hit ID | TAIR ID | Log2 (AC/Control) | TAIR Description |
|---------------|---------|-------------------|------------------|
| Auxin         | ESQ49882 at3g02260 | 2.34 | TIR3 |
|               | XP_002862339 at1g77690 | 3.30 | LIKE AUX1 3 (LAX3) |
|               | ESQ53440 at1g23080 | 4.32 | PIN-FORMED7 (PIN7) |
|               | AFD01316 at3g61830 | 2.42 | Auxin response factor18 (ARF18) |
|               | AFD01294 at5g62000 | 2.22 | Auxin response factor2 (ARF2) |
| ABA           | ESQ44396 at3g56850 | −2.71 | ABA-responsive element binding protein3 (AREB3) |
| ET            | BAJ34438 at5g25350 | 2.69 | EIN3-binding F-box protein2 (EFB2) |
| IA            | BAJ34249 at1g76690 | 2.98 | 12-oxophytodienoate reductase2 (OPR2) |
| BR            | ESQ45459 at3g50750 | −2.41 | Brassinosteroid signalling positive regulator-related |
|               | ESQ31959 at5g24150 | −2.68 | Squalene monooxygenase |
|               | O65726 at5g24150 | −2.95 | Squalene monooxygenase |

Table 4. Differentially expressed genes related to phytohormone in response to successful and unsuccessful *B. juncea*-aphid interactions.
For example, all the differentially expressed flavonoid biosynthetic genes were down-regulated in LE, but not in AC-infested sample (Table 5).

Several transcripts showing differential expression in both LE and AC data set were mapped to glucosinolate (GSL) metabolism. Our results showed that all the differentially expressed transcripts annotated for GSL biosynthesis such as branched-chain aminotransferase4 (BCAT4), cytochrome P450 79F1 (CYP79F1), cytochrome P450 83A1 (CYP83A1), UDP-glucosyl transferase 74B1 (UGT 74B1), and Sulfotransferase17 (SOT17) were down-regulated in case of LE infestation. This clearly indicated attenuating effect of L. erysimi on GSL biosynthesis leading to susceptible plant-aphid interaction. In contrary, enzymes involved in GSL breakdown such as nitrile-specifier protein 2 was up-regulated in case of both LE and AC, while thioglucoside glucohydrolase1 (TGG1) was up-regulated only in AC-infested sample (Table 5). Several hydrolases are involved in breakdown of glucosinolates into more toxic compounds related to defense. Taken together, the results show that the L. erysimi (host) infestation suppressed many of the secondary metabolite biosynthetic genes, more profoundly GSL biosynthetic genes; whereas the number of related transcripts showing activation was more in case of A. craccivora infestation.

Table 5. Differentially expressed genes related to secondary metabolite biosynthesis in B. juncea in response to infestation by L. erysimi and A. craccivora, respectively.
insect pests towards transcriptional reprogramming of host31,32. The two aphid species altered the gene-expression of B. juncea limited extent. For example, about 50% of A. craccivora compared to B. juncea A. craccivora in defense against aphid39. Activation of molecular response involved in -B. juncea infested sample (AC) indicating that more quantitative of species to plant-aphid interactions30. Overall, in plant-aphid interaction which further influence the expression of several transcripts. Further, accumulation of 14-3-3 protein was high in incompatible than compatible interaction with infecting pathogens38. Feeding fitness of the two aphid species on two different aphid species, one being successful insect-pest and the other being unsuccessful on it have been studied. In case of unsuccessful interaction, elicitation of endogenous defense and downstream metabolic changes limit the insect herbivores35. Feeding fitness of the two aphid species on B. juncea plants was assayed by quantification of honeydews based on ninhydrin staining (Fig. 1). The results showed significantly low rates of phloem diversion by A. craccivora compared to L. erysimi when released on B. juncea plants. Retarded feeding also led to low survival rates and highly inhibited fecundity of A. craccivora compared to L. erysimi. Earlier studies showed that on nonhost plants aphids do probe, survive and reproduce but only to a limited extent. For example, about 50% of A. pisum and 60% of R. padi adult aphids survived for 3 to 4 days in survival experiments on nonhost Arabidopsis plants16,17. Molecular responses of B. juncea against two of these aphid species L. erysimi (sample LE) and A. craccivora (sample AC) were assessed in terms of changes in transcriptome of B. juncea leaves at 24h post infestation with reference to key defense related pathways.

Statistical analysis of the transcriptome data identified a large number of differentially expressed genes related to plant-aphid interactions36. Overall, in L. erysimi infested sample (LE) the number of up- and down-regulated transcripts were more compared to the A. craccivora infested sample (AC) indicating that more quantitative molecular response involved in B. juncea-L.erysimi interaction. It also suggested more potential of successful insect pests towards transcriptional reprogramming of host11,24. The two aphid species altered the gene-expression of several host transcription factors (Supplementary file 6), which are the important regulators of gene-expression during various biotic and abiotic stresses33,34.Both the aphid species up-regulated the bHLH TF family transcripts such as MYCs which play an important role in JA-mediated plant defense against herbivores and pathogens34. NAC transcription factors play diverse roles in response to biotic and abiotic stresses and in growth and development35. For example, transcripts of Arabidopsis ATAF1 were increased in response to drought, high salinity, ABA, JA, wounding and Botrytis cinerea infection36. Our results also showed the involvement of NAC TF family in plant-aphid interaction which further influence the expression of several transcripts. Further, accumulation of starch plays a defensive role against aphid infestation37. Interestingly, in our data the abundance of BES1 family TF genes encoding for starch catabolism such as beta-amylases (BAM1, BAM3) were suppressed in LE sample, whereas no differentially expressed genes were observed in case of AC sample. Protein14-3-3 and e2f-dp TF genes encoding for starch catabolism such as beta-amylases (BAM1, BAM3), trypsin and protease inhibitor family protein) and four up-regulated transcripts (bHLH101, OPR2, ABCG36) infested samples when compared to uninfested control. Thus, the qRT-PCR results confirmed reliability and quality of the transcriptome data and the estimation of genes expressions based on FPKM values.

Discussion
Mustard aphid, L. erysimi is a specialized aphid species which heavily infests most of the rapeseed-mustard members including Indian mustard, B. juncea. Quantitative resistance to mustard aphid is unavailable among the cultured germplasms of rapeseed-mustard38. Mustard aphid has evolved with mechanisms for attenuating defense machinery of the host plants and rapid colonization39. In contrary, the legume specialist cowpea aphid Aphis craccivora fails to establish a rapid infestation on B. juncea which is not a natural host. In the present study, differential host response of B. juncea to two different aphid species, one being successful insect-pest and the other being unsuccessful on it have been studied. In case of unsuccessful interaction, elicitation of endogenous defense and downstream metabolic changes limit the insect herbivores35. Feeding fitness of the two aphid species on B. juncea plants was assayed by quantification of honeydews based on ninhydrin staining (Fig. 1). The results showed significantly low rates of phloem diversion by A. craccivora compared to L. erysimi when released on B. juncea plants. Retarded feeding also led to low survival rates and highly inhibited fecundity of A. craccivora compared to L. erysimi. Earlier studies showed that on nonhost plants aphids do probe, survive and reproduce but only to a limited extent. For example, about 50% of A. pisum and 60% of R. padi adult aphids survived for 3 to 4 days in survival experiments on nonhost Arabidopsis plants16,17. Molecular responses of B. juncea against two of these aphid species L. erysimi (sample LE) and A. craccivora (sample AC) were assessed in terms of changes in transcriptome of B. juncea leaves at 24h post infestation with reference to key defense related pathways.

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In Arabidopsis, Atmyb44 mutants are highly susceptible to M. persicae which indicates involvement of MYB44 in defense against aphid38. Activation of MYB44 in response to A. craccivora only, supports resistance of B. juncea to A. craccivora. Similarly, DNA-binding protein AtWRKY22 promotes susceptibility to green peach aphid M. persicae, and modulates SA and JA signalling39. Global gene expression analysis of M. persicae-infested wrky22 mutants revealed the up-regulation of genes involved in SA signalling and down-regulation of genes involved in plant growth and cell-wall loosening suggesting that WRKY22mediated susceptibility is associated with
suppression of SA-signalling. We also observed up-regulation of WRKY21 in case of L. erysimi, and WRKY19 in A. craccivora infestations. Further, the WRKY70 transcription factors an important node in the convergence between SA and JA signalling was also highly up-regulated in AC sample. One of the TFs associated with WRKY TF family is TGA transcription factors. They are important regulators of SA-induced expression of PR genes. TGAs are also implicated in the activation of JA and ET-dependent defense genes in the absence of SA signal. We identified that TGA4 and TGA7 were activated in response to A. craccivora infestation (Supplementary file 6). Thus, the results suggested involvement of several TFs in nonhost type resistance of B. juncea against A. craccivora.

ROS such as superoxide, hydrogen peroxide and singlet oxygen are generated during plant responses to both abiotic and biotic stresses. Plants concomitantly generate several ROS scavenging or detoxifying enzymes for preventing cellular damages during oxidative stresses. Expression of several ROS scavenging transcripts was affected in B. juncea in response to both the aphid species (Table 3). Down-regulation of larger number of ROS homeostasis related transcripts indicated less propensity of ROS generation in B. juncea in case of L. erysimi infestation. Similar results were also observed in cotton where infestation by cotton aphid down-regulated several ROS scavenging transcripts and in Arabidopsis, in which aphid-feeding down-regulated several H2O2 concentration modulating genes. In contrary, genes involved in oxidative signal transduction such as peroxidase and catalase were up-regulated in resistant wheat plants infested by the Russian wheat aphid.

The importance of JA pathway in defense against aphids have been demonstrated by induction of JA-dependent pathways by exogenous application of JA. Further, down-regulation of COI1 in LE sample indicated likely disruption in signal transduction process of JA signalling. EAD3 which was activated in LE sample was demonstrated to have an impact on SA- and JA-mediated defense signalling in Arabidopsis. Further, we also observed the involvement of Auxin, ABA and BR pathways during the infestation by L. erysimi and A. craccivora (Table 4). The role of auxin signalling in plant-aphid interactions is unknown. However, recently it has been shown that PIN5, an auxin transporter involved in regulating intracellular auxin homeostasis and metabolism may be involved in plant susceptibility to aphids as Arabidopsis pin5 mutants were more resistant to M. persicae and M. cerasi when compared to wild-type plants. Down-regulation of auxin homeostasis genes UGT74B1 and PIN4 by L. erysimi whereas all other auxin related transcripts were up-regulated by A. craccivora, demonstrated influence of host-defense suppression by L. erysimi on the auxin biosynthetic pathway. The role of ABA in plant-aphid interactions has been emerged from studies involving Arabidopsis mutants impaired in ABA biosynthesis or signalling genes. The M. persicae fecundity was decreased on the leaves of Arabidopsis mutants that were defective either in ABA synthesis (aba1) or in the negative regulation (abi1) of the ABA signalling pathways. The role of BR in plant immunity against bacterial pathogens and viruses was demonstrated in mutant Arabidopsis defective in BR signalling by BAK1. Further bak1 mutants of Arabidopsis supported longer survival rate of pea aphid on Arabidopsis which is a nonhost. Mustard aphid down-regulated the expression of BR signalling genes BAK1 and BRS1; however, the BR receptor BRI showed increased transcript level (Table 4). In the case of cowpea aphid, for which mustard is a nonhost, all the BR-related genes were down-regulated.

Secondary metabolites play important role in plant defense against pathogens and herbivores. The role of flavonoids in plant defense against pathogens, herbivores, and environmental stresses has been well established. Interestingly, all the transcripts coding for flavonoids biosynthesis were down-regulated in case of L. erysimi infestation which reinstates the proposition of host defense suppression in case of successful infestation. However, the lignin biosynthesis genes were up-regulated by A. craccivora, suggesting the involvement of cell wall refortification to limit its infestation on nonhost B. juncea. The biosynthetic genes of phenylalanine-derived lignins and flavonoids were also down-regulated by cabbage aphid, Brevicoryne brassicae in Arabidopsis. In chrysanthemum, enhanced expression of lignin biosynthesis genes and lignin accumulation by over-expressing ComMYB19 transcription factor resulted in limited invasion by the aphids and increased aphid tolerance of chrysanthemum. Along with other secondary metabolites the transcriptome data also indicated more profound suppression of GSL biosynthetic genes by L. erysimi (Table 5). Defensive glucosinolates abundant in Brassicaceae members are stored in specialized cells and when tissue damage occurs, they are hydrolysed by myrosinases to produce various products which are toxic and deterrents to herbivores. In LE sample, we observed down-regulation of the transcripts related to biosynthesis of aliphatic glucosinolates. Similar results were observed in Arabidopsis where infestation by aphids down-regulated GSL-metabolic genes. The transcripts of myrosinase (TGG1), involved in breakdown of glucosinolates and NSP2, involved in glucosinolate hydrolysis with the help of myrosinase were also differentially regulated by both the aphids in B. juncea. It was intriguing to note that the cowpea aphid A. craccivora had a little impact on the expression of glucosinolate biosynthesis and breakdown genes, suggesting a possible role of glucosinolate-myrosinase pathway in limiting A. craccivora from colonizing on B. juncea plants.

Conclusion
Members of Brassicaceae family including rapeseed-mustard are rich reservoir of defensive phytochemicals including glucosinolates. While these defensive metabolites are responsible for resistance to a large number of herbivores and pathogens, mustard aphids, a specialist aphid species rapidly colonize most of the rapeseed-mustard crops. Thus, it was intriguing to identify the mechanistic differences in defense activation when the B. juncea plants deter an aphid species as not being a natural host of it. Identification of important genes and pathways leading to nonhost defense vis-a-vis their counter suppression in case of susceptibility as host is likely to provide important clues for developing varietal resistance. In future perspective, the present work supplemented the limited resource of transcriptome base, much needed to validate various defense pathways and their differential regulations under host-type and nonhost-type defense response, in B. juncea.
Materials and Methods

Plant material and insect-infestations. Growing conditions of Indian mustard, *Brassica juncea* cv. Varuna and maintenance of mustard aphids were carried out as described previously by Koramutla et al. The cowpea aphid, *Aphis craccivora* was maintained on cowpea plants grown and maintained in a growth chamber, set at 24 ± 1 °C, 65–70% relative humidity and 16/8 h (light/dark) photoperiod. Four-week old *B. juncea* plants were used for aphid-infestation experiments. One hundred adult aphids on each plant were released for infestation and allowed to settle and feed on the plants. After 24 h of infestation, aphids were removed gently with the help of a paint brush, leaf samples were collected in liquid N₂ and stored at -80 °C until further analysis. Similarly, mock brushed leaves were collected from the uninfested plants as controls.

Aphid performance on *B. juncea*. To evaluate the performance of the aphid species *L. erysimi* and *A. craccivora* on the mustard plants, five adult aphids were confined on a leaf using clip-cage. Total number of nymphs produced, and survival of the adult aphids were recorded after 4 days post infestation. The experiments were performed independently on three biological and five technical replicates. The data was analyzed on Microsoft excel using one-way ANOVA, mean separations and significance were tested using Student's t-test (*p* < 0.05).

Ninhydrin staining and quantification of aphid honeydew. *B. juncea* leaves were infested with 100 individuals each of *L. erysimi* (LE) and *A. craccivora* (AC) in independent experiments. Honeydews were collected on the 3MM whatman paper discs in Petri dishes (90 × 15 mm), placed under infested *B. juncea* leaves. Similar arrangement was replicated for uninfested *B. juncea* leaves. Whatman paper discs were collected and soaked in 0.1% (w/v) ninhydrin prepared in acetone and dried at 65 °C in a hot air oven for 30 min. The whatman paper discs with purple colour spots were scanned for documentation. For quantifying the aphid honeydews, the paper discs were cut into pieces and the stains were extracted in 10 ml of 90% (v/v) methanol for 1 h with periodical shaking. The samples were centrifuged at 6000 rpm for 5 min, and the supernatants were measured at 500 nm against 90% methanol as blank.

Library construction and deep sequencing. Total RNA was extracted from leaf samples infested with aphids for 24 h using RaFlex total RNA isolation kit (GeNei, India) according to the manufacturer’s instructions. The total RNA pooled from three biological replicates were used forlibrary preparation, sequencing and unigene identification outsourced to Xcelris Labs Limited (www.xcelrisgenomics.com). The paired-end cDNA sequencing library was prepared using Illumina TruSeq RNA Library Preparation V2 kit as per manufacturer’s protocols. Briefly, mRNA was enriched and fragmented enzymatically. These short fragments were used for first and second strand cDNA synthesis, followed by end repair, A-tailing and adapter ligation, and finally to index PCR amplification of adaptor-ligated library. Library quantification and qualification was performed by using a HT DNA High Sensitivity Assay kit. The mean fragment sizes of the libraries were in the range of 450–675 bp. Finally, the library was sequenced using Illumina MiSeq/NextSeq.

Bioinformatics analyses. The raw reads obtained from the Illumina were filtered to exclude low quality reads and the reads containing adapter sequences. The resulting clean reads were assembled separately for each library with CLC Genomics Workbench (version 6.0). The assembled contigs were validated by mapping reads back to the assembled contigs. The coding sequences (CDS) were predicted from Control, LE and AC-infested assembled contigs using ORF-Predictor with default parameters. The predicted CDS were annotated by blastx against the NCBI non-redundant or The Arabidopsis Information Resource (TAIR10) protein databases with an E-value threshold of < 1 e-6. Blast2GO program was used for Gene Ontology (GO) and KEGG annotation of the CDS. The calculation of transcript expression used the FPKM method. After FPKM calculation, common hit accessions based on BLAST against non-redundant databases were identified for differential gene expression analysis. The transcripts whose log2 ratio ± 2 (four-fold change) and *P* < 0.05 between the uninfested control and aphid-infested samples were considered as differentially expressed. The differentially expressed genes (DEGs) were subjected to analysis of metabolic pathways and plant transcription factors. The MapMan application software was used to visualize the DEG involved in the metabolic pathways.

Validation of gene expression using qRT-PCR. Twelve differentially expressed genes were selected for validation using qRT-PCR. The primers were designed using IDT primer quest software (https://www.idtdna.com/). cDNA was synthesized from DNase treated total RNA (2 μg) using PrimeScript 1st strand cDNA synthesis kit (TaKaRa Bio Inc, Japan) as per the manufacturer’s instructions and diluted 20 times with nuclease free water. The qRT-PCR was performed on StepOne Plus Real-Time PCR (Applied Biosystems, USA) in a final volume of 20 μL containing 2 μL diluted cDNA, 10 μL 2X SYBR Premix Ex Taq (TaKaRa Bio Inc, Japan), 0.4 μL ROX reference dye, 0.4 μL each of forward and reverse primer (10 μM), and 6.8 μL RNase-free water as described in Koramutla et al. The thermal cycling conditions were as follows: 95 °C for 1 min followed by 40 repeated cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. Relative gene expression was determined using 2^−ΔΔCT method by normalizing to the GAPDH gene expression. The primers used for qRT-PCR validation were listed in Supplementary Table S1.

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**Author contributions**

R.B. and L.D. conceived the idea, designed the experiments and wrote the manuscript; L.D., M.K.K. conducted the experiments, collected and analysed the data; S.S., R.C. supplied experimental material and collected data.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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