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Transcriptomic signature of Fusarium toxin in chickpea unveiling wilt pathogenicity pathways and marker discovery

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1. Introduction

Chickpea is the second most important food legume in terms of gross production and acreage and is grown in over fifty countries of the world representing all the continents [1]. It is a self-pollinated, diploid (2n = 2x = 16), cool season pulse crop with a moderate genome size of 740 Mb. It is not only an excellent source of nutritive dietary protein for mass of undernourished people throughout the developing world, but also plays an important role in improving soil health, fertility and sustainability of agro-ecosystems [2]. The global chickpea production is about 13.1 Mt from an area of 13.54 Mha with India being the largest producer contributing about 67.3% of the total world production (FAOSTAT 2013) [3]. Chickpea yield is severely curtailed by several abiotic (drought, heat, cold and salinity) and biotic (Ascochyta blight, fusarium wilt, dry root rot and pod borer) constraints of which fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* is one of the serious threats. Annual yield losses worldwide from this disease account for 10–15% with total loss under specific conditions [4]. *Fusarium oxysporum* is well known for causing vascular wilt and root diseases in a wide range of agricultural and ornamental plants [5]. *F. oxysporum* f. sp. *ciceris*, is a soil or seed borne root pathogens that colonizes and proliferates in the vascular tissue disrupting the translocation of water thereby causing typical wilt symptoms, which include foliage chlorosis, pseudostem longitudinal splitting, necrosis and ultimate death.

The best way, at present to control fusarium wilt disease (FWD) and enhance crop productivity in chickpea is through molecular breeding for resistant cultivars [6]. The traditional breeding program can be supplemented with introgression of resistance genes from resistant varieties into high yielding susceptible varieties. Such introgression needs identification of key genes involved in FWD resistance. Transcriptome study of two contrasting genotypes for wilt resistance by challenging them with *Fusarium* can decipher differentially expressed genes (DEGs) leading to genes/markers discovery. Though transcriptome studies have been reported in chickpea, they had been confined to general aspects like root and shoot tissue [7], shoot apical meristem and flower development [8,9] different developmental stages [10], root nodulation [11], comparative transcriptome with other legume [12]. A few chickpea transcriptome studies are also reported for abiotic stresses [13], salt stress [14] and heat stress [12] and biotic stresses like Fusarium wilt [14,15].

Though, the greenhouses and sick plots have been used widely for wilt studies, the uniform inoculum density of *Fusarium oxysporum* is the major limiting factor. Towards that end, the screening and selection of wilt resistance in chickpea using pathotoxin was carried out [16]. However, they sprayed the plants with Fusaric acid, unlike the purified toxin from the culture of *Fusarium oxysporum* which has been used in the present study. This is because wilting mechanism is a biochemical reaction rather than physical blockage of xylem by mycelia. To best of our knowledge, there is no report on use of uniform concentration of toxin to challenge resistant and susceptible varieties of chickpea. Thus investigation of differential expression profiling is critically imperative to get candidate genes and their associated markers. Though attempt was made by Ashraf et al., 2009 [15], on stress responsive transcriptome analysis of chickpea wilt by making cDNA library subtraction followed by EST assembly and microarray to delineate plant immunity pathway but gene regulatory network and hub proteins are yet to be reported. In their study, SNP markers were identified but SSR, indel markers and

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isoforms were not reported. Also, the chickpea seedlings in this study were inoculated with Fusarium spore rather than purified uniform Fusarium toxin. Kohli et al., 2014 challenged the chickpea seedlings with Fusarium toxin for miRNA discovery but no reports are available on DEGs which are controlled by miRNA so that silencing technology can be explored for wilt management [14].

The present work aims at investigation of DEGs in shoot by transcriptional profiling depicting gene regulatory network of contrasting chickpea genotypes challenged with uniform toxin concentration. In addition, we report the discovery of molecular markers (STRs, SNPs and indel markers), isoforms and identification of DEGs controlled by miRNA in wilt disease.

2. Materials and methods

2.1. Plant growth and wilt stress

The seeds of contrasting genotypes WR315 (wilt resistant) and BG256 (wilt susceptible) were surface-sterilized with Tween 20 and 0.1% $HgCl_2$ for 5 min each followed by thorough washing with sterile distilled water under aseptic conditions. The sterilized seeds were allowed to germinate on wet Whatman 3 filter paper (GE Healthcare) at 37 °C in dark and grown hydroponically under sterile conditions in culture tubes containing 10 ml autoclaved Hoagland's nutrient solution in an environmentally controlled growth room maintained at 25±2 °C, 55 ± 5% humidity under 16 h photoperiod (270 μmol $m^{-2}s^{-1}$ light intensity) for two weeks before imposing wilt stress. One seedling was grown per culture tube.

2.2. Production and storage of F. oxysporum spores

A virulent, wilt causing isolate of the fungus *F. oxysporum* f. sp. *ciceris*, from Delhi, India was isolated from the chickpea seedling with obvious wilt symptoms. Collar regions from the wilted seedlings were excised, surface sterilized for 2 min with sodium hypochlorite, rinsed twice in distilled water and plated on petri plates containing Komada's medium (KM) specific for the growth of *F. oxysporum* (Komada, 1975). Plates were incubated at 25 ± 2 °C in an illuminated incubator with a 16 h photoperiod for 5 days. Fungi developing from the diseased tissue of chickpea were then subcultured and single spored on the petri plates containing potato-dextrose agar (PDA). For liquid culture, Czapek-Dox cation liquid medium (CDCLM) described by Hamid & Strange (2000) consisting Czapek-Dox liquid medium (Oxoid) supplemented with $ZnSO_4 \cdot 7H_2O$, 0.05 g l^{-1} $CuCl_2 \cdot 6H_2O$, 0.02 g $l^{-1}MnCl_2 \cdot 4H_2O$, 0.02 g l^{-1} $CaCl_2 \cdot 2H_2O$, 0.1 g l^{-1} $CoCl_2 \cdot 6H_2O$, 0.02 g l^{-1} was prepared and 30 ml aliquots of this medium was distributed in a 250 ml flask and inoculated with the spores (2 mm disc) of *F. oxysporum* isolate. Flasks were incubated at 20 °C for 5 days with constant shaking after which the mycelium was removed by filtration through four layers of muslin cloth and the spores pelleted by centrifugation. Spores were then re-suspended in sterile distilled water, centrifuged twice, finally re-suspended in sterile distilled water at a final concentration of 10^7spores ml^{-1} and stored in liquid nitrogen.

2.3. Toxin production

To obtain filtrates of the fungal culture, 1 L Roux bottles containing 100 ml CDCLM were inoculated with 100 μl spore suspension of *F. oxysporum* (10^7spores ml^{-1}) and incubated statically for 12 days at 20 °C in horizontal position to provide maximum surface area for the culture. Culture was then filtered through four layers of muslin to remove the mycelium and the filtrate centrifuged at 10,000 g for 30 min to remove spores. The resulting supernatant was again filtered through four layers of muslin before filtering through a 0.2 mm filter (Nalgene, Rochester, USA). Filtrate was reduced to one third volume under rotavapor at 45 °C. The concentrate was extracted with n-butanol and the solvent removed under vacuum. The concentrated butanol extract was subjected to preparative thin layer chromatography.

2.4. Toxin purification

Preparative thin layer chromatography was performed on silica gel coated glass plates (20 × 20 cm) of 0.2 mm thickness (Merck Sehuchardt, Germany). TLC plates were developed, air dried and visualized with UV light (254 nm). The bands positive to UV light were scraped and extracted with high purity HPLC grade methanol and the solvent evaporated under vacuum. For toxin isolation protocol described by Gopalakrishnan et al., 2005 was followed [17].

2.5. Tissue collection

For wilt stress, Hoagland solution in one set of tubes was replaced by 10 ml of pure toxin isolated from the fungus whereas in control set of tubes Hoagland solution was replaced by 10 ml of fresh Hoagland solution under sterile conditions. Wilt stress response was measured by determining the chlorophyll content of the stressed and control plants of both the genotypes following the procedure described by Arnon in 1949 [18]. Shoot tissues were collected from the stressed and control seedlings of both the genotypes 48 h post infection. At least three independent biological replicates of each tissue sample were harvested, immediately frozen in liquid nitrogen and kept at −80 °C.

2.6. RNA isolation and quality controls

Total RNA was extracted from all tissue samples using TRI Reagent (Sigma Life Science) according to manufacturer's instructions. The quality and quantity of each RNA sample were assessed using Nanodrop (Nanodrop Technologies) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Singapore). The integrity of RNA was also checked by agarose gel electrophoresis. The high quality RNA samples (RNA integrity number > 7) were used for analysis. Total RNA from three biological replicates were pooled for mRNA purification followed by library preparation and sequencing was done using the Roche 454 GS FLX system and titanium series sequencing reagents (Roche 454 Life Sciences, Branford, CT, USA). Generated NGS data was submitted in NCBI (BioProject PRJNA302574).

2.7. Transcriptome data analysis

2.7.1. Data description, preprocessing and quality check

De novo assembly approach was adopted rather than reference based. De novo assembly has advantage in predicting gene families even from uncovered regions of chickpea reference sequence presently having 73% coverage [19]. De novo assembly performs better in such situation in terms of sensitivity and accuracy [20]. For the above discussed data, analysis was performed in combination of three sets: i.e., *de novo* assembly of each of the data obtained from wilt stressed and control shoot samples of BG256 and WR315 and the combined *de novo* assembly of the data generated from control and stressed shoot samples of both the varieties. The *de novo* all these assemblies were done using robust transcriptome reconstruction tool, Newbler 2.9–1 [21]. Fig. 1 illustrates the workflow of transcriptome pipeline for the study.

The raw sequence reads were filtered and examined for quality before approaching for assembly. This was done by first removing

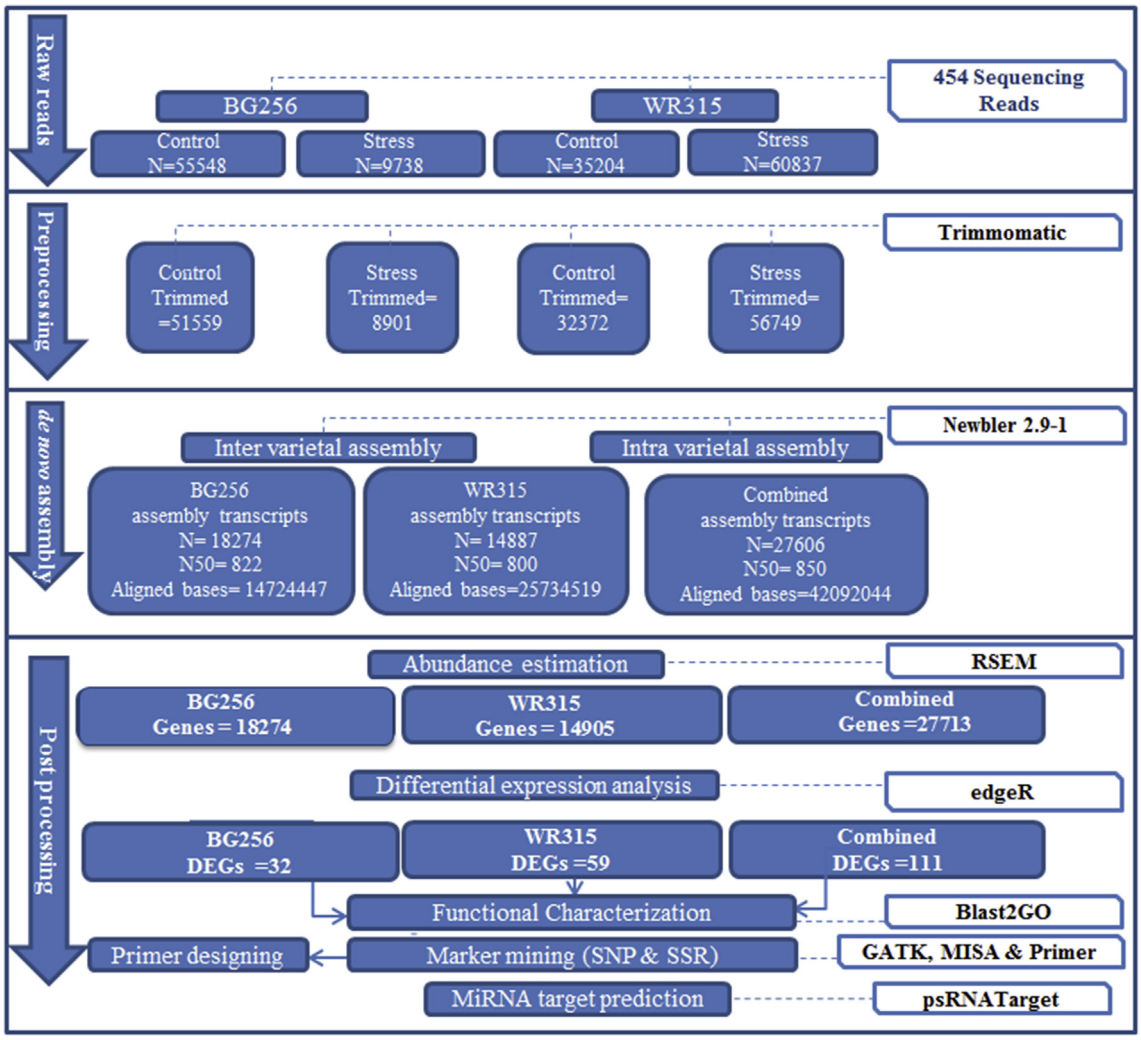

Fig. 1. Transcriptome analysis pipeline for this study.

the adapters using FASTXtoolkit [22] version 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/), followed by discarding low quality reads. Trimmomatic [23] was used to trim and clean illumina data. After this, read quality was examined using FastQC [24] (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic uses sliding window approach to trim low quality reads and encodes quality of fastq files to either base33 or base64.

2.7.2. *de novo* transcriptome assembly

For *de novo* reconstruction of transcriptome, Newbler 2.9–1 was used to assemble trimmed reads into isotigs and isogroups. Isotig from Newblerassembly contains information of individual transcripts. Isotigs from same Isogroup are splice variants of a given transcript. The generated 454matrix file provided statistics of assembly, reports counts of transcripts and statistics based on all transcript. The N50 value of assemblycan be used to calculate the contig distribution in assembly. 454 reads identified as singletons were used with isotigs for downstream analysis.

2.7.3. *Differential expression analysis*

2.7.3.1. Sample specific transcript quantification. Sample specific transcript quantification is an important step in RNA-Seq analysis. It identifies the reads that are mapped to assembly and then estimates the abundance of genes and isoforms. RSEM [25]i.e. RNA-Seq by expectation maximization was used to measure transcript abundance level of genes and isoforms for each dataset. Assembled transcripts and reads were aligned using Bowtie [26]. This alignment was used by RSEM to generate gene and isoform matrices which comprise of transcript_id, gene_id, length, effective_length, effective_count, TPM, RPKM [27], and IsoPct values. EdgeR [28] was used for expression analysis of transcripts from inter-varietal and intra-varietal assemblies. Differentially expressed transcripts/isoforms were extracted from EdgeR by running utility perl script to generate heatmap, MA plot and volcano plot, describing the variation in transcript components. Isoforms were also identified from BG256, WR315 and the combined dataset. Differentially expressed genes were selected based on Bonferroni adjusted p-values.

2.7.4. *Functional characterization and annotation of differentially expressed transcripts*

Sequences of the differentially expressed transcripts were extracted from the output file using in-house perl script. Blast2GO [29] Pro ver 3.1 was used for aligning extracted sequences to all reading frames of the transcripts against protein database followed by mapping, enzyme coding and pathway analysis. Mapping and pathway analysis of sequences describe the associated GO terms and pathways functioning in stress conditions.

2.8. Identification of miRNA controlled DEGs

Post-transcriptional regulation by microRNAs (miRNAs) is an important response to biotic and abiotic stresses [30]. In order to obtain DEGs controlled by miRNAs, all mature miRNAs of legumes were downloaded from miRBase [31] version 21 and aligned using psRNATarget [32] programme in all the three datasets.

2.9. STR and variant detection

Mining of STR markers was performed from the assembled transcripts using MISA [33] i.e. MIcroSatellite identification Tool which identified different STR types *viz*., mono-, di-, tri-, tetra-, penta-, hexa-nucleotide and compound repeats. From all the mined STRs, five best pairs of forward and reverse primers were designed using Primer3 tool (http://primer3.sourceforge.net/releases.php). Detection of variants (SNPs and indels) was performed using BWA-Picard-GATK pipeline. For this, desi chickpea variety was taken as reference genome from NCBI-Genomes (http://nipgr.res.in/CGAP2/download/genome_sequencing/genome_sequence/). All the three *de novo* assemblies were aligned to reference genome using BWA tool. Picard tool was used to preprocess the alignment/map files (SAM/BAM) for sorting, duplicate removal, read group addition and build BAM index for the BAM file. These indexed BAM files were further used in Genome Analysis Tool Kit (GATK) for variant calling [34]. Variant annotation and effect prediction were carried out using SnpEff [35] and SnpSift [36] toolbox.

2.10. Gene regulatory network

For the DEGs of BG256, WR315 and combined BG256 and WR315, protein-protein interaction network (PPIN) was performed. Networks were visualized using Cytoscape [37] version 3.2.1, an open source software platform for visualizing complex networks. Expression Correlation [38], a java based plug-in for cytoscape was also used, where similarity matrix is computed using the Pearson Correlation Coefficient. *Network Analyzer* was used for the analysis and network centrality that computes specific node centrality parameters and describes the network topology.

Genes within a network with higher number of connectivity play a central regulatory role, and is important in detecting highly connected gene in a network. The node *degree*, *betweenness centrality* (BC) and *closeness centrality* (CC) values were used to characterize the whole network. Node and edges filtration was carried out based on the centrality level. Edges were filtered and retained for network based on EdgesBetweenness with values $\geq$ 2, 2.6 and 3.3, for BG256 and WR315 and combined network respectively. Hub genes of complex networks were also obtained according to analysis of degree betweenness and stress. The genes at the top of degree, betweenness and stress distribution in the significantly perturbed sub-networks were defined as *hub genes* [39].

3. Results

3.1. Sequencing and sequence assembly

Four cDNA libraries for transcriptome analysis were prepared from the mRNA of the shoots of the susceptible cultivar 'BG256' and the resistant cultivar 'WR314' under control and stressed conditions. A total of 161327 single end raw reads were generated through Roche sequencing. To facilitate sequence assembly, repetitive, low-complexity and low quality reads were filtered out using trimmomatic and FastQC generating a total of 149581 high quality reads with an average length of 350 bp (Table 1).

A total of 51559, 8901, 32372 and 56749 high quality reads comprising of 18077958, 3071633, 11250815 and 20570811 nucleotide bases for BG256 (control), BG256 (stress), WR315 (control) and WR315 (stress), respectively were retained for further analysis. The length distribution of the high-quality reads shows that most of them are more than 300 bp in length. Inter varietal and intra varietal assembly by *de novo* assembler assembled the reliable reads into 614, 556 and 1261 contigs with an average length of 824, 763 and 804 for BG256, WR315 and combined dataset, respectively. The N50 length of the contigs was found to be 822, 800 and 850 for BG256, WR315 and combined dataset, respectively. The number of singletons obtained was 17660, 14331 and 26435 for BG256, WR315 and combined dataset, respectively. After clustering, we finally obtained 27606 distinct sequences that cannot be extended on either end. Such sequences were defined as unigenes, including 1261 clusters and 26435 singletons (Table 2)

Table 1
Summary of raw and trimmed reads.

	BG256		WR315	
	Control	Stress	Control	Stress
Raw reads	55548	9738	35204	60837
After Trimming	51559	8901	32372	56749
Bases	18077958	3071633	11250815	20570811
No of aligned reads	34130	6128	21013	49238
No of aligned bases	12551864	2172583	7478912	18255607

3.2. Abundance estimation and differential expression analysis

The contigs obtained after assembly of high quality reads from BG256, WR315 and combined dataset were subjected to abundance estimation through RSEM for calculation of effective length, expected count, TPM (Transcript Per Million), FPKM (Fragment Per Million Read) and IsoPct (Isoform Percentage) values. Distribution of FPKM values in transcripts are shown in Fig. 2. The expression values were normalized and heatmap created for differentially expressed genes (DEGs) using edgeR package [28] (Figs. 3–5).

Total of 32, 59 and 111 differential expressed genes were obtained for BG256, WR315 and combined dataset respectively at p-value cutoff 0.05 and 2 fold changes (S1 Table). Out of 32 DEGs in BG256, 15 were upregulated with maximum log fold change value of 10.378 while 17 were downregulated with maximum log fold change value of 8.688. Among 59 DEGs in WR315, 34 were upregulated with maximum log fold change value of 9.505 and 25 downregulated with maximum log fold change value of 8.023. For the combined assembly, out of 111 DEGs, 58 were upregulated with maximum log fold change value 9.759 and 53 downregulated with maximum log fold change value 8.990. Graphical representation of differential expression in BG256, WR315 and combined data is described by MA plot and volcano plot. MA plot describes the differential expression by plotting log transformed expression values with mean average values, while volcano plot scales significance value at y axis and fold change at x axis (Fig. 6). There was one

Table 2
Statistics of *de novo* assembly.

	BG256	WR315	All
No. Isotigs	614	556	1261
No. Singletons	17660	14331	26435
No. Assembled reads	37422	64951	105415
Average isotig size	824	763	804
N50 isotig size	822	800	850
Largest isotig size	7107	4074	4431
Unigenes	18274	14887	27606

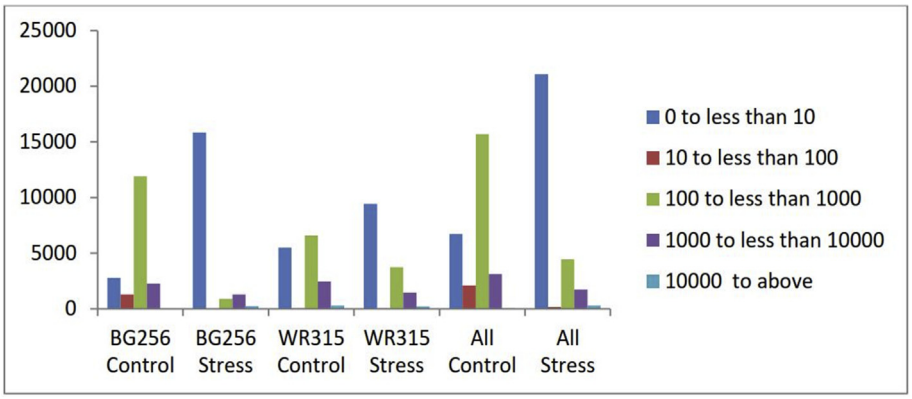

Fig. 2. Distribution of Isoform in FPKM (Fragment per million read).

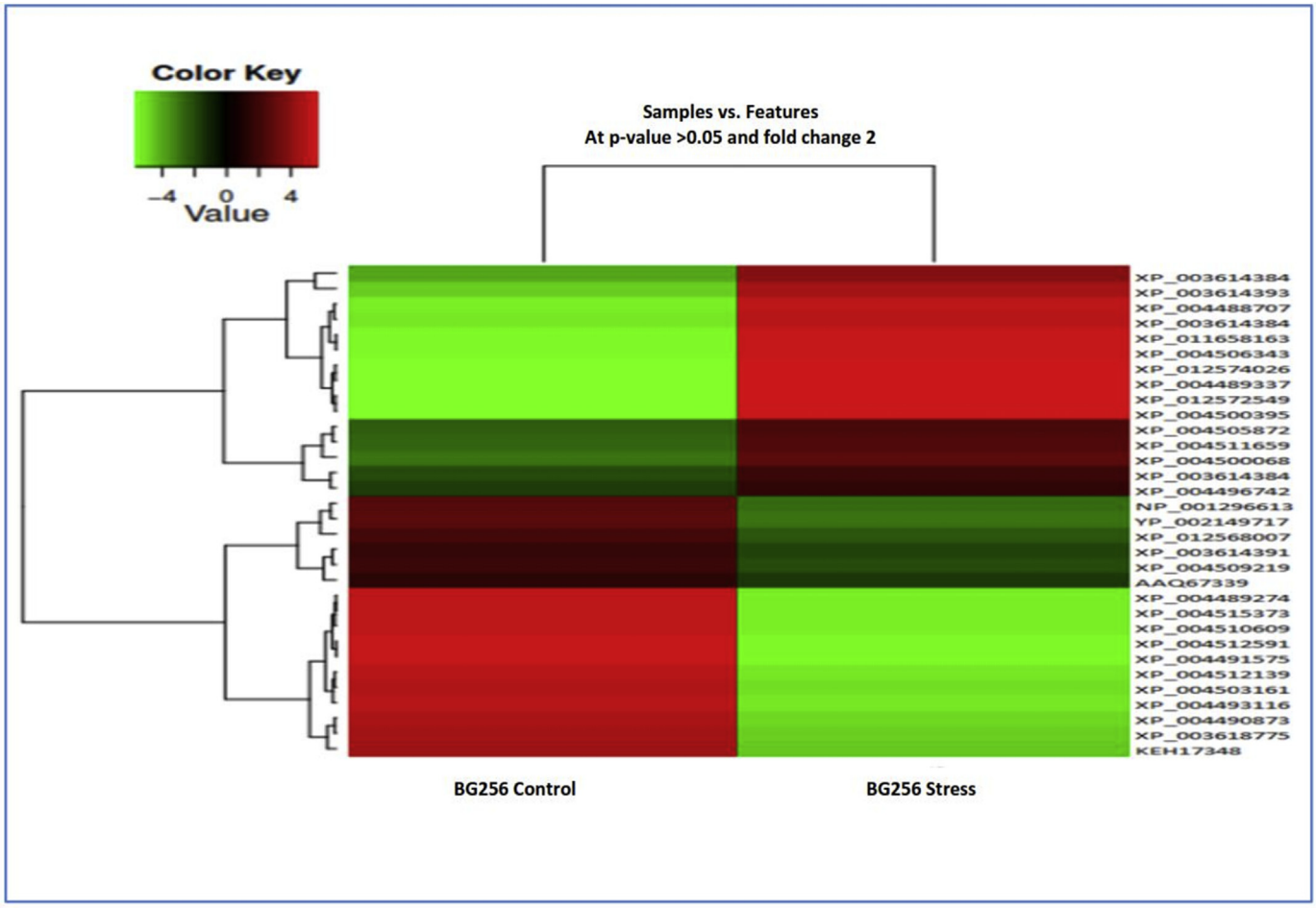

Fig. 3. Heat map of differential expression of BG256control vs. Bg256stress.

differentially expressed gene with 6 isoforms in BG256 and two isoforms in WR315, whereas there were four differentially expressed genes with two isoforms each in combined assembly S2 Table.

3.3. Functional annotation

Differential expressed transcripts of BG256, WR315 and combined dataset were annotated using Blast2GO pro version program. Blastx was performed with expected value 1e-3 against non redundant database, followed by mapping and annotation to annotate all unigenes. A total of 28, 56 and 102 unigenes were mapped to at least one GO term for BG256, WR315 and combined dataset respectively. Similarly, 9, 19 and 32 enzyme code hits were found for BG256, WR315 and combined respectively. It was found that for BG256, maximum GO distribution was for metabolic process, followed by cellular process and binding function. Similar trend was observed in WR315 genotype and combined dataset (Figs. 7–9).

All these genes showed their role in different metabolic pathways. A total of 10, 17 and 26 pathways were involved in differential expression of BG256, WR315 and combined dataset of chickpea

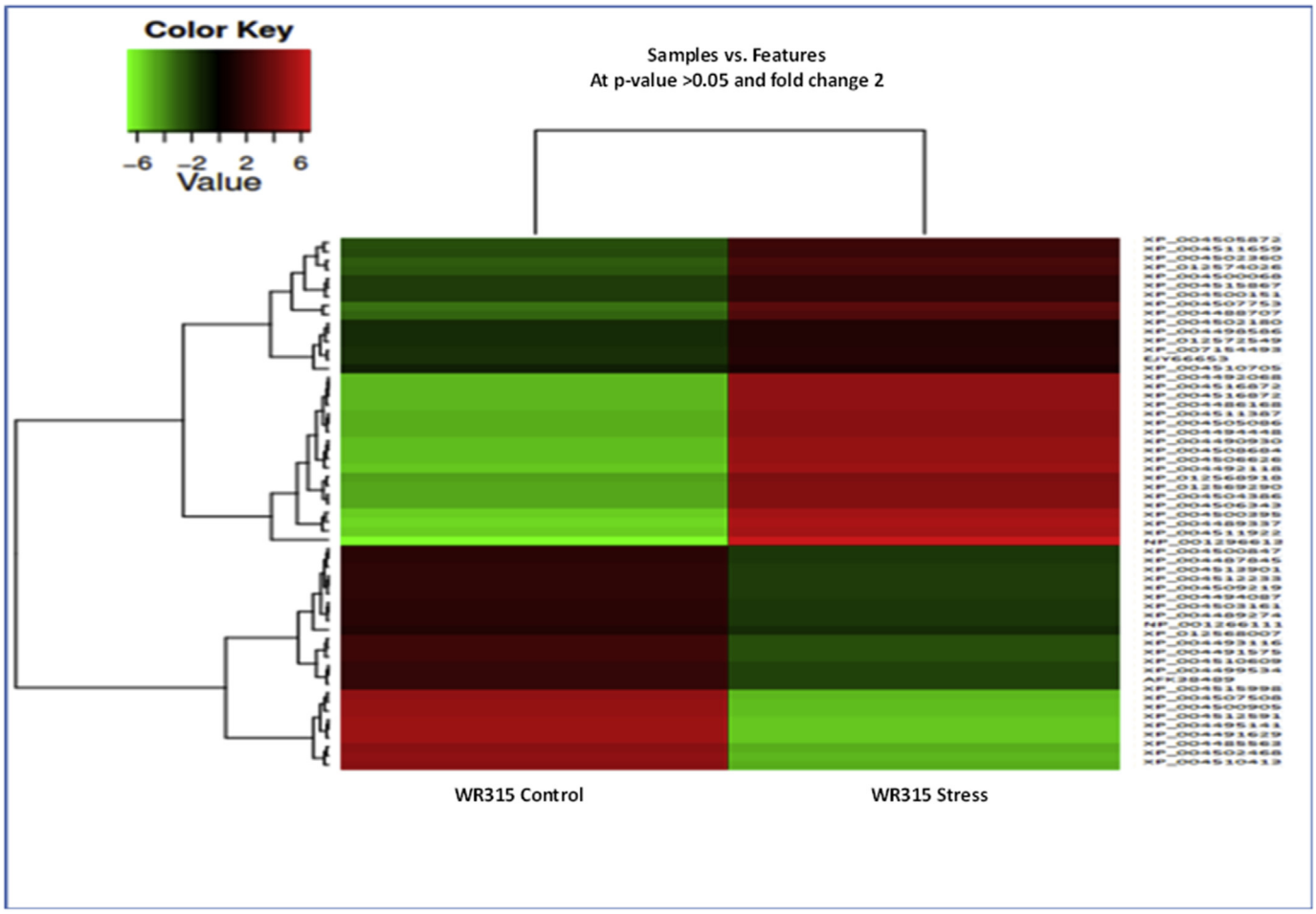

Fig. 4. Heat map of differential expression of WR315control vs. WR315stress.

respectively (S3 Table).

3.4. Identification of miRNA controlled DEGs

Alignment of all reported legume miRNA with DEGs revealed that for BG256 (32), WR315 (59) and combined set (111), only limited number (2, 14 and 32 respectively) of them are controlled by miRNA (S4 Table).

3.5. STR and variant detection

Mining of STRs showed the abundance of tri-nucleotides, followed by di-nucleotides in all the three datasets. BG256 showed maximum number of STRs, i.e. 276 out of which 3 were from differentially expressed genes. Similarly, for WR315, 31 STRs were tri-nucleotides of which 2 were from DEGs. For the combined dataset, abundance of tri-nucleotides (441) were reported with 5 STRs from DEGs (Table 3).

The details of primers from mined STRs are provided in S5 Table. Variant detection for SNPs and indels were performed. A total of 221 SNPs and 12 indels were found in BG256; 246 SNPs and 21 indels were detected in WR315 whereas combined data showed the presence of 337 SNPs and 40 indels (Table 4). Details of the SNPs and indels with their positions in chickpea genome have been provided in S6 Table.

3.6. Gene regulatory network

In GRN, maximum degree value of BG256 and WR315 was found to be 29 and 56, respectively. The node having degree centrality $\geq$28 and $\geq$ 50 (approximately 20%) for BG256 and WR315, respectively were considered as hub gene (Figs. 10 and 11). These included senescence-associated protein, polyubiquitin, chlorophyll *a-b* binding protein, ferredoxin-NADP, translation factor sui1, carbonic anhydrase, ribulose bisphosphate carboxylase, oxygen-evolving enhancer, elongation factor 1-alpha, Post translational modification genes. Post translational modification, protein turn over, chaperones and Photosynthesis were the common hub genes in both BG256 and WR315 genotype. Detailed role of each of the hub gene involved in gene regulatory network of toxin induced wilting effect has been described in Table 5. S7 Table shows the network centrality parameters of BG 256 and WR 315 varieties of chickpea. Other major functional classes included carbohydrate metabolism, lipid metabolism, energy metabolism, cellular redox state and transcription. These include genes encoding cytochrome P450, thioredoxin, superoxide dismutase and glutathione-S-transferase that are known to play critical role in plant stress [40,41]. Genes encoding this pathway is present in both susceptible and resistant genotypes. Hub genes in a given GRN has strong tendency to exhibit pleiotropic effect. Such study can identify tissue and time specific hub gene regulators [42].

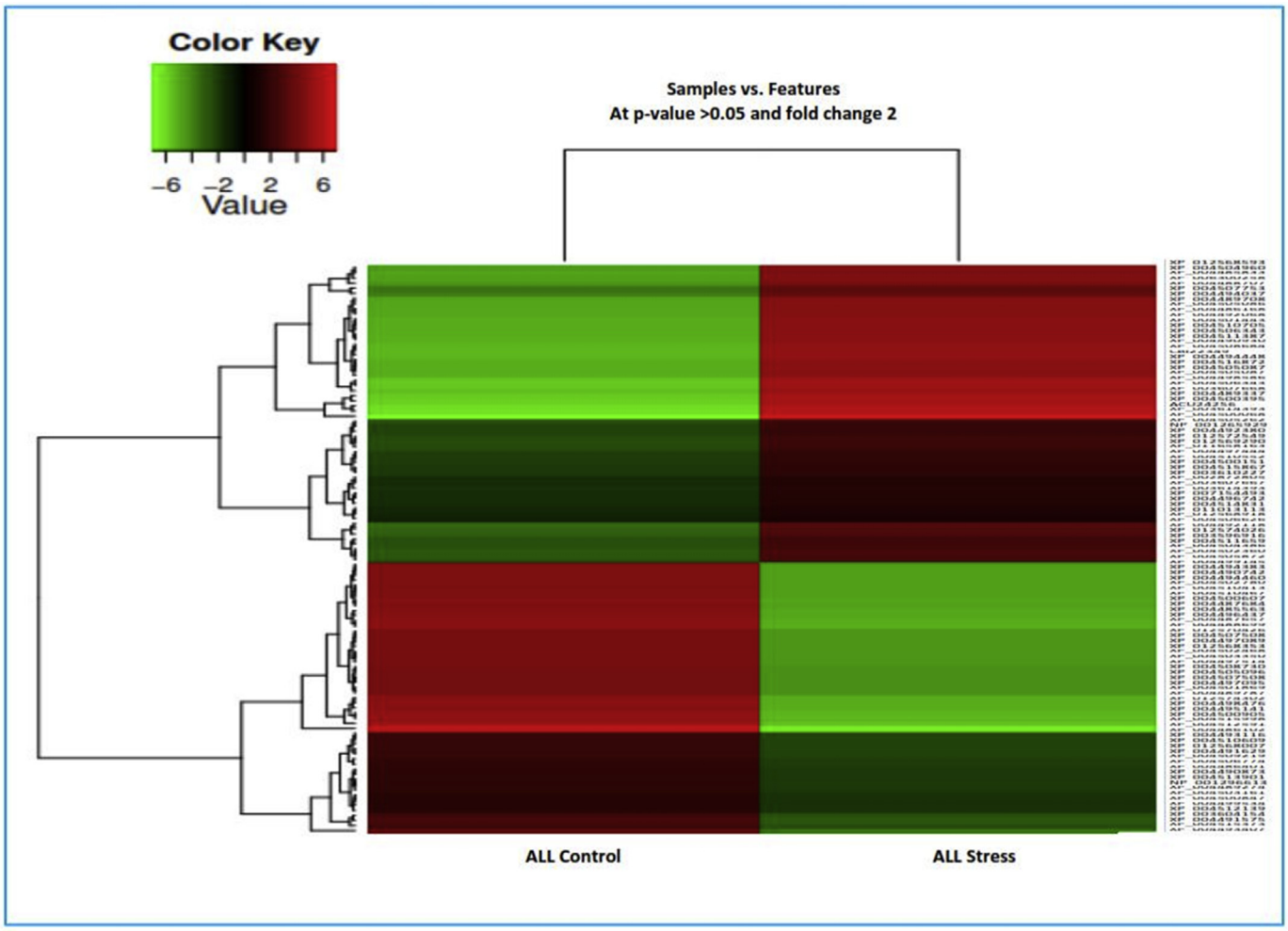

Fig. 5. Heat map of differential expression of combined control and stress samples from both varieties.

4. Discussion

Our transcriptional profiling clearly exhibits that two contrasting genotypes are a good source to reveal genes and pathways involved in chickpea wilt disease. We found 202 differentially expressed genes. Asraf et al., 2009 has reported 973 contigs having 209 known gene families [15]. Present study reports 822 contigs having 202 gene families. Since there were just 86 common gene families in these two studies, thus we report 116 novel gene families associated with toxin response by chickpea. This difference in transcriptomic profile might be due to cardinal difference in host–parasite interaction (HPI) and direct toxin challenge. Fungus mediates pathogenicity along with secondary metabolites production during HPI [56]. During such interaction fungal growth and colonization is involved [57]. This step is missing in case of direct toxin challenge. Toxin mediates a selective pathway having characteristics of programmed cell death [58]. In our experiment toxin was present in hydroponic system which was directly absorbed by roots and through xylem it reached directly to shoot tissues. Since there is no interaction of two different germplasm (fungi and chickpea) in form of intercellular/interspecies interaction by step of fungal colonization, thus we observed effect of toxin on host plant directly at cellular level obviating several steps involved in systemic response. Many of the host plant interaction pathways having systemic response in defence might be either missing or would be different.

In our transcriptome data, we found upregulation of R genes in stressed plants which is related to protein in post translational modification, protein turn over, chaperons development, storage, dormancy and senescence pathways. R genes act as candidate genes for resistance to *Fusarium* in *Arabidopsis* [59], melon [60] and tomato [61] which is widely known. In susceptible genotype under stressed condition, leucine rich repeat (LRR) domains were found to be upregulated. These are referred as R genes which are involved in protein-protein interaction as well as ligand binding [62]. In both susceptible and resistant genotypes, glutathione S-transferases (GSTs) were upregulated under stressed condition which clearly reflects that toxin challenge directly influences cell cycle and cell division. Since GSTs are known to be involved in detoxification of a wide variety of xenobiotic compounds [63], hence both varieties are showing similar response. For pathways like cell cycle control and cell division controlled by antioxidant genes is important defence mechanism against biotic stress resistance. In our study, we also found upregulated antioxidant genes controlling cell cycle and cell division. Similar observation was also reported in Arabidopsis [64] and chickpea [65]. We found changes in carbohydrate transport and metabolism via beta-galactosidase pathway leading to glycan structure degradation to maintain osmotic balance as reported in other legume [66]. In response to toxin challenge, there is down regulation of photosynthesis by differential expression of reductase as reported in other abiotic stresses [67]. We found up regulation of antibiotic synthesis pathway (in at least five

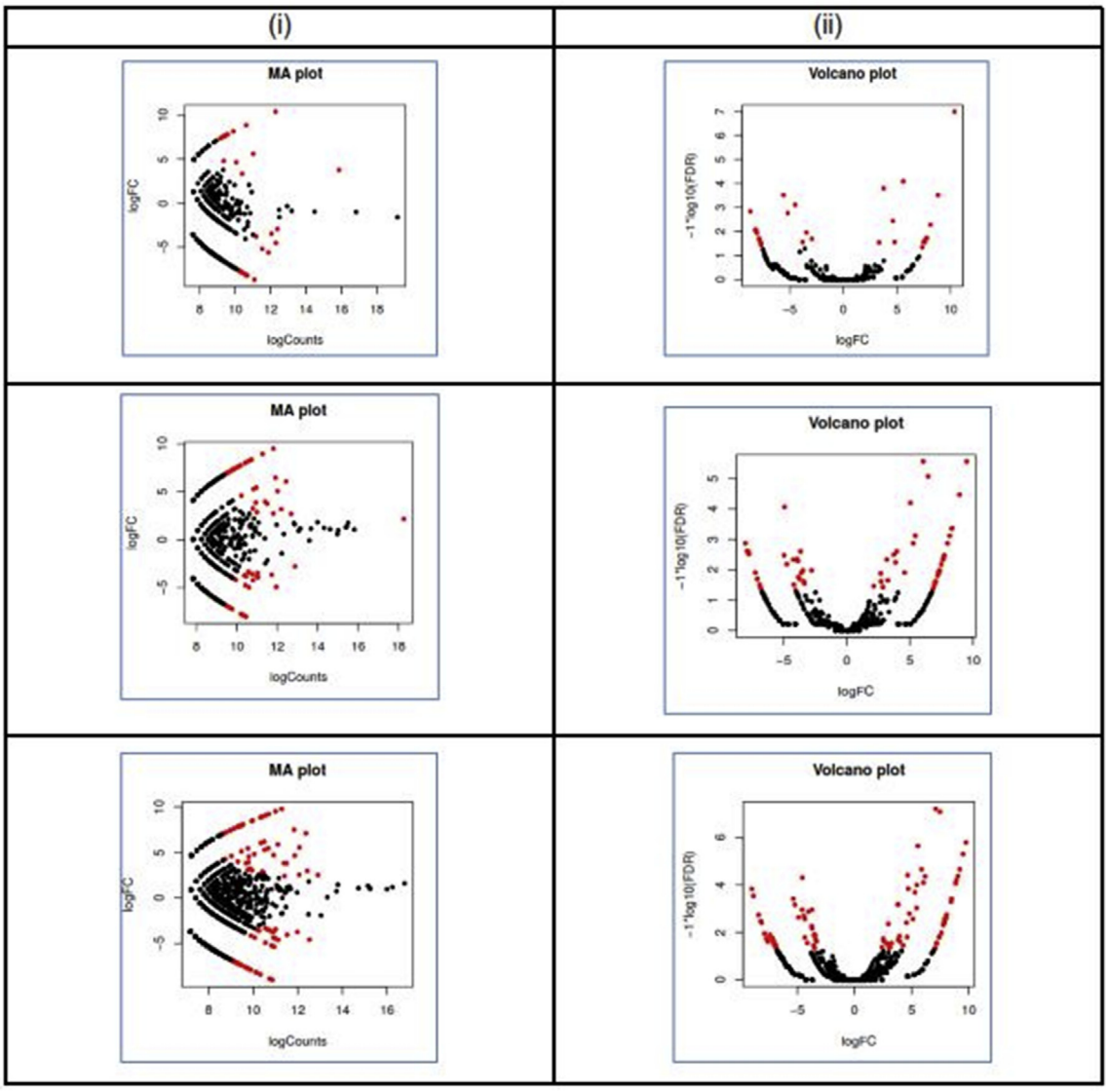

Fig. 6. MA plot and Volcano plot of Differential expression: (i) MA plot scale the log transformed expression (M = log ratios) with Mean average (A); (ii) Volcano plot scale the significance values to fold change.

transcripts) in our study in response to toxin challenge. This pathway is involved in resistance mechanism against fungal challenge as reported in other plants [68]. The upregulated alanine, aspartate and glutamate metabolism pathway reveal that there is change in plant homeostasis and glutamate signalling which also leads to root apical meristem activity [69]. Upregulation of glutathione metabolism pathway leads to metabolic detoxification in plant in response to environmental stresses to counter the cellular damage [70]. In our analysis, we also found upregulation of caprolactam degradation pathway which is known for defense response by plant against fungal toxin [71]. At least three differentially expressed transcripts were involved in upregulation of glyoxylate and dicarboxylate metabolism known for performing detoxification of both aldehydes and redox balance in response to stress [72]. Upregulation of aldolase activity as observed in our result, is known to represses glycolysis and activate gluconeogenesis in response to fungal infection as reported in maize [73].

Signal transduction pathway plays a very important role in coordinating overall activity for plant defense mechanism. In our study too, jasmonic acid (JA) and salicylic acid(SA) controlling genes were found to be differentially expressed. In combined analysis of both the genotypes, SA and JA pathways were found to be downregulated. Auxin, known to mediate plant defence response in interaction with SA and JA pathway [74,75] was found to be downregulated. Similar results were also observed in *Arabidopsis* [76,77] and tomato [76]. The lipoxygenase gene expressed in response to wounds was found to be upregulated as also reported in soybean [78].

In addition to response against abiotic stress, ABA signalling plays important role in regulation of innate immunity. It acts as negative regulatory compound by inactivating other defense signalling pathways involving SA and JA [79]. In our study, we found downregulation of ABA pathway genes similar to *Arabidopsis* and upregulation of NADPH oxidase. NADPH oxidase is known to play important role in resistance against *Fusarium* as also reported in banana [80]. Signal transduction components like mitogen-activated protein (MAP) kinases (MAPK) were found to be upregulated. This enables fungal pathogen to overcome on plant defence response leading to manifestation of wilt disease [81].

Isoforms are known to generate phenotypic variability from a

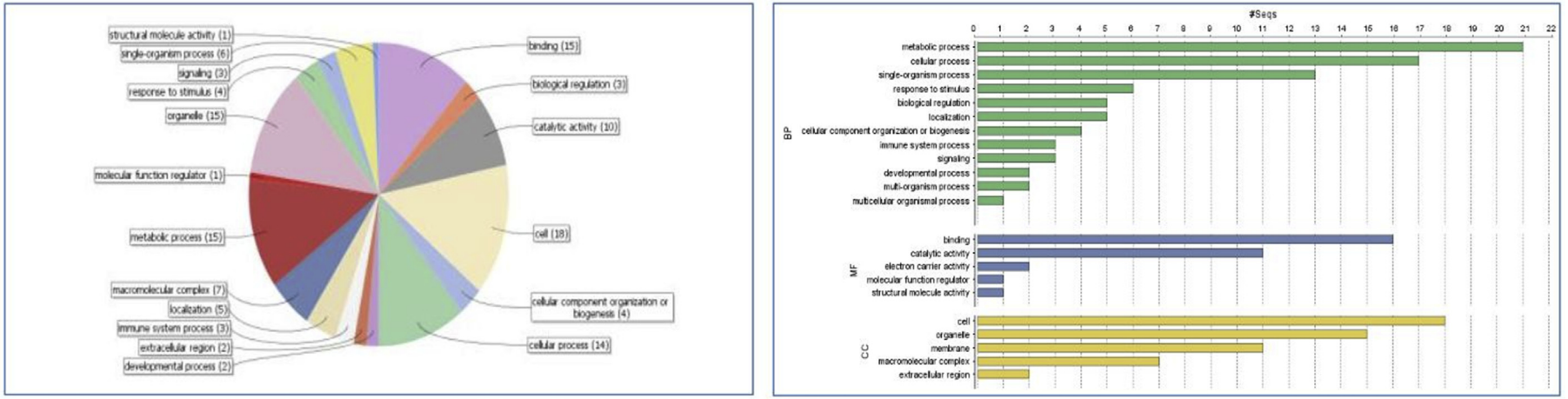

Fig. 7. GO terms and GO distribution of differentially expressed genes of BG256 variety.

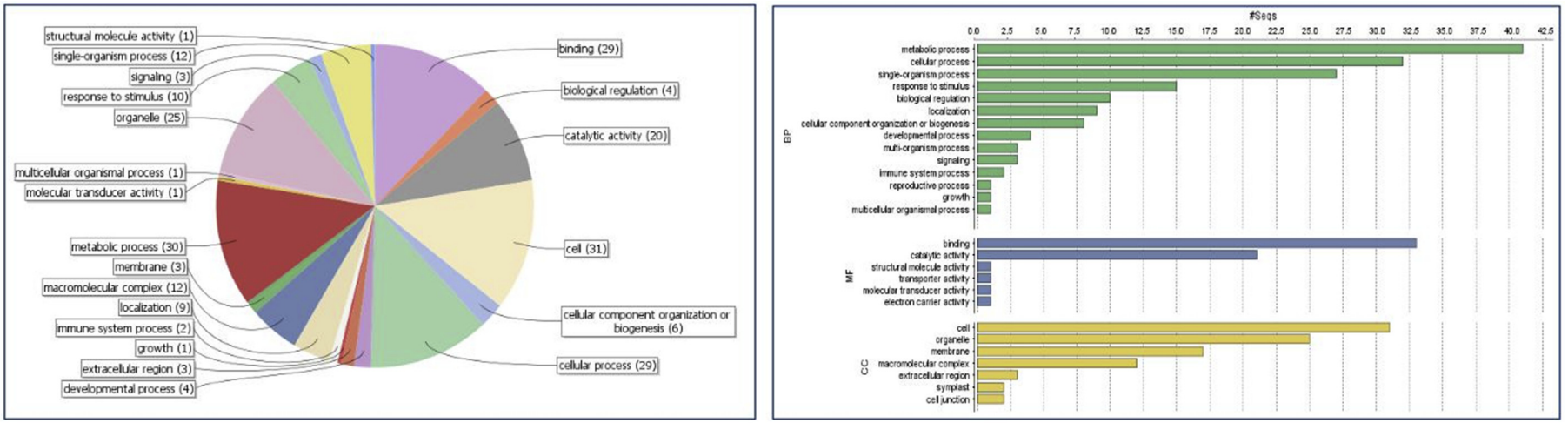

Fig. 8. GO terms and GO distribution of differentially expressed genes of WR315 variety.

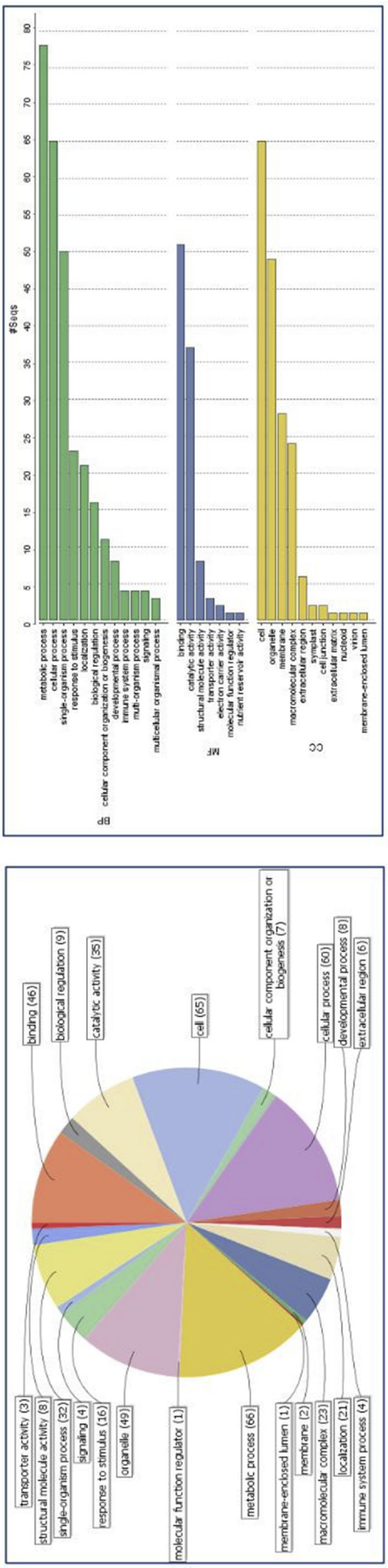

Fig. 9. GO terms and GO distribution of differentially expressed genes of all.

Table 3
Number of STRs mined from the three sets of assemblies.

STR Type	BG256	WR315	BG256 + WR315
Mono	3	4	4
Di	126	94	197 (3)[a]
Tri	276 (2)[a]	31 (2)[a]	441 (4)[a]
Tetra	8	1	10
Penta	6	2	8
Hexa	5	5	9
C	21	21	41

[a] Numbers in brackets denotes the STRs from DEGs.

Table 4
Number of SNPs mined from the three sets of assemblies.

	BG256	WR315	All
SNP	221	246	337
Indel	12	21	40
Total	233	267	377

single gene [82]. We found one differentially expressed gene having 6 isoforms in case of susceptible genotype and two isoforms in resistant genotype, whereas there were four differentially expressed genes each with two isoforms in combined analysis. Manifestation of senescence in leaf by SAP (senescence associated protein) is reported to have both up and downregulated gene pathways [83]. Similarly in susceptible genotype, we also found senescence related protein gene transcript having 3 isoforms of which 2 were upregulated (8 and 10 fold) and one downregulated (3 fold). In resistant genotype, the upregulated carbonic anhydrase (CA) gene family had at least two isoforms playing role in process of photosynthesis, respiration, pH homeostasis and ion transport to provide resilience to fungal attack [84]. CAs are ubiquitous enzymes involved in fundamental processes like photosynthesis, respiration, pH homeostasis and ion transport. In overall comparison, we found ubiquitin gene having at least two isoforms with three fold high expression in stressed condition. It clearly signifies that initially in both wilt resistant and wilt susceptible genotypes under stress condition. Ubiquitin protein degrades the cellular protein leading to catabolic pathway where ubiquitination is a well-established imperative step [85]. In plants, ubiquitin/proteasome pathway of protein degradation has been implicated in defence [86]. These findings suggest that regulated protein synthesis, modification and protein turnover may play central role in enabling plants to alter their proteome to maximize their chances of survival under adverse conditions [15]. We found that chloroplastic gene fructose-bisphosphate aldolase 1 with two isoforms was downregulated by 7 folds which can further be correlated with lower metabolite production, lowering of antimicrobial activity and subdued photosynthetic activity as expected in wilting tissue. Interestingly, heat shock protein gene, both nuclear (heat shock protein 18.5 kDA Class I like) and mitochondrial (heat shock protein 22kDA) with two isoforms showed 7 fold increase in expression in stress. This is obviously expected it acts as molecular chaperone involved in molecular folding in order to protect the cell [87].

DEGs controlled by miRNAs can be of much relevance as, gene silencing by miRNA in plants has been reported [88]. Those miRNA controlling signalling pathway can be of immense use in wilt management. Such reports are available in other crops with reference to wilt disease caused by *Fusarium oxysporum* [89] and *Verticillium* wilt disease [90]. DEGs identified in the study may be targeted by gene silencing technology to control wilt. Such Host-induced gene silencing (HIGS) of fungal genes has already been reported for barley powdery mildew caused by *Blumeriagraminis*, pathogen [91–93]. Recently such use of RNA interference

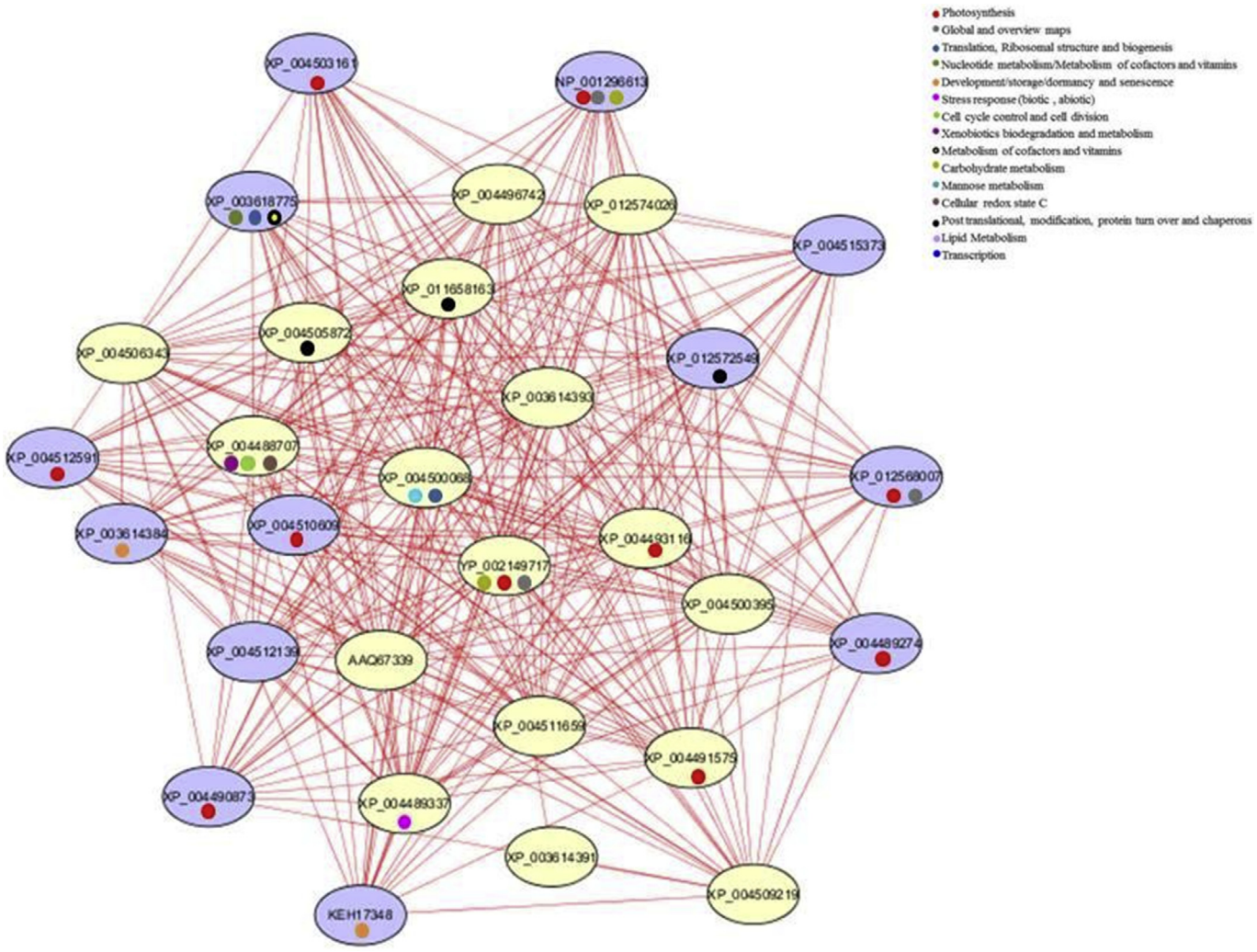

Fig. 10. Gene Network Analysis in BG-256 genotype: Co-expression network of 32 global DEGs. Network depicting relationships among differentially expressed genes involved in BG-256 genotype. Nodes represent the 32 global DEGs (Yellow Colour) associated with Edges (Red Colour). Purple nodes represent important hub gene. Different colour of Dots represents the gene involved in different Pathways. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

technology has been successfully used as non-transgenic control of plant diseases by external spray for example maize crop disease caused by sugarcane mosaic virus [93] and tobacco crop disease caused by tobacco mosaic virus or Potato virus Y [94]. RNA silencing mediates resistance in plant against fungal pathogens is already reported [95]. Such approach does not have harmful effect on plants. Present finding of miRNA can be used to artificially synthesised aiRNA having improved target specificity in silencing the non-desirable biological pathway [96].

Besides genome-wide, transcript-based mining of SSR markers called SSR-Functional domain markers (SSR-FDM) were also reported [97]. Since they are from coding region thus their variability may represent associated functional variations. These transcript-based SSR markers can be used in linkage mapsing as well as genetic variability and functional diversity analysis [98]. Use of such SSR-FDM are reported in various crops for example tomato and pepper [99], sugarcane [100], holy basil (tulsi) [101], sesame [102], African oil palm [103] and tea [104]. Interestingly we could also observe, limited genotype specific DEG having SSRs which could be potentially used as direct FDM (functional domain markers). For example, in case of susceptible genotype (BG256) gene carbonic anhydrase which is involved in nitrogen metabolism pathway is also reported in maize in similar contrasting genotype transcriptome analysis in response to Fusarium ear rot [105,106]. Another gene ferredoxin NADP leaf chloroplast gene involved in photosynthetic pathway (enzyme: reductase) which is reported to be associated with reduction in photosynthesis in Arabidopsis thaliana [107]. In case of resistant genotype (WR315) we could see gene Heat Shock 83 which is involved in Nitrogen metabolism (enzyme: dehydratase), this gene is reported as DEG in QTL region having candidate gene associated with resistance to fusarium ear rot and fumonisin contamination in maize [108]. Similarly another gene thiosulfate sulfotransferase which is reported to express differentially in response to powdery mildew in wheat Triticum aestivum which has a role in increasing resistance against similar fungal challenge [109].

SNP mined from these transcripts (Table 4) can directly be used as functional domain marker. SNP markers from UTR regions were also reported in other crop also like rice [110], fruit tree crop, Longan [111]. Reported STR/SNP FDM needs population screening in order to get degree of polymorphism/to practically qualify as marker. In addition, key genes and associated markers of wilt disease pathway genes can be used to transfer resistance genes (donor) into high yielding susceptible (recipient) variety of

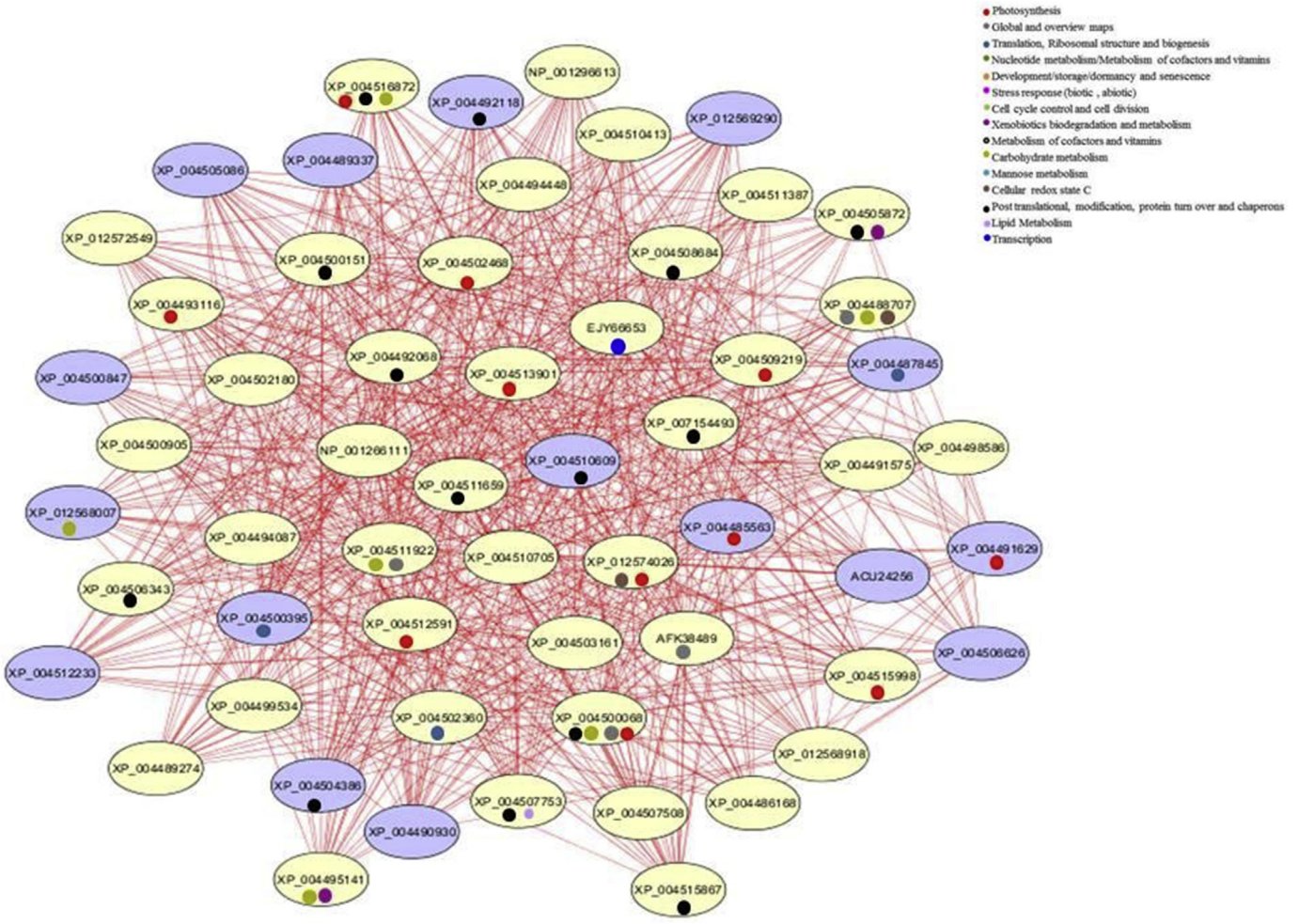

Fig. 11. Gene Network Analysis in WR-315 genotype: Co-expression network of 59 global DEGs. Network depicting relationships among differentially expressed genes involved in WR-315 genotype. Nodes represent the 59 global DEGs (Yellow Colour) associated with Edges (Red Colour). Purple nodes represent important hub gene. Different colour of Dots represents the gene involved in different Pathways. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Role of hub genes controlling gene regulatory network in response to toxin in chickpea.

Hub Gene s	Role of Hub Genes	Reference
Heat Shock Chaperones	This gene is responsible for protein folding, assembly, translocation, degradation and control of cellular processes involved in wilt stress induced by toxin.	[43]
Galactocinol sucrose galactosyl transferase	This mediates as signalling molecule in wounding process and accumulates in vegetative tissues in response to various abiotic stresses.	[44]
Polyubiquitin	In ubiquitin–proteasome system, it works as central modifier of plant signalling mediating targeted protein degradation in plant senescence in response to stress.	[45]
Comparative Analysis of Zinc Finger Proteins Involved in Plant Disease Resistance	They are involved in plant disease resistance especially in LRR region and *R-Avr* interaction.	[46]
Elongation Factor Hub gene	In case of Arabidopsis thaliana, it has been reported for NB-LRR-dependent signalling and MAMP/PAMP-mediated signalling in microbial plant interaction.	[47]
Carbonic Anhydrase Hub gene in chick pea	It mediates reduction in energy production by repression of P700 chlorophyll a-apoprotein and NADH-plastoquinone oxidoreductase, cytosolic fructose 1,6-bisphosphatase and splicing factor-like protein. This is also involved in signalling cascades responsible for sensing and relaying osmotic stress signals which is required to maintain the ionic balance.	[48]
Carbonic anhydrase and redox based signalling	It mediates pathogen/pathogen toxin induced changes in the cellular redox environment which are sensed by this key regulatory protein, modulating plant immunity.	[49]
SAP	Leaf senescence involves programmed cell death mediated by SAP genes. This is mediated by a network of genes altering chlorophyll and pigment content with reduced photosynthesis followed by hydrolysis and dismantling of cellular organelles	[50]
Hub gene 60 S	This mediates osmotic stress tolerance against various abiotic stress.	[51]
Ferridoxin NADP	This hub gene controls network of photosynthetic electron transport to chloroplast redox metabolism against oxidative stress	[52]
Hub gene Oxygen Evolving Enhancer (OEE):	It regulates network of various process involved in photosynthesis, energy balance in response to dehydration stress like drought and salinity	[53]
Translation Factor sui1	It mediates biological regulatory network against various abiotic stress including salt stress.	[54]
Xenobiotic bio	It mediates process of biodegradation	[55]

chickpea [2,112–116] as well as in other crops.

5. Conclusion

This is the first transcriptomic profiling report with gene regulatory network of chickpea challenging purified fusarium toxin in extreme genotypes. This also resolves the issue of asymmetric toxin uniformity which is always compromised in physically challenged fusarium.

Our study shows that transcriptome analysis of contrasting genotypes reveals signature of wilt disease. We report 111 DEGs of 26 pathways, 9 hub protein, 6 genes having 16 isoforms, 32 genes controlled by miRNA. In marker discovery, we report 441 SSR-FDMmarkers, 337 SNPs and 40 indels. The reported DEGs which are controlled by miRNA can be a promising genomic resource for exploration of silencing technology in disease management. Present finding of DEGs and isoforms can be targeted for SNP discovery in large population. Reported functional domain markers can be of immense use in development of wilt resistance varieties. Elucidated key pathways and its genes can be targeted for future marker discovery and association studies to be used in the endevour of chickpea productivity and sustainable wilt management.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.pmpp.2017.09.006.

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