Development of PR genes panel for screening aphid-tolerant cultivars in *Brassica juncea*

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Abstract The exorbitant yield loss incurred by Indian farmers every year (10–90%) in rapeseed–mustard (*Brassica juncea*) is chiefly attributed to the progressive infestation of mustard fields by *Lipaphis erysimi* (Kalt.), a major insect pest belonging to the family of Homoptera. Currently there are no successful tolerant cultivars developed by conventional means in *Brassica juncea* with systemic plant responses in the form of direct or indirect defenses against aphid attack. Lack of specific methods for screening large numbers of genotypes required in breeding for selection of tolerant cultivars in mustard is one of the main causes of slow progress in developing resistant varieties of *Brassica juncea*. Traditional phenotype-based breeding has to be augmented with recent molecular approaches for potential genotype selection and cultivar development in *Brassica juncea*. In current study a pathogen-responsive gene panel was developed which could be used for expression-assisted breeding program in mustard for selection of tolerant types against aphid infestation, minimizing the huge crop losses suffered by farmers every year.

Keywords *Brassica juncea* · *Lipaphis erysimi* · Pathogen-responsive genes · Gene expression

Introduction

Indian mustard (*B. juncea*) is the major oilseed crop of the Indian sub-continent belonging to the rapeseed–mustard group. It accounts for nearly 27% of total edible oil production of the country. India ranks third in the world and first in Asia, both in terms of acreage and production of mustard; however, India's average yield of mustard is low in comparison to other mustard-growing countries chiefly due to aphid pest causing excessive losses in the yield of the crop (Pandey et al. 2013; Rao et al. 2014).

The mustard aphid, *Lipaphis erysimi* (Kalt.), causes damage to the crop in the range of 9–96% across different agro-climatic conditions of India (Hasan and Singh 2011). Patel et al. (2004) reported *Lipaphis erysimi* as the most serious pest affecting mustard crop and causing yield loss in the range of 35.4–91.3% under different thermal environments in eastern central India. Kular and Kumar (2011) conducted field studies to assess the losses in seed yield of different *Brassica* species (*B. juncea*, *B. napus*, *B. carinata*, *B. rapa* and *Eruca sativa*) due to infestation of insect pests. The loss in seed yield, due to mustard aphid and cabbage caterpillar, varied from 6.5 to 26.4%. Bakhietia (1984) observed a 57.8–80.6% yield loss due to mustard aphid. In Haryana, Singh et al. (1993) reported 38.20–46.56% yield losses in susceptible cultivars under unprotected conditions.

The nymph as well as the adult form of *L. erysimi* covers the entire surface of flower buds, shoots and pods in large numbers and feeds on different parts of the plants by sucking the cell sap (Hasan and Singh 2011). Malik and Deen (1998) observed that infestation of *L. erysimi* on *B. juncea* causes reduction in plant height, number of branches per plant, siliqua per plant, grains per siliqua, seed yield, oil content and oil yield. The aphid damages the crop

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from the seedling stage to the maturity stage. *L. erysimi* was also found infesting the roots at depths of 2–15 cm. Infestation of 38–63 aphids on the roots of individual plants caused 42% plant mortality in the first week of January (Singh and Singh 1988). Singh et al. (1983) also reported reduction in yield of Indian mustard by 1.5 kg/ha for each increase of 1 aphid beyond 25 aphids/plant in Punjab. Weather plays an important role in the appearance, multiplication and disappearance of mustard aphid (Kumar et al. 1999; Vekaria and Patel 2000). A large number of field and laboratory studies indicate that insect abundance increases with rising temperature (Cannon 1998; Bale et al. 2002). In warm humid climate, the time to attain peak population was relatively shorter than in cool climate (Rao et al. 2014).

Plant traits which help in basal defenses against aphid infestation are cell wall modifications, proteins or secondary metabolites that have antixenotic antibiotic properties and plant volatiles that repel aphids or attract their natural enemies. Aphids also induce transcripts associated with plant hormones known to modulate disease resistance, including jasmonic acid (JA), salicylic acid (SA), ethylene, abscisic acid (ABA) and gibberellic acid (Goggin 2007; Wang et al. 2009).

Taj et al. (2011) elucidated co-expression and *in silico* interaction studies for interlinking the activation of *MAPK3* and *LOX* genes during the pathogenesis of *Alternaria brassicae* in *Brassica juncea*. The study suggested that the induction of JA biosynthesis required *MAPK3* activation and pointed out that interactions of *MAPK3* genes with *LOX* and its proposed role in biosynthesis of JA/JA induced the expression of defense genes in mustard. Bhardwaj et al. (2015) carried out a transcriptomic study in *Brassica juncea* cultivar Varuna to detect genome-wide changes in its transcriptome against high temperature or drought stress. The study revealed that the maximum number of upregulated transcription factors in high temperature and drought stress belonged to heat shock factors (*HSFs*) and dehydration-responsive element-binding (*DREB*) families, respectively. Bandopadhyay et al. (2013) performed a transcriptomic study to identify the genes involved in resistant response in wild crucifer *Rorippa indica* during *L. erysimi* infestation. Plant disease resistance involves the SA-dependent signaling which is effective against biotrophs and JA/ET-dependent signaling against necrotrophs. Aphids use decoy defense strategy by induction of less effective SA-dependent defense response and suppression of JA-dependent pathways, but incompatible interaction studies revealed JA dominance in later phase in *R. indica* leading to canceling of decoy trap and effective resistance.

In the present study, 14 candidate genes were selected for the development of PR genes panel to estimate the intensity of aphid infestation in *B. juncea*. The expression

level of 14 candidate genes in different samples of *B. juncea* having varying levels of aphid infestation were analyzed by real-time PCR.

Materials and methods

Sample collection

Aphid-infested plant parts of GM 3 variety of *Brassica juncea* were collected from a field in Pethapur (23°16.676N and 72°39.664E), Gandhinagar, Gujarat. Pod, stem and leaf samples having varying level of aphid infestation and control samples were collected and the number of aphids per centimeter of plant part was counted for pod, stem and leaf samples (Prasad et al. 1983).

Aphid identification through sequencing

DNA was extracted from aphid using DNeasy Blood and Tissue Kit (Qiagen) and quantitated on QIAxpert (Qiagen). PCR amplification was carried out in 20 µl volume using ReadyMix™ Taq PCR Reaction Mix (Sigma), 10 µM of LCO_1490 (GGTCAACAAATCATAAAGATATTGG) and HCO_2198 (TAAACTTCAGGGTGACCAAAAAATCA) primers and 50 ng of extracted DNA. PCR was performed under the following conditions: 2 min at 94 °C; five cycles of 1 min at 94 °C, 1.5 min at 45 °C and 1.5 min at 72 °C; 35 cycles of 1 min at 94 °C, 1.5 min at 50 °C and 1 min at 72 °C and a final cycle of 5 min at 72 °C. End point analysis of PCR-amplified product was done on 2% agarose gel. The 658 bp product was purified using Exosap (Affymetrix) and sequenced using LCO_1490 and HCO_2198 primers using 3500XL Genetic Analyzer (Applied Biosystems), with BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

RNA extraction and cDNA preparation

The samples were placed in RNAlater tubes to capture and preserve all the transcripts during aphid infestation in field. RNA was isolated from control and aphid-infested leaf, pod and stem samples using RNeasy Plant Mini Kit (Qiagen). The extracted RNA was quantified on QIAxpert (Qiagen) and 300 ng was used as the final concentration for the preparation of cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems).

Selection of genes for development of PR genes panel and qPCR

All the primers used in the study for the development of PR genes panel were selected from the works of Taj et al. (2011),

Table 1 List of genes selected for PR panel development

Sr. No.	Gene	Gene name	Function	References
1	<i>AP2</i>	Apetala2	Nuclear transcription factor, and controls flower and seed development	Sun et al. (2012)
2	<i>TIR1</i>	Transport inhibitor response 1	Ubiquitination of transcription repressors AUX/IAA, promoting expression of auxin repressor gene	Sun et al. (2012)
3	<i>SNAP</i>	Senescence-associated protein	Induced during senescence and pathogen infections	Sun et al. (2012), Espinoza et al. (2007)
4	<i>MAPK</i>	Mitogen-activated protein kinase	Plant MAPK cascades regulate stress and hormonal responses, innate immunity, and developmental programs	Rodriguez et al. (2010), Taj et al. (2011)
5	<i>GP3</i>	Germin-like protein	Zygotic and somatic embryogenesis, salt stress, pathogen elicitation, heavy metal stress	Sun et al. (2012)
6	<i>PRH43</i>	3'-phosphoadenosine-5'-phosphosulfate reductase homolog	Involved in sulfate assimilation	Sun et al. (2012)
7	<i>DREB2B</i>	Dehydration-responsive element-binding proteins	Induces the expression of cold-responsive gene, which leads to enhanced cold/frost tolerance in plants; highly expressed during drought stress	Bhardwaj et al. (2015)
8	<i>60S</i>	60S ribosomal subunit	Protein synthesis	Sun et al. (2012)
9	<i>HSP101</i>	Heat shock protein 101	Involved in refolding of proteins which form aggregates under heat stress	Bhardwaj et al. (2015)
10	<i>LEA1</i>	Late embryogenesis abundant proteins	Regulation of plastid organization and biosynthesis, expressed during high temperature and drought conditions	Bhardwaj et al. (2015)
11	<i>SAC9</i>	SAC-domain containing protein	SAC9 phosphatase is involved in modulating phosphoinositide signals during stress response	Sun et al. (2012)
12	<i>LOX</i>	Lipoxygenase	Enzyme of JA pathway associated with A number of developmental stages, mobilization of lipids during germination, defense against pest, antimicrobial activity	Taj et al. (2011)
13	<i>RRTF</i>	Ethylene-responsive transcription factor 109	Mediates cross talk between JA signaling and auxin biosynthesis to regulate lateral root formation	Sun et al. (2012)
14	<i>HSFA7A1</i>	Heat shock factor A7A1	Highly expressed during high temperature stress conditions	Bhardwaj et al. (2015)
15	<i>ACT3</i>	Actin	Ubiquitous component of plant cytoskeleton. Involved in cell division, plane localization, cell elongation and cell shape determination	Sun et al. (2012)

Bhardwaj et al. (2015) and Sun et al. (2012) (Table 1). Primer sequences of all the genes used in the panel development study are furnished in Table 2. Real-time PCR was performed to quantify the fold-level change in all the genes during aphid infestation. The cycling condition was set as per the manufacturer's protocol mentioned in the Power SYBR Master Mix kit (Applied Biosystems).

Relative quantification

Analysis for the relative quantification data for all the targets was done using DataAssist bioinformatics tool. DataAssist™ software utilizes the comparative CT ($\Delta\Delta CT$) method (Livak and Schmittgen 2008) to quantitate relative gene expression across a number of genes and samples. DataAssist platform was used to create the RQ plot of 14 candidate genes. RQ is the fold change in

expression of a gene in a sample compared to the calibrator sample. The calibrator has an RQ value of 1. For graphical representation, log 10 value of fold change was plotted against the sample. Heat map genes were hierarchically clustered by Pearson correlation coefficient using complete linkage. Box plot displays the overall range of CT distribution for each sample from all genes in the experiment. Different samples give the average CT value, minimum CT value and maximum CT value of the sample corresponding to its respective gene.

Analysis for PR genes panel elucidation

Selection of genes for PR gene panel from 14 candidate genes was carried out based on genes registering maximum representation in various sample types. Heat map graphically displays the results by hierarchical clustering. Distances

Table 2 Primer sequences of genes selected for PR gene panel development

Sr. No.	Gene	Primer sequence	
1	<i>AP2</i>	F	GATTCGCCGATTCTTGAT
		R	TCTGCTTCTTTCCTGGTCG
2	<i>DREB2B</i>	F	GCTCCAGATCAAGAGGATG
		R	CGAGTCAGTCTGAGGTG
3	<i>LEA 1</i>	F	GCATGCTGAGGTCAGC
		R	CCAGCTTCATGCTGCG
4	<i>LOX</i>	F	AGAAATGCTTGCTGGGCTAA
		R	GCTCAGTGTCGTTCTGGACA
5	<i>MAPK3</i>	F	CGTACACTTCGCGAGATCAA
		R	ATTCATTGCGTTGCTGCTCT
6	<i>HSP101</i>	F	CAAGGATATCTACATACCTCGTAAC
		R	CATAGCCATCCAAATGATACTTTG
7	<i>HSFA7A1</i>	F	CTGTCCCAAGAGAAACCCCTC
		R	GTCGCTGCGGATTTCATTC
8	<i>TIR1</i>	F	AAGGCTCAGACGGAACA
		R	TGCTGGTCTTGAGGTGCT
9	<i>GP3</i>	F	CAGCGAGTCTTGATTGTC
		R	CGATTTCCGTCTTGGTCT
10	<i>PRH43</i>	F	GAAGTGGAACCCCTTGGC
		R	CATCCATCGTCCCAAGAA
11	<i>SAC9</i>	F	GCCAATCTGCCTCCAGTTC
		R	GATACGTCAAGTCTTCACTGC
12	<i>SNAP</i>	F	GCGGTATACCTGTAAACGATAACG
		R	CCACAAGCCAGTTATCCCTGTG
13	<i>RRTF</i>	F	TTACAGCCTAAACAACCATC
		R	TTCTCCAGCAATAGCACAG
14	<i>60S</i>	F	CAGTGATCTTCACAGCCTTCT
		R	TTATGAATCTTCCCAGCAACT
15	<i>ACT3</i>	F	GGCTACTCTTTCACCACGAC
		R	GGATACCAGCATTCTCCATAC

between samples and assays are calculated for hierarchical clustering based on the ΔCT values using Pearson's correlation distance. Heat map portrays the expression of the 14 pathogen-responsive genes; the color scale illustrates the relative expression level of pathogen-responsive genes, representing ΔCT . Green indicates genes with relatively decreased levels of expression, whereas red indicates genes with relatively increased levels of expression.

Results

Aphid infestation load

Aphid load was estimated by counting the number of aphids per centimeter of plant part (Prasad et al. 1983). Aphid infestation load is furnished in Table 3.

Sequencing of mustard aphid

The *COI* gene of mustard aphid was amplified and sequenced. The sequence analysis using nucleotide BLAST revealed the most identical homology match with two sequences (Table 4): *Lipaphis pseudobrassicae* with accession number EU701711.1 and *Lipaphis erysimi* with accession number KP189473.1.

Gene expression of PR genes

Compared to the control leaf (L1) sample in L2 sample, *60S*, *AP2*, *DREB2B*, *MAPK*, *GP3* and *TIR1* genes were upregulated in the fold range between 2.46 and 36.01, and *HSFA7A1*, *LEA1*, *LOX*, *PRH43*, *RRTF*, *SAC9*, *SNAP* and *HSP101* genes were downregulated in the fold range between 0.00 and 0.57. In the L3 sample compared to the

Table 3 Aphid load in different plant parts

Sr. No.	Sample type	Sample code	Aphid/cm	Average
1	Stem	S1	0	41
		S2	14	
		S3	30	
		S4	80	
2	Pod	P1	0	25
		P2	15	
		P3	20	
		P4	40	
3	Leaf	L1	0	40
		L2	10	
		L3	40	
		L4	70	

Table 4 BlastN results which hold homology with the sequenced amplicon

Sr. No.	Accession	Description	Total score	Query coverage (%)	E value	Max ident (%)
1	EU701711.1	<i>Lipaphis pseudobrassicae</i>	1109	93	0	97
2	KP189473.1	<i>Lipaphis erysimi</i>	1098	93	0	96

control L1, *AP2*, *GP3*, *LOX*, *MAPK*, *SNAP* and *TIR1* genes were upregulated in the fold range between 1.2 and 363.06, while *HSFA7A1*, *60S*, *DREB2B*, *LEA1*, *PRH43*, *RRTF*, *SAC9* and *HSP101* genes were downregulated in the fold range between 0.00 and 0.4. In the L4 sample compared to the control, L1, *AP2*, *GP3*, *LOX*, *MAPK*, *PRH43* and *TIR1* genes were upregulated in the fold range between 3.73 and 91.23, while *HSFA7A1*, *60S*, *DREB2B*, *LEA1*, *SNAP*, *RRTF*, *SAC9* and *HSP101* genes were downregulated in the fold range between 0.01 and 0.39.

Compared to the control stem (S1) in the S2 sample, *AP2*, *DREB2B*, *HSFA7A1*, *HSP101*, *LEA1*, *MAPK*, *PRH43*, *RRTF*, *SAC9*, *SNAP* and *TIR1* genes were upregulated in the fold range between 1.23 and 655.94, while *60S*, *GP3* and *LOX* genes were downregulated in the fold range between 0.15 and 0.84. In the S3 sample compared to the control S1 sample, *AP2*, *MAPK* and *SNAP* genes were upregulated in the range of 1.09–82.61, while *DREB2B*, *HSFA7A1*, *HSP101*, *LEA1*, *PRH43*, *RRTF*, *SAC9* and *TIR1* genes were downregulated in the fold range between 0.00 and 0.94. In the S4 sample compared to the S1 sample, *AP2*, *MAPK*, *SNAP* and *TIR1* genes were upregulated in the fold range between 1.47 and 13.57, while *DREB2B*, *HSFA7A1*, *HSP101*, *LEA1*, *PRH43*, *RRTF* and *SAC9* genes were downregulated in the fold range between 0.00 and 0.97.

Compared to the control pod (P1) sample in the P2 sample, *60S*, *AP2*, *DREB2B*, *GP3*, *MAPK*, *PRH43*, *RRTF*, *SAC9*, *SNAP*, *TIR1*, *HSP101* and *LEA1* genes were upregulated in the fold range between 1.15 and 41.23, while *HSFA7A1* and *LOX* genes were downregulated in the

fold range between 0.07 and 0.21. In the P3 sample compared to the P1 sample, *60S*, *AP2*, *DREB2B*, *GP3*, *PRH43*, *SAC9*, *SNAP*, *TIR1*, *HSP101* and *LEA1* genes were upregulated in the fold range between 1.3 and 43.11, while *HSFA7A1*, *LOX*, *MAPK* and *RRTF* genes were downregulated in the fold range between 0.06 and 0.65. In the P4 sample compared to the control P1 sample, *AP2*, *GP3*, *PRH43*, *SNAP* and *TIR1* genes were upregulated in the fold range between 1.25 and 70.82, while *60S*, *DREB2B*, *HSFA7A1*, *HSP101*, *LEA1*, *LOX*, *MAPK*, *RRTF* and *SAC9* genes were downregulated in the fold range between 0.15 and 0.97 (Table 5; Figs. 1, 2, 3).

PR gene panel elucidation

Cluster analysis using heat map

Based on ΔCT value assessed in an assay-centric fashion, 14 genes were divided into two major nodes. In the first major node, there were six sub-nodes which include genes *PRH43*, *RRTF*, *LEA1*, *SAC9*, *HSFA7A1* and *HSP101*. The second major node encompassed nine sub-nodes including genes *LOX*, *MAPK*, *SNAP*, *60S*, *DREB2B*, *TIR1*, *ACT3*, *AP2* and *GP3*. Genes *GP3*, *AP2* and *TIR1*, *PRH43* capture the differential expression pattern ranging from higher fold levels (red coloration) to lower fold levels (green coloration), making them best suited for PR gene panel development. *MAPK*, *DREB2B* (green gradient) and *SNAP* (red gradient) show gradient pattern in coloration, allowing them to capture the differential response of plant tissues during aphid infestation (Fig. 4).

Table 5 Fold change expression levels of pathogen-responsive genes

Assay	L2	L3	L4	S2	S3	S4	P2	P3	P4
<i>60S</i>	2.73	0.09	0.39	0.15	0.02	0.67	10.89	7.54	0.97
<i>AP2</i>	2.62	1.20	15.52	1.99	1.09	1.47	13.42	1.62	2.98
<i>DREB2B</i>	2.73	0.17	0.22	1.23	0.56	0.53	41.23	28.61	0.37
<i>GP3</i>	22.93	1.34	41.49	0.30	0.94	0.97	4.54	3.53	5.17
<i>HSFA7A1</i>	0.03	0.40	0.07	151.61	0.10	0.03	0.21	0.20	0.25
<i>HSP101</i>	0.00	0.03	0.37	74.75	0.84	0.03	1.15	1.76	0.50
<i>LEA1</i>	0.02	0.04	0.05	655.94	0.63	0.21	6.96	2.18	0.37
<i>LOX</i>	0.57	4.10	9.30	0.84	0.05	0.02	0.07	0.17	0.37
<i>MAPK</i>	2.46	3.06	6.30	469.35	9.55	2.09	4.47	0.65	0.15
<i>PRH43</i>	0.00	0.00	3.73	90.05	0.00	0.72	27.23	43.11	70.82
<i>RRTF</i>	0.00	0.06	0.01	28.03	0.02	0.10	1.59	0.06	0.18
<i>SAC9</i>	0.07	0.05	0.01	28.56	0.04	0.00	2.31	1.30	0.21
<i>SNAP</i>	0.30	3.27	0.34	170.98	82.61	13.57	3.96	1.52	1.25
<i>TIR1</i>	36.01	363.06	91.23	14.50	0.22	6.71	3.85	3.76	3.00

Fig. 1 RQ plot of 14 PR genes for leaf samples having varying levels of aphid infestation

Selection of genes for the development of PR gene panel

Selection of genes for the development of PR gene panel was done based on their expression in aphid-infested and control samples. Among the upregulated genes, *AP2*, *TIR1*, *SNAP*, *MAPK*, *GP3*, *PRH43* and *DREB2B* are the best candidate genes for the development of the PR gene panel, as these genes are expressed in most of the sample tissues (Table 6).

Discussion

Aphids reproduce at a higher rate during the early vegetative stage of mustard plants when the developmental period is shortest and the production of winged morphs is lowest. The nymphs and adults cause damage by sucking away the plant sap, often covering the entire surface of the shoots, floral buds and pods. The insect pests cause enormous yield and economic losses in *Brassica* crop

Fig. 2 RQ plot of 14 PR genes for stem samples having varying levels of aphid infestation

Fig. 3 RQ plot of 14 PR genes for pod samples having varying levels of aphid infestation

production every year, and are a threat to global agriculture. Several studies have shown that weather plays an important role in the appearance; multiplication and disappearance of mustard aphid (Kumar et al. 1999; Vekaria and Patel 2000; Hossain et al. 2006) and a large number of field and laboratory studies indicate that insect abundance increases with rising temperature (Cannon 1998; Bale et al.

2002). The effect of global warming on plant insect interaction is projected as the reason for increase in herbivore pressure on plant (Coley 1998). In the present investigation, the aphid infestation load in leaf, stem and pod was enumerated to be greater than 25 numbers/cm of plant body, signifying high infestation of pest on mustard plants (Singh et al. 1983).

Fig. 4 Heat map analysis of 14 PR genes in assay-centric fashion

Table 6 Pathogen-reponsive gene panel ranking

Sr. No.	Assay	Upregulated sample number	Ranking
1	AP2	9.0	1
2	T1R1	8.0	2
3	MAPK	7.0	3
4	SNAP	7.0	3
5	GP3	6.0	4
6	PRH43	5.0	5
7	DREB2B	4.0	6
8	60S	3.0	7
9	HSP101	3.0	7
10	LEA1	3.0	7
11	SAC9	3.0	7
12	LOX	2.0	8
13	RRTF	2.0	8
14	HSFA7A1	1.0	9

The present methods of aphid control in mustard are primarily based on synthetic chemical insecticides. These chemicals, besides aggravating environmental pollution, can also be toxic to friendly insects. A resistant cultivar is always a more sustainable and environment-friendly option for managing insect pests. Hence, development of an insect-resistant cultivar having a heritable and transferable resistance is the most sustainable option (Stoner and Shelton 1988; Ahuja et al. 2010). The development of resistant cultivar through breeding programs for insect resistance is conducted mainly by first surveying for possible sources of insect resistance (antixenosis, antibiosis, tolerance) in varieties and strains

locally available and hybridization to combine genes for resistance with desirable agronomic characters followed by screening for resistance characters in hybrid progenies (Warwick 2011; Smith and Chuang 2014). The evaluation of resistance is mainly carried out using parameters such as seedling survival, aphid injury to plants, aphid infestation, aphid fecundity and yield evaluation (Kavitha and Reddy 2012). These screening parameters are carried out in field and are not economical in time and effort. Hence, fast and more accurate detection methods for measuring the basal defense responses in breeding progenies has to be used in combination with visual parameters of screening for resistance in hybrid breeding programs. In the present study, expression of 14 defense-responsive genes assimilated from studies of Taj et al. (2011), Bhardwaj et al. (2015) and Sun et al. (2012) was quantified in stem, leaf and pod sections of GM 3 variety of mustard (*Brassica juncea*) sampled from an aphid-infested field located at Pethapur, Gandhinagar, Gujarat. Non-infested stem, leaf and pod sections were taken as calibrator control samples during relative quantitation study with *ACT3* gene as the endogenous control.

Goggin (2007) implicated plant traits involved in basal defenses such as cell wall modifications, proteins or secondary metabolites that have antixenotic or antibiotic properties and plant volatiles that repel aphids or attract their natural enemies. Aphids also induce transcripts associated with plant hormones known to modulate disease resistance, including jasmonic acid (JA), salicylic acid (SA), ethylene, abscisic acid (ABA), auxins (AUX) and gibberellic acid. Bandopadhyay et al. (2013) elucidated some pathogen-responsive genes involved during wild crucifer *Rorippa indica* resistance response on infestation of mustard with aphid *Lipaphis erysimi*. The time course relative expression level profiles of 24 genes induced in *R. indica* on forced infestation with *L. erysimi* at different time points, viz. 6, 12, 24, 48 h post-infestation were analyzed by quantitative real-time analysis. Candidates comprising the largest group of genes during aphid-induced defense signaling were that of SA-dependent signaling, which is effective against biotrophs and JA/ET-dependent signaling against necrotrophs. In the present study, genes involved in the above-mentioned pathways were included for the development of the PR gene panel for capturing the role of phytohormonal cross talk in plant defense signaling during aphid attack.

The main aim of the study was to elucidate a fast and accurate PR gene panel which could be used for molecular screening of commercial, wild relatives and mutant lines of mustard plant in breeding programs. The development of aphid-tolerant mustard plants is better option than developing plants with high levels of antibiosis resistance, as the latter will promote the development of aphid virulence. Hence in the present investigation, major focus was given to genes involved in basal defense mechanisms. Based on the results of the

Fig. 5 Candidate PR genes showing up/downregulation and downregulation in *Brassica juncea*

relative expression stipulated in the RQ plot, heat map and ranking of genes in different sample tissues AP2, TIR1, SNAP, MAPK, PRH43 and DREB2B were constantly found upregulated. The final panel of six PR genes also signifies a combinatorial effect of biotic stress and abiotic stress prevalent in the field. The aphid count analysis also showed the highest infestation levels in the stem and leaf followed by pod. An interesting observation made during the study was even when the aphid load was less in the pod section of the mustard plant, the number of upregulated genes in the pod was highest compared to the stem section which had the highest infestation of aphid. This reveals that defense mechanisms are in their prime functionality in pod samples, as they constitute the most important parts of mustard plants (Fig. 5). Aphid virulence in newly developed mustard hybrids can also be derived by correlating the upregulation of genes to the intensity of aphid attack which can be quantified in a seasonal study.

Conclusion

The present study is the first attempt in Gujarat to quantify selected genes of biotic and abiotic stress defense pathways in mustard plant during aphid infestation. The study also further establishes the reason for huge loss incurred by the

farmers while growing GM 3 variety of mustard in the field, as it shows low resistance to aphid attack in Gujarat. The study also points to the urgent need for development of tolerant varieties of mustard to combat aphid attack and seasonal screening of developed varieties using PR gene panel to check the development of aphid virulence. The PR gene panel explicated in the present study could be used to screen commercially available accessions of mustard along with land races via sick plot studies to select tolerant types, leading to the expression-assisted breeding of mustard, tolerant to aphid infestation, thus minimizing the huge crop losses incurred by farmers every year.

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Compliance with ethical standards

Conflict of interest The author(s) declare no competing financial interests and there is no conflict of interest.

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