



Transcriptomic dissection of cross kingdom crosstalk in *Brassica juncea*-*Sclerotinia sclerotiorum* pathosystem

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Abstract

Sclerotinia sclerotiorum is a highly destructive phytopathogen with a broad host range. It causes severe productivity losses in food and vegetable crops worldwide. Understanding host-pathogen interactions is essential for developing effective control strategies. In this study, we investigated transcriptomic changes in stem lesions caused by the virulent isolate PAU (SsLd1-White), of *S. sclerotiorum* on resistant and susceptible pools derived from *Brassica juncea* advanced generation introgression lines, 24 hours post-inoculation (hpi), alongside controls. Gene Ontology enrichment and KEGG pathway analyses helped to characterize transcriptional dynamics between the two pools. Comparative transcriptome analysis revealed 3,627 and 10,524 differentially expressed genes (DEGs) uniquely expressed in the resistant and susceptible pools, respectively, along with 1,807 DEGs commonly expressed in both pools at 24hpi. Several DEGs were associated with key physiological and metabolic processes, including response to wounding, systemic acquired resistance, oxidative and osmotic stress, fungal defense, signal transduction, and jasmonic acid signaling. The study provides novel insights into the molecular basis of the *B. juncea*-*S. Sclerotiorum* pathosystem, contributing to a deeper understanding of host-pathogen interactions and informing future resistance breeding and management strategies.

Keywords: *Brassica juncea*, differentially expressed genes, *Sclerotinia sclerotiorum*, resistance, RNA-sequencing

Introduction

S. sclerotiorum is a highly destructive necrotrophic phytopathogen with a broad host range, causing severe losses in a wide range of economically important crops (Bolton *et al.*, 2006, Saharan and Mehta 2008, Liang and Rollins 2018, Derbyshire *et al.*, 2022, Mehta *et al.*, 2023). Despite extensive research on its pathogenicity, the infection mechanisms employed by this fungus remain complex and not fully understood. Furthermore, the formation of long-lived sclerotia enables the pathogen to persist in soil over long periods, making disease management particularly challenging (Willetts and Wong 1980, Zhu *et al.*, 2013, Derbyshire and Denton-Giles 2016). As a result, current control strategies are often inadequate, highlighting the need for novel and effective approaches to reduce the impact of *S. sclerotiorum*. Thus, a deeper understanding of host-pathogen interactions is critical for developing improved management strategies for Sclerotinia stem rot disease.

S. sclerotiorum deploys an array of pathogenicity mechanisms, including the secretion of plant cell wall-

degrading enzymes (PCWDEs), poly-galacturonases, cutinase, proteases, oxalic acid (OA), and effectors, to destroy host cells and manipulate the host's cellular processes (Li *et al.*, 2004, Yajima *et al.*, 2009, Amselem *et al.*, 2011, Bashi *et al.*, 2012, Liang and Rollins 2018, Ding *et al.*, 2019, Chittem *et al.*, 2020). Recent research has increasingly elucidated the functional characterization of genes and their roles in the pathogenesis and/or virulence of *S. sclerotiorum* (Rollins 2003, Yarden *et al.*, 2014, Seifbarghi *et al.*, 2017, Yu *et al.*, 2017, Yang *et al.*, 2018, Wang *et al.*, 2019, Rana *et al.*, 2021, 2022, Chittem *et al.*, 2020, Hossain *et al.*, 2023). For instance, Chittem *et al.*, (2020) highlighted that peroxisome-related processes, cell wall degradation, and detoxification of host-derived metabolites are central to the pathogenicity of *S. sclerotiorum* in *B. napus*. Building on this, Sarfraz *et al.*, (2026) employed integrative transcriptomic analysis across resistant and susceptible *B. napus* cultivars, uncovering 1,176 differentially alternatively spliced (DAS) genes between infection-induced and cultivar-specific transcriptome groups. Marked induction of genes such as *SEC14*-like lipid transfer protein (SFH8), *FKBP17*, and

transcription factors including *HCA2*, *VAL2*, and *WRI4* was indicative of their role in immune signaling and hormonal pathways. Collectively, these findings underscore the complex interplay of metabolic regulation, alternative splicing, and transcriptional control in shaping host resistance and pathogen adaptation.

We had previously identified sources of resistance to *S. sclerotiorum* in wild crucifers such as *B. fruticulosa* and *Erucastrum cardaminoides*, and developed *B. juncea*-*B. fruticulosa* introgression lines through interspecific hybridization, followed by backcrossing, selfing for seven generations, an additional generation of crossing with commercial checks, and three subsequent cycles of selfing resulting in the development of tertiary introgression lines ($F_1 \rightarrow BC_1 \rightarrow S_7 \rightarrow BC_2 \rightarrow S_4$). In this study, resistant (R) and susceptible (S) sets of these tertiary introgression lines were challenged with a highly virulent isolate of *S. sclerotiorum* (Ss-white), followed by RNA sequencing at 24hpi. Transcriptome analysis revealed distinct transcriptional dynamics between the two pools, with GO enrichment and KEGG pointing to several DEGs involved in key physiological and metabolic pathways. Importantly, a subset of candidate genes emerged as putative contributors to pathogenicity in *B. juncea*-*B. fruticulosa* introgression lines. These findings provide valuable insights into the molecular basis of host-pathogen interactions and highlight the potential of advanced approaches such as RNAi, and host-induced gene silencing (HIGS) to enhance resistance against *S. sclerotiorum*.

Materials and Methods

Plant materials and fungal strain

In the previous study (Atri *et al.*, unpublished data), the stem resistance against *S. sclerotiorum* was evaluated in population comprised of 224 lines ($F_1 \rightarrow BC_1 \rightarrow S_7 \rightarrow BC_2 \rightarrow S_4$). These lines were developed by backcrossing an initial interspecific hybrid (*Brassica fruticulosa* × *B. juncea* cv. RLC 1), followed by multiple generations of selfing to stabilize traits while retaining donor alleles. Subsequently, selected advanced generation introgression lines were hybridized with another mustard genotype, PBR 357, and advanced through three successive generations using the single seed descent method. Overall, this approach aimed to stabilize resistance-related genes within a superior agronomic background, while reducing linkage drag and progressively enhancing the recurrent parent genome. The developed breeding materials were sown in a randomized complete block design with two replications and repeated evaluated for three consecutive season's

viz. 2020-21, 2021-22, and 2022-23. The relative resistance responses across ILs were evaluated using a modified stem inoculation method (Buchwaldt *et al.* 2005, Bradly *et al.* 2006). Based on the phenotypic data, three resistant *B. juncea* introgression lines IL-R1 (ADJR8*K-57), IL-R2 (ADR16*PBR-357), and IL-R3 (A-825*K-57), having a consistent lesion length (0-<2.5cm) and susceptible *B. juncea* cv. RLC-1, Giriraj and PBR-357 were selected for transcriptomic analysis. The most virulent fungal strain of *S. sclerotiorum* viz.: PAU (Ss-White), was used for pathogenicity assays.

Disease inoculation and tissue sampling

The sclerotia of *S. sclerotiorum* isolate Ss-white were collected from the Punjab Agricultural University (PAU), Ludhiana, India. They were surface-sterilized in 50% (v/v) sodium hypochlorite and 70% ethanol for 4 minutes, followed by two washes in sterile distilled water for 1 minute (Clarkson *et al.*, 2003, Li *et al.*, 2006). The isolates were cultured on potato dextrose agar medium (potato infusion 200g, dextrose 20g, agar 20g, H, O 1L, pH 5.6 ± 0.2) at 20°C under a 12h light/12h dark cycle (Clarkson *et al.*, 2003). Daughter sclerotia were harvested from the incubated plates after four weeks and maintained at 4°C for subsequent inoculations. Plants were grown in pots placed in growth chambers at 22°C under light (fluorescent lamp, 100 lux) and 16°C in the dark. For inoculation, selected *B. juncea* ILs were grown at 25°C with a photoperiod of 10h light/14h dark and used for infection assays. At the end of flowering, three plants from each line were inoculated with 6mm mycelial plugs obtained from actively growing two-day-old *S. sclerotiorum* colonies, using a 1ml pipette tip following a modified stem inoculation method (Buchwaldt *et al.*, 2005, Li *et al.*, 2006, Rana *et al.*, 2017). The inoculated stem area, including lesions, was harvested at 24hpi, flash-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Stem tissues collected from three individual plants across three ILs were pooled to constitute one biological replicate, yielding four samples: R-water, R-white, S-water, and S-white. Statistical analyses were performed using SAS software (SAS Institute Inc., 2013).

RNA extraction, library construction and Illumina sequencing

Total RNA was extracted from pooled inoculated stem samples collected at 24 hpi from three individual plants in each experiment, using the TRIzol reagent kit (Rio *et al.*, 2010) according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The total RNA quality was assessed using Qubit RNA HS Assay (Invitrogen) for concentration estimation

and Agilent TapeStation RNA Screen Tape (Agilent Technologies, USA) for integrity measurement. Samples with adequate RNA integrity (RIN ≥ 7) were taken forward for cDNA synthesis. For each sample, 200-500 ng of total RNA was used for library preparation. Libraries were prepared using the NEBNext Ultra II Directional RNA Library Preparation Kit for Illumina (New England Biolabs, Ipswich, MA, USA), following the manufacturer's recommended protocol. Sequencing was performed using 150bp paired-end chemistry on the Illumina NovaSeq 6000 platform. Library preparation and sequencing were carried out by a commercial service provider (NGB Diagnostics Pvt. Ltd., Noida, India).

Briefly, mRNA was enriched using poly-A selection, followed by fragmentation to achieve an insert size of approximately 300-400bp. Fragmented RNA underwent first- and second-strand cDNA synthesis, after which the double-stranded cDNA was end-repaired, A-tailed, and ligated with indexed Illumina adapters. The ligated products were purified and enriched through PCR to generate the final RNA-Seq libraries. Library quantification was performed using a Qubit Fluorometer (Invitrogen, Life Technologies, Grand Island, NY, USA), and library size distribution was assessed using the High Sensitivity D5000 kit on an Agilent TapeStation. The indexed RNA libraries were pooled in equimolar ratios and loaded onto the Illumina NovaSeq 6000 system, where sequencing was conducted using 2 $\times$ 150bp paired-end chemistry. Post-sequencing, samples were demultiplexed and indexed adapter sequences were trimmed using CASAVA v1.8.2 software (Illumina Inc.).

Mapping of reads to the reference genome and differentially expressed genes (DEGs)

The paired-end RNA-Seq reads from four libraries (two each from R and S-pools, stems inoculated with *S. sclerotiorum*) were processed using Trimmomatic v0.39 (Bolger *et al.*, 2014) to remove adaptor sequences, duplicates, empty reads, and low-quality sequences, with the parameters: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 SLIDINGWINDOW:4:15 TRAILING:3 MINLEN:40. The clean reads were retained in FASTQ format. High-quality reads were then mapped to the reference genome using STAR (Dobin *et al.*, 2013) with default parameters, except that the mapping quality threshold (Q) was set to 100. The two-reference genome were used for this analysis: *B. juncea* cv. Varuna (*Brassica juncea* genome assembly UDSC Var 1.1-NCBI-NLM) and *S. sclerotiorum* (https://fungidb.org/common/downloads/Current_Release/Ssclerotiorum1980UF-70/).

The featureCounts (Liao *et al.*, 2014) were used to assign reads to exonic regions of genes. DEGs were identified using the edgeR package (Robinson *et al.*, 2010), retaining only those with a detection threshold of ≥ 1 count per million (CPM). DEGs were filtered using a false discovery rate (FDR) < 0.05 and an absolute log₂ fold change ($|\log_2FC|$) > 2 . Pairwise comparisons were conducted between R and S pools inoculated with *S. sclerotiorum* Ss-white isolate.

GO and KEGG enrichment analysis of DEGs

Functional annotation of the DEGs was performed by BLASTx against *Arabidopsis thaliana* protein sequences. GO enrichment and KEGG analysis was carried out using DAVID (Sherman *et al.*, 2024), and the results were visualized with SRplot (Tang *et al.*, 2023). Additionally, Venn diagrams of upregulated, downregulated, and shared DEGs in R and S comparisons across Ss-white isolate were generated using SRplot.

Results and Discussion

Disease development

The ILs, comprising 224 lines including resistant and susceptible checks, were investigated for their responses to stem inoculation against *S. sclerotiorum* over three consecutive years. Based on stem lesion lengths, the ILs were categorized into five resistance groups: highly resistant (HR), resistant (R), moderately resistant (MR), susceptible (S), and highly susceptible (HS), with lesion ranges of 0- <2.5 cm, 2.5- <5.0 cm, 5.0- <7.5 cm, 7.5- <10.0 cm, and >10.0 cm, respectively, as described by Garg *et al.*, (2010) and Rana *et al.*, (2017). The results revealed significant variation in resistance responses to stem inoculation among the ILs ($P < 0.001$; Fig 1A). From these, three resistant ILs and three susceptible genotypes were pooled to form the R-pool and S-pool, respectively, which consistently exhibited HR and S responses across the three seasons. Across the three seasons, the S-pool-comprising *B. juncea* cv. RLC-1, Giriraj, and PBR-357 exhibited mean lesion lengths of 12.1 cm (season I), 12.49 cm (season II), and 12.56 cm (season III). The overall mean lesion length across seasons was 12.39 ± 0.46 cm, indicating a consistently high level of susceptibility with minimal variation across environments. In contrast, the resistant introgression lines IL-R1, IL-R2, and IL-R3 displayed significantly lower mean lesion lengths of 1.08, 0.95, and 0.74 cm, respectively. The overall mean lesion length across the resistant lines was 0.93 ± 0.22 cm, reflecting a strong and stable resistance response (Fig 1A and 1B).

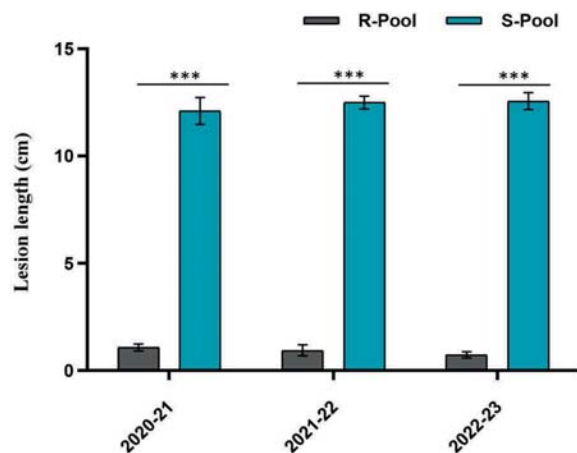


Fig. 1: Resistance response of *B. juncea* introgression lines against *S. sclerotiorum*. (A): Hypersensitive resistance (HR) response with stem lesion size <2.5 cm (B and C): Highly susceptible (HS) with stem lesion size >10.0 cm.

RNA-Seq data and differentially expressed genes analysis

The libraries of four samples (R-water, R-white, S-water, and S-white) were sequenced on the Illumina NovaSeq 6000 platform using 2×150bp paired-end chemistry, generating an average of 18.4 million clean reads per sample (Table 1). In the Ss-white condition, the total reads mapped to the reference genome (*B. juncea* cv. Varuna) were 82.77% (14,492,191 reads) in the R pool and 70.66% (13,450,210 reads) in the S pool, while uniquely mapped reads accounted for 78.53% and 66.18%, respectively. In contrast, the mapping percentages were 97.01% and 95.34% for the R and S pools under Ss-water conditions, at 24 hpi, respectively. In Ss-white, the total reads mapped to *S. sclerotiorum* were 0.09% (15,770 reads) and 1.48% (278,906 reads) at 24 hpi (Table 1). Differential expression analysis identified 3,627 *B. juncea* DEGs uniquely

expressed in the R pool and 10,524 DEGs uniquely expressed in the S pool, along with 1,807 DEGs commonly expressed in both pools at 24 hpi (Fig 2).

GO and KEGG enrichment analysis of DEGs

A total of 3,559 upregulated and 1,875 downregulated DEGs were identified in the R-pool of *B. juncea*, whereas the S-pool exhibited 2,635 upregulated and 9,696 downregulated DEGs after 24 hpi. GO enrichment analysis revealed that several biological processes were enriched in the R-pool (water vs. Ss-white), including response to wounding, systemic acquired resistance, response to oxidative stress, response to abscisic acid, defence response to fungus, signal transduction, regulation of transcription, regulation of defence response, response to osmotic stress, circadian rhythm, response to auxin, leaf senescence, and response to jasmonic acid—all of which were predominantly upregulated. In the S-pool, processes such as developmental growth, response to water deprivation, response to light stimulus, defence response to osmotic stress, cellular response to oxygen-containing compounds, salt stress, protein metabolic processes, protein phosphorylation, and the abscisic acid signalling pathway were significantly upregulated after 24 hpi (Fig 3A, 3B). Notably, defence response to osmotic stress, response to wounding, and cell wall biogenesis were significantly upregulated in the S-pool (water vs. Ss-white).

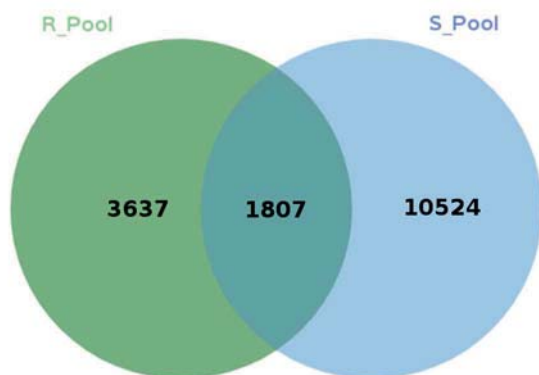


Fig. 2: Venn diagram depicting the number of common and unique DEGs between R-pool and S-pool of *B. juncea* infected with *S. sclerotiorum* white (Ss-W) at 24hpi

The KEGG pathways between the R and S pool include metabolic pathways, biosynthesis of secondary metabolites, pentose phosphate pathway, glyoxylate and dicarboxylate metabolism, inositol phosphate metabolism, plant hormone signal transduction, carbon fixation in photosynthetic organisms, phenylpropanoid biosynthesis, plant-pathogen interaction, fatty acid



Fig. 3: Analysis of specific DEGs: GO biological processes between R-pool and S-pool of *B. juncea* infected with *S. sclerotiorum* white (Ss-W) at 24hpi, (A): R-pool, water vs Ss-white (B): S-pool, water vs Ss-white

biosynthesis, sulphur metabolism, steroid biosynthesis, and carbon metabolism. Several other KEGG pathways such as photosynthesis-antenna proteins, biosynthesis of secondary metabolites, phenylalanine, tyrosine and tryptophan biosynthesis, MAPK signalling pathway, biosynthesis of cofactors, glutathione metabolism, fatty acid metabolism and biosynthesis, plant hormone signal transduction was significantly enriched in S-pool (water vs Ss-white) after 24hpi (Fig. 4A, 4B).

Recent advances in transcriptomic analyses have enabled the identification and functional characterization of numerous pathogenicity-associated genes and effector proteins contributing to virulence (Seifbarghi *et al.*, 2017, Ding *et al.*, 2019, Chittem *et al.*, 2020, Rana *et al.*, 2021, 2022, Sarfraz *et al.*, 2026). Furthermore, the availability of high-quality genome sequences for *B. juncea* cv. Varuna and the fungal pathogen *S. sclerotiorum* has been greatly enhanced by the advent of cost-effective sequencing

technologies (Amselem *et al.*, 2011, Derbyshire *et al.*, 2017, Paritosh *et al.*, 2021, Gupta *et al.*, 2022). However, the utilization of introgressed resistance against this disease remains limited due to associated linkage drag. Therefore, there is a need for more targeted approaches, such as cross-kingdom RNA interference and host-induced gene silencing (HIGS), to develop broad-spectrum resistance. These strategies enable precise manipulation of host resistance genes and targeted silencing of key pathogenicity or virulence factors in the pathogen (Baulcombe 2015, Weiberg *et al.*, 2015, Derbyshire *et al.*, 2019).

In the present study, *B. juncea*-*B. fruticulosa* introgression lines exhibited significant variation in resistance responses across three seasons. Three introgression lines viz. IL-R1, IL-R2, and IL-R3 were categorized as HR, based on their consistently small lesion lengths, and grouped to form the R-pool. In contrast, *B.*

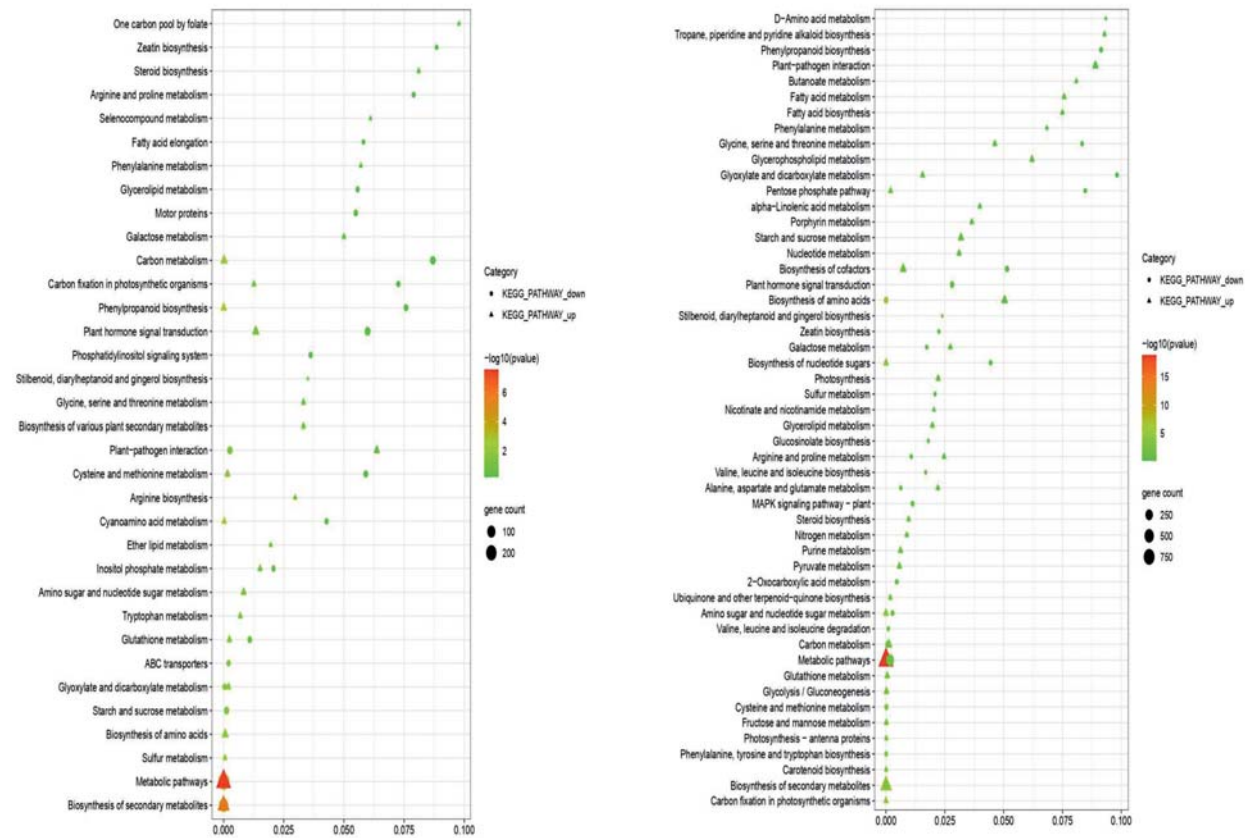


Figure 4: KEGG pathways significantly enriched between R-pool and S-pool of *B. juncea* inoculated with *S. sclerotiorum* isolate white at 24 hpi, (A): R-pool, water vs Ss-white (B): S-pool, water vs Ss-white

Table 1: Summary of sequence read alignments to the reference genomes of *B. juncea* cv. Varuna and *S. sclerotiorum*

Sample	R-Pool		S-Pool	
	Ss-White	Ss-Water	Ss-White	Ss-Water
Clean Reads	175,085,28	204,140,27	190,325,45	167,724,57
Total mapped to <i>B. juncea</i> cv. Varuna	14492191 (82.77%)	19803763 (97.01%)	13450210 (70.66%)	15990814 (95.34%)
Uniquely mapped to <i>B. juncea</i> cv. Varuna	13749992 (78.53%)	14718651 (72.10%)	12596205 (66.18%)	12513811 (74.61%)
Total mapped to <i>S. sclerotiorum</i>	15770 (0.09%)	70208 (0.35%)	278906 (1.48%)	86386 (0.52%)
Uniquely mapped to <i>S. sclerotiorum</i>	3453 (0.02%)	131 (0.01%)	222874 (1.18%)	912 (0.01%)

juncea cv. RLC-1, Giriraj, and PBR-357, which showed susceptible responses, were grouped into the S-pool. Further, transcriptome analysis was performed to systematically characterize the transcriptional dynamics between the R and S pools inoculated with *S. sclerotiorum*. RNA sequencing generated an average of 18.4 million clean reads per sample (Table 1).

Under *Ss-white* inoculation, 82.77% of reads from the R-pool and 70.66% from the S-pool successfully mapped to

the *B. juncea* cv. Varuna reference genome. In contrast, only 0.09% and 1.48% of reads mapped to *S. sclerotiorum* at 24 hpi in the R- and S-pools, respectively. When RNA is extracted from infection sites containing both plant and fungal tissues, mapping efficiency to the host reference genome is reduced because pathogen-derived transcripts fail to align. This lowers overall mapping rates and diminishes the effective sequencing depth for host transcripts. To overcome these limitations, a dual RNA-Seq strategy-either by using a combined host-pathogen

reference genome or through sequential mapping-can be employed. This approach enables accurate read assignment and facilitates clear separation of host and pathogen transcriptomes. In total 3,559 upregulated and 1,875 downregulated DEGs were identified in the R-pool, and 2,635 upregulated and 9,696 downregulated DEGs were observed in the S-pool after 24 hpi.

Functional annotation, including GO enrichment analysis, identified several key biological processes (BP), cellular component (CC), and molecular function (MF), that were actively regulated at 24 hpi. In the R-pool, GO enrichment analysis identified GO BP terms such as response to wounding, regulation of defense response, response to oxidative stress, response to abscisic acid and jasmonic acid, defense response to fungus, signal transduction, cellular response to oxygen, metabolic processes and systemic acquired resistance were predominantly upregulated, indicating an active and coordinated defense response at 24 hpi of *S. sclerotiorum* infection. In contrast, the S-pool exhibited significant alterations upon colonization by *S. sclerotiorum*, including changes in transcriptional activity, protein metabolic processes, protein phosphorylation, response to salicylic acid, ethylene and the abscisic acid signaling pathway. This was further supported by extensive transcriptional reprogramming in the S-pool, with significant enrichment of processes related to, metabolic, cellular catabolic, and biosynthetic processes, water deprivation, transcriptional regulation, protein metabolism, cell wall biogenesis, and leaf senescence during pathogenesis.

Conclusion

Present study identified several DEGs in *B. juncea* with potential roles in defense response to fungus, signal transduction, transcriptional regulation, protein metabolic processes, and response to abscisic acid and jasmonic acid. This study provides valuable insights into the underlying molecular mechanism, contributing to a better understanding of *B. juncea*-*S. sclerotiorum* interaction.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request. The transcriptome data set of all the samples has been deposited in the IBDC-INDA database (Bio project Accession no. PRJEB110406).

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