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Optimization of an Efficient Protein Extraction Protocol Compatible with Two-Dimensional Electrophoresis and Mass Spectrometry from Recalcitrant Phenolic Rich Roots of Chickpea (Cicer arietinum L.)

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Two-dimensional electrophoresis and mass spectrometry are undoubtedly two essential tools popularly used in proteomic analyses. Utilization of these techniques however largely depends on efficient and optimized sample preparation, regarded as one of the most crucial steps for recovering maximum amount of reliable information. The present study highlights the optimization of an effective and efficient protocol, capable of extraction of root proteins from recalcitrant phenolic rich tissues of chickpea. The widely applicable TCA-acetone and phenol-based methods have been comparatively evaluated, amongst which the latter appeared to be better suited for the sample. The phenol extraction-based method further complemented with sodium dodecyl sulphate (SDS) and pulsatory treatments proved to be the most suitable method represented by greatest spot number, good resolution, and spot intensities. All the randomly selected spots showed successful identification when subjected to further downstream MALDI-TOF and MS/MS analyses. Hence, the information obtained collectively proposes the present protein extraction protocol to be an effective one that could be applicable for recalcitrant leguminous root samples.

1. Introduction

Presence of intricate photosynthetic machinery, cell wall and other organelles, complex primary and secondary metabolic processes, and their cellular regulation adds to the complexity of functional biology of plants. In recent years, proteomics has become one of the most enthralling fields in molecular biology as it targets the molecular link in the information chain from protein to its coding sequence and its manifestation in the form of phenotype. In contrast to the relative ease of mRNA isolation, c-DNA synthesis and analysis, protein extraction presents numerous challenges due to its heterogeneous nature, structural complexity and instability. Such features dramatically complicate their extraction, solubilization, handling, separation, and ultimately identification. Moreover no technology currently exists that is equivalent to PCR, which can amplify low abundance proteins [1].

The most critical step in any proteomic study is protein extraction and sample preparation. However, the difficulties involving plant protein extractions especially from roots are quite complicated as compared to other organisms. Root tissues are highly vacuolated with relatively low protein content. They are often rich in proteases, storage polysaccharides, lipids, phenolics and a broad array of secondary metabolites [2–4]. Such contaminants cause major obstacles for two-dimensional electrophoresis (2DE) resulting in horizontal and vertical streaking, smearing, and reduction in the number of distinctly resolved protein spots [5].

The present investigation deals with protein extraction from chickpea roots. Chickpea is the most important legume crop in India and ranks third in the world’s list of important
legumes. Its production is greatly hampered by different abiotic and biotic factors. Major yield loss is caused by root invading pathogens like *Sclerotium rolfsii* (collar rot), *Fusarium solani* (black root rot), *Thielaviopsis basicola* (black streak root rot), *Phytophthora sp.* (*Phytophthora* root rot), *Fusarium sp.* (*Fusarium* root rot), *Fusarium oxysporum* *f.sp.* *ciceris* (*Fusarium wilt*), and so forth. Hence, root proteins serve to be excellent target to study early signaling in plant-pathogen interaction involving root invading pathogens in particular.

Most common and basic protocols used for protein extraction from plant tissue are TCA-acetone and phenol-based extraction methods. TCA-acetone precipitation was initially developed by Damerval et al. [6]. This method increases the protein concentration and helps removing contaminants, although some polymeric contaminants are often coextracted. This appears as a problem with tissues that are rich in compounds such as soluble cell wall polysaccharides and polyphenols. Another method involves protein solubilization in phenol, with or without using SDS followed by precipitation with methanol and ammonium acetate and subsequent resolubilization in IEF (isoelectric focusing) sample buffer [5, 7, 8]. This method can efficiently generate protein extracts from resistant tissues such as wood [9], olive leaves [10], maize roots [11], and hemp roots [12], and so forth. Similar studies also suggested that phenol-based method reduces protein degradation during extraction and helps in solubilizing membrane proteins and glycoproteins [5, 13]. However, requirement of extensive time appears to be the major limitation of this method. Thus, these extraction protocols demand optimization for particular organisms, tissue or cell compartment.

In current study attempts were made to optimize the phenol SDS method along with sonication for protein extraction from small amount of recalcitrant chickpea roots. Evaluations of other different extraction methods were also done in comparison to the optimized phenol SDS sonication method and its compatibility with high throughput method like mass spectrometry analysed.

2. Materials and Methods

2.1. Plant Material. Experiments were performed using chickpea seeds (JG62) obtained from International Crops Research Institute for the Semi Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. Seeds sown in a mixture of sand and synthetic soil (1:1) were allowed to grow in natural green house conditions suited for the crop [14]. Roots of 15–20 days old seedlings were thoroughly washed, frozen in liquid nitrogen, and stored at −80°C prior to extraction of protein.

2.2. Extraction Protocols

(A) TCA-Acetone Precipitation Method. TCA-acetone precipitation was carried out according to Damerval et al. with some modifications [6]. One gram of root material was ground in a precooled mortar in the presence of liquid nitrogen. Approximately 100–150 mg of ground tissue powder was precipitated overnight with freshly prepared 2 mL of 10% TCA, 0.07%β-mercaptoethanol in cold acetone. Following precipitation the set was centrifuged at 10,000 g for 15–20 min at 4°C and the supernatant discarded. The obtained pellet was rinsed twice in ice-cold acetone with 0.07%β-mercaptoethanol. An additional modification was introduced between the rinsing steps by incubating the sample for 60 min at −20°C [15]. The pellet was air dried, resuspended in 100 μL sample buffer (8 M Urea, 2% CHAPS, 50 mM DTT, 0.2% Biolyte 3/10 Ampholyte, 0.001% Bromophenol Blue) (Biorad), and vortexed for 1 hour at room temperature. The supernatant was used for downstream analyses (Figure 1).

(B) Phenol Extraction Method. Phenol extraction method was used both singly and in combinations of extraction buffer and SDS along with variations of with and without sonication (Figure 1).

(B.1) Phenol-SDS Buffer Extraction with Sonication (PSWS). Phenol extraction of proteins was carried out as described by Hurkman and Tanaka [7] in the presence of SDS buffer designated as phenol-SDS extraction by Wang et al. [10]. One gram of root tissue was ground in a mortar in the presence of liquid nitrogen and extracted with 3 mL of SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris-Cl, 5% β-mercaptoethanol, and 1 mM phenyl methyl sulfonyl fluoride (PMSF), pH 8.0). The extract was sonicated 6 times for 15 seconds at 60 amps. Following sonication 3 mL of Tris buffered phenol was added to the mixture and vortexed for 10 mins at 4°C. The set was centrifuged at 8,000 g for 10 min at 4°C, phenolic phase collected and reextracted with 3 mL SDS buffer and shaken for 3–10 min. Centrifugation was further repeated using the same settings, phenolic phase collected and precipitated overnight with four volumes of 0.1 M ammonium acetate in methanol at −20°C. Precipitate obtained by centrifugation at 10,000 g for 30 min at 4°C was washed thrice with cold 0.1 M ammonium acetate and finally with cold 80% acetone. The pellet was dried and resuspended in 100 μL sample buffer (Biorad) and used for further analyses.

(B.2) Phenol-SDS Buffer Extraction without Sonication (PSWOS). This method was same as mentioned in case of PSWS only with the elimination of the sonication step.

(B.3) Phenol-Extraction Buffer with Sonication (PEWS). One gram of frozen root tissue was homogenized in liquid nitrogen and was extracted with ice-cold extraction buffer (500 mM Tris-Cl, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, pH 8.0) at 4°C. The extract was sonicated 6 times at 60 amps for 15 sec and further extracted with Tris buffered phenol as described in PSWS.

(B.4) Phenol-Extraction Buffer without Sonication (PEWOS). Protein extraction was carried out in the same way as described in case of PEWS with elimination of the sonication step.
Tissue powder was homogenized with extraction buffer (4°C) with or without sonication (60 amp, 15 seconds, 6 times) PSWS. Tissue powder was homogenized with extraction buffer with SDS (4°C) with or without sonication (60 amp, 15 seconds, 6 times) PEWS. With sonication resulted in PSWOS. Without sonication resulted in PEWOS.

Grind plant root tissue in precooled mortar and pestle with liquid nitrogen. 100 mg of tissue powder was precipitated overnight with 10% TCA and 0.07% β-ME in cold acetone. Acetone (−20°C) wash was added with 60 min incubation in between. Addition of IEF buffer to the dried pellet and vortexing for 1 hr (room temperature).

Samples were extracted with Tris buffered phenol and centrifuged at 8000 rpm for 10 minutes at 4°C.

Phenolic phase was removed and precipitated with 0.1 M ammonium acetate in methanol by centrifuging at 10,000 rpm for 30 minutes. Pellet obtained was washed finally with acetone, air dried, and solubilized with minimal amount of IEF sample buffer (Biorad).

Solubilized samples were estimated and were used for two-dimensional gel electrophoresis.

Figure 1: Schematic representation of extraction of protein from chickpea roots using TCA-acetone and phenol based extraction protocols.

(B.5) Phenol-Extraction Buffer with SDS. This protocol was similar to phenol extraction method. The buffer composition was the same as mentioned in PEWS pH 8.0 with 2% SDS as an additional component. However appearance of a white precipitate following SDS addition to the basal phenol extraction buffer prevented further processing of the sample using this buffer (Figure 1).

2.3. Protein Quantification. Protein concentrations were quantified using the Bradford protein assay method using BSA as a standard [16].

2.4. Two-Dimensional Electrophoresis (2DE). IPG strips (11 cm, 3–10 nonlinear, Readystrip, Biorad) were passively rehydrated overnight with rehydration sample buffer containing 250 μg of isolated protein. IEF was carried out on PROTEAN IEF Cell (Biorad) at field strength of 600 V/cm and 50 mA/IPG strip. The strips were focused at 250 V for 20 mins, 8000 V for 2 hours 30 mins with linear voltage amplification, and finally to 20,000 volt hour with rapid amplification. Following IEF, the strips were reduced with 135 mM DTT in 4 mL of equilibration buffer (20% [v/v] glycerol, 0.375 M Tris-Cl, 6 M urea, 2% [w/v] SDS, pH 8.8) for 15 mins and alkylated with 135 mM iodoacetamide in 4 mL equilibration buffer for 15 mins. The 2DE was performed using 12% polyacrylamide gels (13.8 cm × 13.0 cm × 1 mm) in an AE-6200 Slab Electrophoresis Chamber (Atto Biosciences and Technology, China) at constant volt (200 V) for 3 hours 30 mins in Tris glycine-SDS running buffer. All 2DE gel separation was performed in triplicates for all the methods. The gels were stained with 0.1% (w/v) coomassie brilliant blue R-250 (Sigma) overnight, destained, and stored in 5% acetic acid at 4°C for further analysis.

2.5. Image Analysis of 2D PAGE Gels. Coomassie stained 2-D gels were visualized using Versa Doc (Model 4000) Imaging System (Biorad) and analyzed with PD Quest Advanced 2-D Analysis software (version 8.0.1, Biorad). Spots were detected automatically by the Spot Detection Parameter Wizard using the Gaussian model with standard parameters. Comparison between spot quantities across gels was performed accurately, and normalization was done using local regression model. Only spots present in each of the three replicate gels, with high and low intensity, were randomly chosen for subsequent analyses. Selected protein spots were subjected to in-gel digestion for identification by MALDI-TOF MS and MS/MS analyses.

2.6. MALDI-TOF MS and MS/MS Analysis and Database Search. Spots were excised from protein gels, and in-gel digestion was performed as described by Shevchenko et al.
Table 1: Protein yield/fresh weight of root tissue (μg/gm) using Bradford method.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Protein yield (μg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSWS</td>
<td>603 ± 6.08</td>
</tr>
<tr>
<td>PSWOS</td>
<td>406 ± 5.77</td>
</tr>
<tr>
<td>PEWS</td>
<td>302 ± 5.51</td>
</tr>
<tr>
<td>PEWOS</td>
<td>408 ± 7.64</td>
</tr>
<tr>
<td>TCA</td>
<td>73 ± 2</td>
</tr>
</tbody>
</table>

Table 2: Total number of spots using different methods.

<table>
<thead>
<tr>
<th>Methods</th>
<th>Average number of spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSWS</td>
<td>446 ± 9.07</td>
</tr>
<tr>
<td>PSWOS</td>
<td>287 ± 6.43</td>
</tr>
<tr>
<td>PEWS</td>
<td>338 ± 6.11</td>
</tr>
<tr>
<td>PEWOS</td>
<td>348 ± 1.53</td>
</tr>
</tbody>
</table>

with minor modifications [17]. Proteins were digested in gel using porcine trypsin (Promega) and were extracted using 25% acetonitrile and 1% trifluoroacetic acid. One microlitre of sample and matrix (α-cyano-4-hydroxy cinnamic acid, HCCA) (Bruker, Daltonics) was loaded in a Anchor Chip MALDI Plate (Bruker, Daltonics).

Mass spectra were obtained on an Autoflex II MALDI TOF/TOF (Bruker, Daltonics, Germany) mass spectrometer equipped with a pulsed nitrogen laser (λ=337 nm, 50 Hz). Then the spectra were analysed with Flex Analysis Software (version 2.4, Bruker, Daltonics). The processed spectra were then searched using MS Biolog ((version 3.0) program, against the taxonomy of Viridiplantae (green plants) in the MSDB database using MASCOT search engine (version 2.2). The peptide mass fingerprinting parameters included peptide mass tolerance (≤100 ppm); proteolytic enzyme (trypsin); global modification (carbamidomethyl, Cys); variable modification (oxidation, Met); peptide charge state (1+) and maximum missed cleavage 1. The significance threshold was set to a minimum of 95% (P ≤ 0.05). The criteria used to accept protein identification were based on molecular weight search (MOWSE) score, the percentage of the sequence coverage, and match with minimum five peptides. MS/MS was performed to confirm the identification with matched peptides, selected on the basis of suitability for fragmentation (signal strength and relative isolation).

3. Results

3.1. Protein Quantification

3.1.1. TCA-Acetone Precipitation Method. Protein yield using the classical TCA-acetone precipitation method was extremely low (data not shown). However a modification of incubating the sample at −20°C for 60 minutes in-between the rinsing step yielded a measurable amount of protein. Approximately seventy-three micrograms of protein were obtained from one gram of root tissue using this method (Table 1). However, when the obtained protein was subjected to electrophoresis in SDS PAGE (polyacrylamide gel electrophoresis) gel, no banding profile was visualized (data not shown). Hence, this protocol was eliminated from further downstream analysis.

3.1.2. Phenol-Based Methods. In case of phenol-based methods, protein yields obtained from PSWS, PSWOS, PEWS, and PEWOS were 600 μg, 406 μg, 408 μg, and 300 μg, respectively, (Table 1). One gram of fresh chickpea roots yielded maximum amount of protein with PSWS method as compared to protein obtained by methods PSWOS, PEWS, and PEWOS.

3.2. Data Analysis of 2DE Gels. The 2DE patterns of extracted protein when compared with equal amount of initial protein load revealed that protein extracted by PSWS method displayed a comparatively good resolution with lesser contamination, whereas proteins extracted with methods PSWOS, PEWS and PEWOS resolved fewer protein spots (Figure 2). Approximately 446 detectable spots (as estimated by PD Quest software) were obtained by PSWS method while 287 spots by PSWOS method, 338 by PEWS, and 348 by PEWOS method were detected (Table 2). The number of spots described in Table 2 is the average number of spots across the triplicates. In addition we also found that many spots were diffused or absent in these methods (PSWOS, PEWS, PEWOS) as indicated in the marked areas (Figures 3A, 3B, 3C, and 3D). Intensities of all the spots randomly selected for downstream MS and MS/MS were more in PSWS method as compared to other methods (Figures 4 and 5).

3.3. MALDI-TOF MS and MS/MS Analysis for Protein Identification. All the 9 spots selected for MALDI analysis (Figures 4 and 5), consisting of both less abundant (sp 36, 80, 212) and more abundant (sp 19, 55, 109, 165, 248, 267) proteins, were successfully identified and listed in Table 3 (Figure 6). Data listed in the table include assigned spot number, spot identity, protein identity (MSDB database), number of peptide matches, sequence coverage (%), MOWSE score, accession number, experimental and theoretical molecular weight and pI.
Table 3: Proteins identified by MALDI-TOF MS analyses.

<table>
<thead>
<tr>
<th>S no.</th>
<th>Spot ID.</th>
<th>Protein identity</th>
<th>Peptides matched</th>
<th>Sequence coverage (%)</th>
<th>MOWSE score</th>
<th>Accession number (NCBI)</th>
<th>Mr(kDa)/pl (theoretical)</th>
<th>Plant species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>sp 165</td>
<td>NADP specific isocitrate dehydrogenase</td>
<td>10</td>
<td>17%</td>
<td>70</td>
<td>Q9XGU7_ORYSA</td>
<td>46.4/6.29 (46.0/6.0)</td>
<td>Oryza sativa</td>
</tr>
<tr>
<td>2</td>
<td>sp 212</td>
<td>Glyceraldehyde 3 phosphate dehydrogenase</td>
<td>9</td>
<td>24%</td>
<td>86</td>
<td>Q6K5G8_ORYSA</td>
<td>36.716/7.68 (37/6.5)</td>
<td>Oryza sativa</td>
</tr>
<tr>
<td>3</td>
<td>sp 109</td>
<td>Triose phosphate isomerase</td>
<td>6</td>
<td>20%</td>
<td>71</td>
<td>Q381W8_SOYBN</td>
<td>27.4/5.87 (25/5.5)</td>
<td>Glycine max</td>
</tr>
<tr>
<td>4</td>
<td>sp 55</td>
<td>Fructokinase-like protein</td>
<td>9</td>
<td>40%</td>
<td>94</td>
<td>Q8LPE5_CICAR</td>
<td>26.26/5.03 (35.5, 4.5)</td>
<td>Cicer arietinum</td>
</tr>
<tr>
<td>5</td>
<td>sp 36</td>
<td>ATP synthase (subunit D chain)</td>
<td>13</td>
<td>36%</td>
<td>88</td>
<td>ATPQ_ARATH</td>
<td>19.4/5.09 (20/5.0)</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>6</td>
<td>sp 267</td>
<td>Porin of Pea, channel protein</td>
<td>2</td>
<td>11%</td>
<td>134</td>
<td>T12558</td>
<td>29.7/8.56 (30/9.5)</td>
<td>Phaseolus coccineus</td>
</tr>
<tr>
<td>7</td>
<td>sp 19</td>
<td>Plasma membrane intrinsic polypeptide</td>
<td>10</td>
<td>38%</td>
<td>74</td>
<td>Q9SMK5_CICAR</td>
<td>23.3/4.95 (24.5/5.0)</td>
<td>Cicer arietinum</td>
</tr>
<tr>
<td>8</td>
<td>sp 248</td>
<td>Unidentified protein</td>
<td>11</td>
<td>35%</td>
<td>80</td>
<td>CAA06491</td>
<td>22.12/9.91 (44/9.9)</td>
<td>Cicer arietinum</td>
</tr>
<tr>
<td>9</td>
<td>sp 80</td>
<td>Putative pyruvate dehydrogenase E1 beta subunit isoform 1 protein</td>
<td>2</td>
<td>6%</td>
<td>55</td>
<td>Q6Z1G7_ORYSA</td>
<td>40.2/5.25 (38.5/5.3)</td>
<td>Oryza sativa</td>
</tr>
</tbody>
</table>

Figure 3: 2DE profiles of chickpea root proteins of JG 62. Profile of proteins isolated using PSWS (a), PSWOS (b), PEWS (c), and PEWOS (d) extraction protocols. Inset A, B, C, D represents a close-up view of an area showing spot resolution: in PSWS (a), PSWOS (b), PEWS (c), and PEWOS (d), respectively.
**Figure 4:** 2DE profiles with marked spots selected for MALDI-TOF MS and MS/MS. (a) 2DE profile using PSWS, (b) 2DE profile using PSWOS, (c) 2DE profile using PEWS, and (d) 2DE profile using PEWOS.

### 4. Discussion

Secondary metabolites are known to play important role in structural composition and defense of plants. These metabolites accumulate in various soluble forms in vacuoles and cause severe interference in protein extraction as well as separation in 2DE gels [18, 19]. Chickpea roots are rich in phenolic compounds like tannic acid, gallic acid, 2-coumaric acid, chlorogenic acid, cinnamic acid; flavanoids, isoflavonoids like daidzein, genistein, as well as tannins, lignins, and carbohydrates [20, 21]. These compounds form hydrogen bonds with proteins. Besides they also form irreversible complexes with proteins by oxidation and covalent condensation which leads to charge heterogeneity resulting in streaking of gels [22]. Carbohydrates block gel pores causing precipitation and prolonged focusing time, which also results in loss of protein spots and streaks in the gels [15]. Although the amount of these secondary metabolites is comparatively low in etiolated tissues like roots, but low protein content and limiting tissue amounts demand for a competent protein extraction method. In our study TCA-acetone method and phenol-based method using two different extraction buffers (SDS buffer and extraction buffer without SDS) with and without sonication were evaluated. Comparison was done on the basis of protein yield, spot focusing, resolution, number of resolved spots, and also intensities of the spot and their downstream analysis using high throughput technology (MALDI/MS) of the optimized method.

Quantitative comparison of protein extracts revealed that phenol-based methods gave higher protein yield as compared to TCA-acetone method. The major reason for low protein yield in TCA-acetone method which constrained it for further downstream processing could probably be attributed to the insolubility of protein pellet in IEF buffer as compared to phenol-based methods [23]. Moreover TCA-acetone protocol is known to be effective with tissues from young plants and was found not to be the best choice for more complex tissues [5, 10, 15].

In case of phenol extraction, the proteins were first homogenized in two different extraction buffers; both the buffers contained sucrose which was added to create phase inversion. These buffers formed the aqueous lower phase containing carbohydrates, nucleic acid, insoluble cell debris, while the upper phenol phase contained cytosolic and
Figure 5: Continued.
Membrane proteins, lipids, and pigment [15]. SDS buffer contained about 30% sucrose which helped in better phase separation as compared to extraction buffer (24%). The high pH buffers inhibit common activity of the proteases [24] and cause ionization of phenolic compounds, thus preventing them from forming hydrogen bonding with the protein [22]. It also neutralizes the acids that are released by disrupted vacuoles. PMSF and β-mercaptoethanol which were used in both buffers in the present study were reported to irreversibly inhibit serine protease action and act as a reducing agent which prevents protein oxidation, respectively. KCl and EDTA were used in case of extraction buffer without SDS (PEWS and PEWOS). KCl facilitates the extraction of proteins by its salting in effect and EDTA inhibits metalloprotease and polyphenoloxidase by chelating metal ions [15]. Although the salting in effect or chelation of metal ions could not improve the protein yield as compared to SDS buffer with sonication, SDS is known to act as an excellent solubilizing agent, which allows the recovery of membrane-bound proteins [10]. The solubilization of protein was found to increase with sonication as evident from the increase in protein yield and spot resolution after sonication in PSWS compared to PSWOS. Sonication results in better disruption of cell membrane and release of intracellular proteins and thus provides explanation for SDS to have efficiently solubilized the protein in PSWS method. In contrary, in case of extraction buffer, sonication could not improve protein yield or resolution, presumably due to the interference with constituents of buffer (KCl or EDTA) or due to lack of better solubilizing agent like SDS and/or both.

The phenol used in this method was buffered to pH 8.0 to ensure that nucleic acids are partitioned to the buffer phase and not to phenol-rich phase [25], and thus proteins in phenol phase were purified and concentrated simultaneously by subsequent methanol ammonium acetate precipitation. Phenol acts as one of the strongest dissociaters known to decrease molecular interaction between proteins and other materials [15]. It can minimize protein degradation resulting from endogenous proteolytic activity [26]. Phenol extraction method though with high clean-up capacity has a little tendency to dissolve polysaccharides and nucleic acids.

We found that in PSWS method the spots obtained were well resolved and showed high intensity (Figures 3 and 5) as compared to PSWOS, PEWS, and PEWOS. About 25% unique spots were obtained in PSWS and the rest 75% spots though existed in PSWOS, PEWS, and PEWOS, however, resolved with variable clarity. Streaking was absent in all the gels. We could see that the difference in number of spots between PSWS and PSWOS was more as compared to PEWS and PEWOS, which confirmed that the effectiveness of SDS increased in presence of sonication. However in the latter case (PEWS, PEWOS) sonication did not have much influence.
Improvisation of the extraction buffer was also made by adding 2% SDS, which resulted in precipitation. Interference between constituents of the extraction buffer and SDS was assumed to be the cause of such precipitation. However, further experimentation needs to be performed for confirmation of such predictions.

All protein spots selected for MALDI-TOF/MS and MS/MS from PSWS resulted in successful identification. High intense spot like sp 55, (fructokinase-like protein) and less intense spot like sp 212, (glyceraldehyde 3-phosphate dehydrogenase) both resulted in high quality spectra with low background noise (Figure 6). These results further indicated the compatibility of PSWS method with both MS and MS/MS and its reliability for downstream processing.

5. Conclusion

The present study emphasizes PSWS as the optimized phenol-based method for chickpea root protein extraction. This method successfully isolated high quality protein suitable for downstream processing. Hence, the data obtained projects this protocol as an effective and efficient one that could be applied for other recalcitrant leguminous root tissues as well. Nevertheless, it should be kept in mind that one generalized protein extraction protocol applicable for global protein profiling of variable tissues irrespective of their origins though theoretically conceivable, but fails to meet practical feasibility.

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References


Analysis of root proteome unravels differential molecular responses during compatible and incompatible interaction between chickpea (*Cicer arietinum* L.) and *Fusarium oxysporum* f. sp. *ciceri* Race1 (Foc1)

Muniya Chatterjee†, Sumanti Gupta†, Anirban Bhar1, Dipankar Chakraborti2, Debabrata Basu1 and Sampa Das1*

**Abstract**

**Background:** Vascular wilt caused by *Fusarium oxysporum* f. sp. *ciceri* Race 1 (Foc1) is a serious disease of chickpea (*Cicer arietinum* L.) accounting for approximately 10-15% annual crop loss. The fungus invades the plant via roots, colonizes the xylem vessels and prevents the upward translocation of water and nutrients, finally resulting in wilting of the entire plant. Although comparative transcriptomic profiling have highlighted some important signaling molecules, but proteomic studies involving chickpea-Foc1 are limited. The present study focuses on comparative root proteomics of susceptible (JG62) and resistant (WR315) chickpea genotypes infected with Foc1, to understand the mechanistic basis of susceptibility and/or resistance.

**Results:** The differential and unique proteins of both genotypes were identified at 48 h, 72 h, and 96 h post Foc1 inoculation. 2D PAGE analyses followed by MALDI-TOF MS and MS/MS identified 100 differentially (>1.5 fold<, p < 0.05) or uniquely expressed proteins. These proteins were further categorized into 10 functional classes and grouped into GO (gene ontology) categories. Network analyses of identified proteins revealed intra and inter relationship of these proteins with their neighbors as well as their association with different defense signaling pathways. qRT-PCR analyses were performed to correlate the mRNA and protein levels of some proteins of representative classes.

**Conclusions:** The differential and unique proteins identified indicate their involvement in early defense signaling of the host. Comparative analyses of expression profiles of obtained proteins suggest that albeit some common components participate in early defense signaling in both susceptible and resistant genotypes, but their roles and regulation differ in case of compatible and/or incompatible interactions. Thus, functional characterization of identified PR proteins (PR1, BGL2, TLP), Trypsin protease inhibitor, ABA responsive protein, cysteine protease, protein disulphide isomerase, ripening related protein and albumins are expected to serve as important molecular components for biotechnological application and development of sustainable resistance against Foc1.

**Keywords:** Chickpea (*Cicer arietinum* L.), *Fusarium oxysporum* f. sp. *ciceri* Race 1(Foc1), Defense response, Root proteomics
Background

Plants are often challenged by different types of biotic and abiotic stress factors. Their immobile nature precludes escape from these stress causing agents. Therefore, they possess preformed and inducible defensive strategies to overcome these stresses. In most cases, the host arrests the invading rival at the site of penetration [1]. Such immune response adapted by the host is termed as pattern triggered immunity (PTI) which include reprogramming of host cellular metabolism, reinforcement of cell wall by callose occlusions and production of antimicrobial compounds that act directly to prevent pathogen invasion [2,3]. However, in some selected cases the invading pathogens secrete effector molecules that try to overcome host immunity, which in the absence of cognate host resistant protein/proteins (R-proteins) lead to effector triggered susceptibility (ETS) [4]. On the other hand, in the presence of cognate R protein/proteins the host mounts a defense response of much greater amplitude known as the effector triggered immunity (ETI), which largely overlaps with that of PTI [5]. However, the defense mechanisms of both PTI and/or ETI are regulated by altered protein synthesis and their time dependent degradation. Hence, qualitative and qualitative changes in protein levels are believed to be probable indicators of the ultimate outcome of any plant-pathogen interaction.

Amongst agronomically important crop plants, legume crops are known for their nutritive value that play very important roles in human nutrition as well as serve as supplement to improve growth of livestock [6]. Besides, they also fix atmospheric nitrogen enhancing soil fertility and boosting the yield of subsequently grown crops [7]. These crops are equally vulnerable towards pathogen. But studies on the molecular interaction involving legume-pathogen case study are significantly limited. Chickpea is the third most important legume crop in the world and the most important one in India (FAO). It is a rich source of digestible protein, and hence is considered globally as a valuable crop. However it is found that it accounts for 10% of digestible protein, and hence is considered globally as a valuable crop. However it is found that it accounts for 10% of digestible protein, and hence is considered globally as a valuable crop. However it is found that it accounts for 10% of digestible protein, and hence is considered globally as a valuable crop. However it is found that it accounts for 10% of digestible protein, and hence is considered globally as a valuable crop. However it is found that it accounts for 10% of digestible protein, and hence is considered globally as a valuable crop. However it is found that it accounts for 10% of digestible protein, and hence is considered globally as a valuable crop. However it is found that it accounts for 10% of digestible protein, and hence is considered globally as a valuable crop. However it is found that it accounts for 10% of digestible protein, and hence is considered globally as a valuable crop. However it is found that it accounts for 10% of diges...
Medicago [24,25]. Information gathered from these model plants definitely boost up knowledge of plant immunity but biological interpretation of this knowledge in crop models require experimental substantiation. Moreover, some features and processes are likely to be unique for crop plants and hence cannot be approached via model plant in totality [26]. The present study focuses on the legume crop chickpea and its early response to infection by Foc1. This study aims to understand the mechanistic basis of susceptibility and/or resistance offered by two different genotypes (JG62 wilt susceptible, WR315 wilt resistant) respectively. Approximately 100 proteins were significantly identified by MALDI-TOF MS and MS/MS which included differentially regulated as well as unique proteins identified from both resistant and susceptible genotype of chickpea at different time points of 48 h, 72 h and 96 h after infection with Foc1. These identified proteins are categorized and their probable roles in plant defense are illustrated through interaction network based studies.

Methods

Plant growth and fungal treatment

Chickpea (Cicer arietinum L.) genotypes JG62 (wilt susceptible) and WR315 (wilt resistant), obtained from ICRISAT (International Crops Research Institute for Semi Arid Tropics), Hyderabad, India were used for experimental analysis. Seeds of both genotypes were grown in a mixture of soil and sand (1:1) under natural greenhouse conditions of 22 to 28°C, 35 to 40% relative humidity and 16 h:8 h photoperiod of day and night respectively [15].

F. oxysporum f. sp. ciceri Race1 (Foc1) was obtained from ICRISAT and further purified according to the protocol of Summerell et al [27]. Spores obtained were harvested and stored at -80°C until further use. Two week old seedlings of both genotypes were inoculated with Foc1 using sick soil method as described by Gupta et al [15]. Plants of both genotypes grown on inoculum free soil served as control samples. Both control and infected plants were kept under same growth conditions. Root samples from control and infected plants at 48, 72 and 96 h post inoculation (hpi) were harvested, instantly frozen in liquid nitrogen and stored at -80°C for further analysis. Proteins were extracted from pooled tissue by following Phenol-SDS buffer extraction method with sonication [29]. One gram of root tissue was pulverized in mortar and pestle with liquid nitrogen and homogenized with 3ml of SDS buffer (30% sucrose, 2% SDS, 0.1M Tris-Cl, 5% β-mercaptoethanol and 1 mM phenyl methyl sulfonyl fluoride (PMSF), pH 8.0). The extract was sonicated (60 amps, 15 secs, 6 times) and further treated with Tris buffered phenol. The phenolic phase obtained by centrifugation at 8000 g for 10 min at 4°C was rinsed with SDS buffer. This final phenolic phase was collected and precipitated overnight with four volumes of 0.1M ammonium acetate in methanol at -20°C. Precipitate was obtained at 10,000 g for 30 min.

Washing of protein pellet was performed thrice at 8,000 g for 10 min with cold 0.1 M ammonium acetate and finally washed with cold 80% acetone. The pellet was then dried and resuspended in 100 µl sample buffer (Biorad) for further analysis. Extracted proteins were quantified using Bradford protein assay method using BSA as standard [30].

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Isoelectric focusing (IEF) was carried out on PROTEAN IEF cell (Bio-Rad, USA) using immobilized pH gradient (IPG) strips. Two hundred fifty micrograms of each sample protein dissolved in 185 µl of rehydration sample buffer (8M urea, 2% CHAPS, 50 mM DTT, 0.2% Biolyte ampholytes) was loaded onto 11 cm immobilized pH 3-10 nonlinear (NL) gradient strips (Bio-Rad, USA) and was passively rehydrated overnight at room temperature. IEF was conducted at field strength of 600 V/cm and 50 mA/IPG strip. The strips were focused at 250 V for 20 min, 8000 V for 2 h 30 min with linear voltage amplification and finally to 20,000 Volt hour with rapid amplification. After focusing the strips were reduced and alkylated using 135 mM DTT and 135 mM iodoacetamide respectively, in 4 ml of equilibration buffer (20% v/v glycerol, 0.375M tris-Cl, 6M urea, 2% w/v SDS, pH8.8) for 15 min. Second dimensional electrophoresis was run with strips transferred to 12% SDS polyacrylamide gels (13.8 cm x 13.0 cm x 1 mm) in an AE-6200 slab electrophoresis chamber (Atto Biosciences and Technology, China) at a constant volt (200 V) for 3 h 30 mins in tris-glycine SDS running buffer. The gels were stained with 0.1% (w/v) coomassie brilliant blue R-250 (Sigma) overnight, destained and stored in 5% acetic acid at 4°C. 2D-PAGE gel separation was performed with both technical and biological replications of three.

Image acquisition and analysis

Coomassie stained 2-D gel images were captured with Versa Doc Imaging system (Model 4000, Bio-Rad, USA) and analyzed with PD Quest Advanced 2-D gel analysis software (version 8.0.1, Bio-Rad, USA). For this study, in total 72 reproducible gels were generated (three replicates, four time points, two genotypes and three
biological replicates). Three technical replicates from three biological replicates at different time points (control, 48 h, 72 h, 96 h) for both genotypes (JG62, WR315) were assembled to create the master gel image (match set). Replicate gels used for making the match set had correlation coefficient value of at least 0.8. Background subtraction between the gels was done using floating ball method. Spots were detected automatically by the spot detection parameter wizard using Gaussian model with advance settings, by choosing faint spot, small spot and large spot cluster. Detected spots were visually checked and manually added when required [31]. Each spot included for analysis were present at least in two of the three replicate gels for a particular time point and also was of high quality. Detected spot volumes were normalized by the spot volume of the entire gel and used as a parameter for quantifying protein abundance. The differential spots which showed statistical significance level of p < 0.05 (Student’s t-test) were selected for analyses. However, the spots selected for downstream MALDI-TOF MS and MS/MS analyses fell under three main categories. Firstly it included the spots showing 1.5 fold changes (above or below) in protein abundance level in infected samples at least in any of the time points as compared to the comparable protein level of both the controls. Second category included spots which were accumulated after infection and present in more than one time point in infected samples but absent in controls. Third category included qualitative spots which are reproducibly present only in one infected variety for a particular time point. Spots which were present only in one replicate were not considered for analysis to minimize the interference of missing value. Experimental molecular mass and pI were calculated using 2D-PAGE gel images of standard molecular mass and pI markers. Data were further analyzed using Statistica v10.0 software (Statsoft Inc) through coefficient of variance calculation (CV), followed by comparison of control and treated values to find out statistical differences by multivariate analysis of variance (MANOVA) and Duncan’s multiple range test (DMRT), at p value 0.05. Protein spots that showed significant difference between treatments through DMRT were further processed for downstream MALDI-TOF MS and MS/MS analyses.

Protein identification using MALDI-TOF MS and MS/MS

Protein spots were manually excised from 2D-PAGE gels, destained and in gel digested according to the protocol mentioned by Shevchenko et al. [32] with minor modifications. In gel digestion of proteins were carried out with porcine trypsin (Promega, USA) and peptides were extracted with 25% acetonitrile and 1% trifluoroacetic acid. One microlitre of sample was loaded along with matrix (1 μl, α-cyano-4-hydroxy cinnamic acid, HCCA) (Bruker Daltonics, Germany) in an Anchor Chip MALDI Plate (Bruker Daltonics, Germany).

Mass spectra were generated in an Autoflex II MALDI TOF/TOF (Bruker Daltonics, Germany) mass spectrometer equipped with a pulsed nitrogen laser (λ:337 nm, 50 Hz) in the m/z range from 500 to 3500 Da. The enzyme used was trypsin with one missed cleavage. The spectra obtained were analyzed with Flex Analysis Software (version 2.4, Bruker Daltonics, Germany) for deletion of matrix peaks and tryptic autolysis peaks. Processed spectra were then searched using MS Biotools (version 3.2) program against the taxonomy Viridiplantae (Green plants) in the MSDB 20060831 (3239079 sequences; 1079594700 residues), NCBI nr 20140323 (38032689 sequences; 13525028931 residues), SwissProt 2013_12 (541954 sequences; 192668437 residues) databases using MASCOT search engine (version 2.2). The standard parameters used in the search included peptide mass tolerance (±0.5 Da); fragment mass tolerance (±0.8 Da); proteolytic enzyme (trypsin); global modification (carnomethyl, Cys); variable modification (oxidation, Met); peptide charge state (1+) and maximum missed cleavage of 1, for MALDI-TOF MS minimum S/N = 10 and for MS/MS minimum S/N =3. The significance threshold was set to a maximum of 95% (p</* = 0.05). The criteria used to accept protein identification were based on molecular weight search (MOWSE) score, and the percentage of sequence coverage. From each samples most intense m/z values were chosen for further fragmentation (MS/MS). Automatic decoy database search was performed by choosing the decoy checkbox on MASCOT search engine. Decoy search was performed to avoid false identification of peptide by matching it to a random sequence from a decoy database. Only the results with 0% false discovery rate were accepted. Final protein identification was done by a combined search of PMF (Peptide Mass Fingerprint) and MS/MS data in MASCOT search engine.

Protein interaction network generation and analysis

Pathway Studio software (version 7.1) (Ariadne Genomics, USA) and Res Net database (version 3.0) was used to study the biological interactions [33] between the identified proteins of the present study. These differential and unique protein sequences identified by PMF and MS/MS studies were subjected to BLAST analyses at TAIR database (The Arabidopsis Information Resource) and their homologous genes (bearing TAIR gene IDs, Additional file 1) used as inputs for network generation. Ambiguities and components without any interactive neighbors were eliminated from the import list. Interaction network was generated using the neighbor joining method with a degree of correlation as 1 (only the immediate
upstream and downstream neighbors having direct relationship to the protein/protein products were considered for analyses). In addition, standard filter parameters and relation types were selected for interaction map generation. Presence of the identified proteins in known biological pathways was analysed using AraCyc and Ariadne Pathway data list. Functional classification of the identified proteins based on gene ontologies (GO) were also studied using Pathway Studio software. In both cases statistical significance (p < 0.05) of the pathway locations and GO classification of the identified protein were calculated.

Quantitative real time pcr (qRT-PCR)
Total RNA was extracted from one gram root tissues of infected and uninfected plants of both genotypes at different time points of 48 h, 72 h and 96 h post infection. RNA was extracted using TRI reagent kit (Himedia, India) as per manufacturers' instruction. For avoiding any DNA contamination RNA samples were treated with RNase free DNase (Fermentas, USA). cDNA synthesized using Revert Aid first strand cDNA synthesis kit (Fermentas, USA), was further used for qRT-PCR. Specific primers were designed based on the corresponding nucleotide sequence of identified proteins from CTDB (Chickpea Transcriptomic Database), DFCI (Medicago truncatula database), PDB (Protein data Bank) and NCBI database using Gene Runner software (version 3.1) and listed in Additional file 2. qRT-PCR was performed on Biorad i cycler (Bio-Rad I-Q5, USA) using SyBr green super mix. A reaction mix of 20 μl was prepared containing 25 ng cDNA, 0.3 μM of forward and reverse primers. The PCR conditions used were 95°C for 5 mins, followed by 40 cycles at 95°C for 30 sec, 50°C-55°C for 30 sec and 72°C for 30 sec [16]. A melt curve was also generated at the end of each PCR cycle to verify primer specificity. Sample variation was minimized by normalization using actin as internal standard [34]. Mean fold change was calculated using 2^−ΔΔct method [35]. All experiments were repeated three times and standard error was calculated.

Results and discussion
Analysis of chickpea root proteome
Chickpea root proteome was studied with a view to understand the molecular mechanism governing the susceptibility and/or resistance of chickpea plant upon pathogen infection. Previous results based on histopathological and transcriptomic analyses performed by our research group as well as others, suggested the time points of 48 h, 72 h and 96 h to be crucial for delineating the early defense responses of chickpea during Foc1 attack [15,16,18]. These previous reports stated 96 h as the onset for xylem vessel colonization in compatible roots, while significant differential transcriptomic alterations were detected at as early as 48 h in both the susceptible and resistant genotypes [15,16,18]. An estimated protein yield for all the samples are provided in Additional file 3. Total root proteins were resolved onto 11 cm IPG strip (pH 3-10 NL). Figure 1(A and B) shows representative 2D experimental gel profiles corresponding to control and infected samples at different time points for both the genotypes.
genotypes, JG62 and WR315 respectively. The experimental design is shown in Additional file 4. Three independent experiments were performed to ensure that the changes in protein abundance at each time point were reproducible and significant. Two dimensional gel analyses indicated differential protein profiles for JG62 and WR315 plants upon Foc1 infection. Further PD Quest software analysis detected a total of 274 spots in the master gel (Figure 1C). The number of total spots detected and the differential spots (quantitative and qualitative) obtained post inoculation with Foc1 for each sample is provided in Additional file 3. To assess the reproducibility of the corresponding protein quantification, the CV was calculated for all protein spots, at all time points examined. The CV of protein spots for each sample type and time points was within 21% which is in accordance with other plant stress related studies [36] indicating stability and reproducibility of the present data. Among the total 206 differential spots obtained 163 spots which fell under the previously described three categories were processed for downstream MALDI-TOF MS and MS/MS analysis. MS/MS analyses was performed with 137 spots of which 100 spots that showed significant scores were taken into consideration for further functional clustering. Differential spots obtained due to differences in genotypes, depicting the natural variation between the susceptible and resistant genotypes (i.e differentially abundant between control samples of JG62 and WR315) were excluded from further downstream analyses in the present study (data not shown). Relevance of such differences between both genotypes that could also add significantly to the understanding of chickpea-Foc1 interaction shall be dealt separately in future studies. Selected protein spots were found to be interspersed at and around the median region of IPG strip suggesting the critical pH range for resolving the differential proteins to be around pH 4-7 (Figure 1C). Finally MS/MS analyses using mascot search engine in the available databases (NCBI, MSDB, Swissprot) led to the successful identification of 100 spots (Figure 1C). The details of these proteins and their peptides identified by MS/MS is provided as a table in Additional file 5. Among these 100 spots, 65 spots showed significant (1.5 fold change) quantitative changes in infected genotypes (JG62 and WR315) as compared to comparable protein level in control and 35 spots showed qualitative changes. Out of these 35 spots, 28 were accumulated after infection in more than one time point of either/or both infected genotypes, absent in controls and 7 spots were unique for any one time point and genotypes. MANOVA followed by DMRT indicated the statistical significance of the data provided in Additional file 6. Means that do not share any common alphabet differ significantly by DMRT at 5% level.

Identification and classification of differential and unique proteins in chickpea during Foc1 infection

The identified proteins were classified into nine functional categories based on their putative biological functions and proteins with unassigned functions were categorized as unclassified group. Metabolism related protein (36%) constitute the most abundant group followed by proteins related to scavenging of reactive oxygen species (ROS) (16%), protein synthesis and degradation related proteins (11%), defense related proteins (7%), signaling proteins (7%), storage proteins (6%), transport proteins (4%), developmental proteins (3%), structural proteins (1%) (Figure 2A). The unclassified group accounts for 9% of total identified proteins. Metabolism related proteins were further classified into glycolysis related proteins (31%), proteins of TCA cycle (17%), ATP synthesis and degradation regulating proteins (14%), proteins related to amino acid metabolism (19%), secondary metabolism (8%) and sugar metabolism (5%). Moreover, 3% proteins were found to be related to electron transport and another 3% were related to cell wall metabolism and transport (Figure 2B). Many defense related proteins

![Figure 2](attachment:Figure_2.png)

**Figure 2** Distribution of functional classification of Identified proteins. Functional classification and relative distribution of proteins altogether identified in JG62 and WR315 chickpea genotypes after infection (A). Classification and categorization of metabolism related proteins (B). The proteins belonging to different categories and their expression level at different time intervals are mentioned in Additional file 5 in detail.
identified had scores below 70. Complete draft genome sequence of chickpea has been recently reported, but functional annotations of genes and gene products are still at initial stages. Chickpea is a legume and its closest completely sequenced neighbor legumes are model plants Medicago and Lotus. However, the functional annotation of these neighbor model legumes are also underway and constantly being updated. Besides, chickpea being a crop legume is expected to have some distinct differences with these model legumes. Such differences are likely to be reflected in the protein identification scores of chickpea when subjected to homology matches with these model legumes. Hence, all the important defense related proteins obtained from the present study were discussed even though their scores were in the range of 40-60. Previous studies conducted with chickpea also reported similar identification scores for protein identification [36,37]. In most of the cases each protein spots were identified as a single, unique protein but in some cases the identified protein spots contained more than a single protein; in such cases, the first hit with maximum score was considered for their protein IDs [38]. In addition to this, multiple spots were also found which were identified as the same protein. The appearance of such proteins probably suggests them being chemically and/or molecularly different products of a single gene and referred to as protein species [39] (Additional file 5). They basically fall under three main categories (i) with same molecular mass and different pI; for example, Kunitz protease inhibitor (sp 2, sp 13), Annexin (sp107, sp 499), Glyceroldehyde 3 phosphate dehydrogenase EC 1.2.1.9 (GAPC) (sp505, 509), (ii) with different molecular mass but same pI; for example Cysteine proteinase (sp 99, sp 45), (iii) or with different molecular mass and pI; for example, Superoxide dismutase EC 1.15.1.1 (sp 67, sp103, sp401), Triose phosphate isomerase EC 5.3.1.1 (sp 109, sp 40, sp 85). The differences in Mr and pI values, suggest that these changes in the proteome are probably due to the post-transcriptional modification. They may belong to different members of the same functional family, indicated by small shift in the pI or are degraded protein products as suggested by significant differences between theoretical and observed Mr values. The slight differences in pI and Mr values probably reflect post translational modifications (like phosphorylation, acetylation, glycosylation, methylation) occurring in vivo or may be the result of modifications such as demidation of the proteins during sample preparation and processing [25]. It is known that the same protein may have different functions in different subcellular compartments. In the present study superoxide dismutase (sp 103, sp 401), triose phosphate isomerase (sp 109, sp 85) and GAPC (sp 505, sp 509) were identified as protein variants present in different cellular compartments like mitochondrida, chloroplast or cytosol. Hence their multiple forms may be attributed to their multiple cellular locations [39]. In most of the stress related studies GAPC showed post translational modification like phosphorylation and was found to be present as multiple protein species. But whether the same observation in the present study indicates same modifications needs validation [40].

Proteins related to direct defense responses against Foc1

Defense related proteins contribute to about 7% of total identified proteins. They include PR1 (pathogenesis related protein 1), BGL, EC 3.2.1.39 (glucan endo 1-3 beta glucosidase), TLP (thamatin like protein) and TPI (trypsin protease inhibitor) (Figure 3, Additional files 5 and 7). Pathway analysis showed the association of these proteins with defense and hypersensitive response related pathways (Additional file 8). Gene ontology (GO) based classification showed their relation with biological processes, molecular function and their cellular location (Additional files 9 and 10). Schematic network showed the interaction of these components with other Foc1 inducible proteins (Figure 4). PR1(sp 145) protein known to be directly involved in plant defense against pathogen attack was found to be accumulated at 48 h and 72 h post infection in resistant plants while in case of susceptible plants protein level was not detectable after infection (Figure 3, Additional files 5 and 7). PR1 expression known to be regulated by salicylic acid (SA) is positively regulated by NPR1 (Non expressor of PR genes) during defense [41]. Besides, ACD (accelerated cell death), known to accelerate cell death in Arabidopsis is also a positive regulator of PR1 [42]. MAP kinase (Mitogen activated protein kinase), EDS4 (Enhanced disease susceptibility 4), PAD2 (Phytoalexin deficient 2) linked to fungal defense response also regulate PR1 expression [43,44]. On the other hand, studies conducted on Arabidopsis thaliana reported EDR2 (Enhanced disease resistance 2), NPR3 and NPR4 to be negative regulators of PR1 [45,46]. PR1 expression is also reported to be altered by phospholipase C and fatty acids [47,48]. In the present study the increase of PR1 protein in resistant plants suggests its direct role in Foc1 induced defense, although the role of SA in modulating resistance in the present case study is still speculative. BGL also known as PR2, are enzymes which mainly act by hydrolyzing 1-3 β D glucosidic linkage of fungal cell wall and hence known to provide resistance in plants. BGL (sp 239) was found to be up accumulated in response to fungal attack in both genotypes. However, the susceptible plants showed highest accumulation at 72h (Figure 3, Additional files 5 and 7) that decreased later. Both BGL and PR1 are known to have SA dependent expressional regulation [49]. Both PR1 and BGL are reported to be upregulated in over expression lines containing EIL (ethylene-insensitive3-like) transcription factor in Vigna mungo indicating a positive role of ethylene in regulating defense response [50]. TLPs
Figure 3 (See legend on next page.)
are pathogenesis related proteins having antifungal activity. TLP, also known as PR5 (sp 83,129) was found to be significantly increased in response to Foc1 in both genotypes (Figure 3, Additional files 5 and 7). However, in resistant plants it showed uniform accumulation while in susceptible plants (sp 129) it was found to be absent at later time points (72 h and 96 h). TLP was found to be up accumulated in Medicago truncatula during Orobanche crenata infection indicating that it may eventually take part in defense mechanism against parasitic infection [25]. TPI are known to participate in the wound induced defense response of plants against herbivores and pathogens. TPI (sp 2, 13, 81) were found to be uniformly enhanced in response to Foc1 induction in resistant plants while susceptible ones showed protein level undulations (except for sp 81, which showed uniform protein accumulation). (Figure 3, Additional files 5 and 7). TPI is positively regulated by JA signaling [51]. WRKY transcription factors coordinating herbivory are also known to regulate TPI expression [52]. The induction of TPI probably indicates the involvement of SA/JA mediated hormonal crosstalk which needs further experimentation. Role of PR proteins (PR1, PR2 and PR5) in modulating defense network were also elaborated by transcriptomic as well as proteomic studies involving wheat (Triticum aestivum L.) and stripe rust fungus Puccinia striiformis f.sp. tritici Eriks. (Pst) [53,54].

Role of ROS scavengers/regulators
Sixteen percent of total proteins were classified as ROS scavengers/regulators. Superoxide dismutases, EC 1.15.1.1 (SOD), Peroxiredoxin proteins, Ascorbate peroxidase, EC 1.11.1.11 (APX), Ferric reductase EC 1.6.2.6, Glutathione S transferase, EC 2.5.1.13 (GST), Peroxidase, Thioredoxin (NTRA, NTRB), Monodehydroascorbate reductase, EC 1.6.5.4 (MDHAR, MDAR), Quinone oxidoreductase, EC 1.12.5.1 etc (Figure 3, Additional files 5 and 7) are the proteins included in this class. Pathway analysis showed association of some proteins (SOD, APX, NTRA and NTRB, MDHAR and MDAR etc) with ROS regulatory processes.

![Figure 3](See figure on previous page.)

**Figure 3** Heat map representation of differentially expressed proteins of JG62 and WR315 chickpea genotypes on infection with Foc1. Heat map was generated with the fold change values considering infected/control ratios. Each column represents a particular time point of infection and each row represents corresponding proteins with their identities. Up regulation or down regulation is indicated by the above scale which shows pale to saturated colors of green and red respectively. Yellow color represents mid-value and white represents no expression.

![Figure 4](See figure on previous page.)

**Figure 4** Schematic representation showing the location and interaction between the different Foc1 induced proteins in chickpea roots. Representation shows the intra and inter relationship between the Foc1 induced proteins and their regulatory biological processes. (Complete names of abbreviated proteins are provided in Additional file 1).
pathways (Figure 4, Additional file 8). GO classification illustrated the roles of these proteins according to their biological processes, molecular functions and cellular components (Additional files 9 and 10). Figure 4 showed their cellular location and their interaction with other Foc1 induced proteins. SODs (sp 67, 103, 401) showed oscillations in protein accumulation post infection in both genotypes (Figure 3, Additional files 5 and 7). SODs are known to provide the first line of defense to infected hosts by scavenging the pathogen triggered ROS [55]. SODs are also reported to induce ROS mediated PRI expression in Nicotiana [56]. APX (sp 134, 87, 323) (Figure 3, Additional files 5 and 7) is an important enzyme participating in anti oxidation metabolism in plants [57]. Besides, they are also reported to be upregulated during heat stress in Arabidopsis [58]. Differential induction of SOD and APX in the present study indicated the role of Foc1 induced ROS in triggering defense responses in chickpea. These observations support previous reports based on transcriptomic studies [16,18]. Ferric reductase plays an important role in maintaining iron homeostasis, disruption of which may lead to generation of toxic free radicals. Ferric reductase (sp 232) also known to be an antioxidant for peroxides, showed enhanced protein level in susceptible plants compared to resistant ones (Figure 3, Additional file 5). GST (sp 324,211,317) showed marginal changes in protein accumulation in resistant plants while susceptible plants showed relatively sharp increments and decrements in protein level post Foc1 induction (Additional files 5 and 7). GSTs are reported to reduce oxidative stress inductive organic hydroperoxides in Nicotiana benthamiana following Colletotrichum destructivum infection [59]. In the present study, steady state protein level of GST in resistant plants may indicate lesser accumulation of oxidative stress components as compared to susceptible plants. NTR (sp 78) showed increment in resistant plants following Foc1 infection while susceptible plants showed sharp decline after 72 h of infection (Figure 3, Additional file 5). Such up accumulation of NTR only in resistant plants indicated their efficient role in regulating oxidative stress tolerance [60]. Previous proteomic studies conducted on wheat showed enhanced accumulation of GST and NTR during incompatible interaction with Puccinia striiformis f. sp. tritici Eriks. (Pst). Besides, level of peroxiredoxin was also found to be induced [54]. In addition transcriptomic studies showed the enhancement of peroxidase transcripts in wheat following Puccinia striiformis f. sp. tritici Eriks. (Pst) infection [53]. MDHAR (sp 199) showed similar protein accumulation levels in both plants post infection (Figure 3, Additional files 5 and 7). Such increment indicated role of MDHAR in JA mediated antioxidation metabolism in the present case study that was found to be similar to previous results reported on Arabidopsis thaliana [61]. Besides, increment of MDHAR also linked to increased lipid peroxidation which is marked as a feature during pathogen mediated membrane injury [62]. Quinone oxidoreductase, known to act as detoxifier of ROS induced oxidative stress along with GST was found to be up accumulated at later time points of infection in susceptible plants compared to resistant ones (Figure 3, Additional file 5).

Role of signaling proteins

Signaling proteins constitute about 7% of total identified proteins. Guanine nucleotide binding protein (AGB), Annexins (ANNATs), ABA responsive protein (RAB), Ran binding protein (RANBP), Auxin induced protein and Zinc binding dehydrogenase are classified under this category (Additional file 5). Pathway analysis based on Arabidopsis homologues showed only the association of AGB with signaling pathway (Figure 4). While GO classified all the proteins in this category (AGB, ANNATs, RAB18 and RANBP) according to their relation with biological processes, molecular function and cellular components (Additional files 9 and 10). Network map showed their interaction with other Foc1 induced proteins (Additional file 8). AGB (sp 233) coupled with other G proteins and GPCRs are known to modulate defense responses in Arabidopsis [63]. Besides, AGB are also known to modulate ABA driven K+ and anion channels thus regulating stomatal movement [64]. In the present study, similar protein accumulation pattern of AGB (Figure 3, Additional file 5) in both plants indicate a common regulation of AGB that is probably directed towards stomatal movement, a significant phenomenon observed during vascular wilt. Annexins (sp 107, 499) (Figure 3, Additional files 5 and 7) are reported to regulate pH mediated cellular responses that are directly influenced by ABA and calcium conductance during stress in Arabidopsis and Zea mays [65,66]. The up accumulation of annexins in both plants probably directs the role of Foc1 in triggering pH alterations as well as ABA driven calcium oscillations during infection that needs to be investigated. RAB (sp 71) was found to be up accumulated only in resistant plants post infection. RAB was reported to be induced during ABA perception that activated calcium influx in Arabidopsis thaliana suspension culture cells [67]. Such induction was further known to be mediated by phospholipase D activation [68]. In the present study induction of RAB only in resistant plants directs towards role of ABA and calcium signaling in modulating defense in chickpea during Foc1 infection. RANBP (Sp16) known to regulate nucleocytoplasmic transport under the control of hormones and light, was found to be uniquely expressed at 72 h post infection in susceptible plants [69]. The relevance of such selective induction in the present study requires further investigation.
Role of metabolism related proteins

Majority of the proteins identified (36%) fell under metabolism related proteins (Figure 2A). This category was further re-categorized into several sub classes (Figure 2B, Figure 3, Additional files 5 and 7). Such large assemblage of metabolism related proteins indicates that pathogens usually target the host metabolism for self survival and reproduction, while on the other hand host puts forth complete effort in shielding their primary metabolism from the devastations of pathogen attack [16]. Pathway analysis showed the association of some of these proteins with metabolic pathways (Figure 4). GO classification grouped them according to their biological processes, molecular functions and cellular components (Additional files 9 and 10). Interaction map further showed the location and interaction of some of these proteins with their neighbors as well as within themselves (Additional files 8). Glycolytic enzymes triose phosphate isomerase, EC 5.3.1.1 (TIM) (sp 109, 85, 40) and glyceraldehyde dehydrogenase phosphate, EC 1.2.1.9 (GAPC) (sp 212,505,509) were found to show similar pattern of protein level undulations in both compatible and incompatible interaction suggesting the common role of glycolytic ATP on pathogen triggered immune response of host [70]. However, enolase EC 4.2.1.11 (LOS) (sp 198) showed sharp decline at later time points of infection in susceptible plants while resistant plants showed steady state protein level (sp 198) or sharp induction (sp 182, 351) at different time points of infection. Enzymes of TCA cycle such as isocitrate dehydrogenase, EC 1.1.1.42 (ICDH) (sp 165), malate dehydrogenase, EC 1.1.1.37 (sp 156) and fumarase, EC 4.2.1.2 (FUM) (sp 271,272) showed elevated or stable protein accumulation in resistant plants as compared to susceptible plants suggesting a constant energy supply, which is required for different processes like photosynthesis, respiration and photorespiration during stress [71-73]. ATP synthase (sp 36, sp 196) and ATPases (VHA) (sp 480,124,168) however showed similar protein level patterns in both plants after infection. This may indicate the need for maintaining energy and soluble homeostasis necessary for protein sorting and cell wall repair that probably aid to cell protection during pathogen progress- ion [74]. Similar interaction studies involving wheat and stripe rust fungus reported the increment of ATP synthase both at transcriptomic as well as proteomic levels [53,54]. Cytochrome c oxidase, EC 1.9.3.1 (COX) (sp 11) which is known for translocation of protons to drive aerobic respiration as well as to regulate stress mediated signals [75] showed elevated protein levels in resistant plant at later time period as compared to susceptible plants. These findings suggest that even though, energy requirement is necessary for both the genotypes during stress, but proper channelization of energy needed for running basic metabolic activities controls resistance, which perhaps is efficiently maintained by the resistant plants. Cysteine protease (RD) (sp 45) showed up accumulation of protein only after Foc1 infection in both plants. RD is known to be important players in plant immunity, especially in regulating resistance response against necrotrophic pathogen [76]. In the present study the selective up accumulation of RD after Foc1 infection predicts the role of RD in regulating biotrophic interaction also. However, such assumption requires further experimental support. Phosphoserine amino transferase, EC 2.6.1.52 (PSAT) (sp 264) was found to be absent at 96 h in susceptible plants while resistant plants maintained a moderate protein accumulation level even after infection suggesting the need of serine biosynthesis which is known to be associated with photo-respiration [77]. S-adenosyl methionine synthetase, EC 2.5.1.6 (SAM) (sp 122) a direct product of methionine catabolism acts as substrate for several transmethylation reactions including those that occur during lignin biosynthesis [78]. SAM was found to be absent at 96 h post inoculation in susceptible plants while resistant plants regained the protein accumulation at 96 h suggesting the role of transmethylation and lignin biosynthesis in somehow regulating repair mechanisms caused by pathogen invasion. Proteins related to secondary metabolism (sp 207, 173, 164) showed differential abundance level post infection in both plants. They are known to regulate defense response during biotic stress [79]. Methylesterase (sp 290) were found to be select- ively enhanced at 72 h post infection in both the plants. Methylesterases are known to be directly or indirectly associated with defense reactions by regulating the degree of methyl esterification of pectin that is known as essential cell wall components [80]. Selective accumulation of methyl esterase after infection in both plants suggests a possible cell wall repair mechanism to be operational, which however may be more efficient in resistant plants as indicated by its elevated level.

Role of proteins involved in its folding, synthesis and degradation

This group of proteins accounts for about 11% of total identified proteins. Pathway analysis showed the association of adenylate kinase EC 2.7.4.3 (ADK, AMK2) with protein synthesis and purine biosynthetic pathways (Figure 4). While all other proteins related to protein synthesis, folding and degradations showed enlistment under categories of GO (biological function, molecular function and cellular component) (Additional files 9 and 10). Network analyses also showed the intra and inter relationship of these proteins with other Foc1 induced proteins (Additional file 8). 26S proteasome
subunits are known to contribute to both basal defense as well as R gene mediated defense in Arabidopsis. Activation of these proteins are known to regulate innate immunity both positively and negatively as appropriate protein degradation are necessary for mounting defense [81]. Besides, studies on Nicotiana reported the induction of 20S proteasome subunits that was found to be linked to HR and SAR [82]. In the present study the differential accumulation of 26S proteasome subunits EC 3.4.25.1. (sp 57, 10, 240) in both the plants post infection suggests the role of protein degradation in regulating defense (Figure 3, Additional files 5 and 7). However, whether such regulation is directed towards positive and/or negative influences needs to be investigated in detail separately for both compatible and incompatible interaction. Adenylate kinase EC 2.7.4.3 (ADK, AMK2) (sp 234) was found to be up accumulated in 48 h in resistant plants while susceptible plants maintained an overall low protein level. ADK, known to be involved in salvage pathways of adenine and adenosine also convert cytokinin and ribosides to corresponding nucleotides. Such cytokinin conversion regulates the hormonal level of plants [83]. Absence of ADKs is known to cause chloroplastic deformity in Arabidopsis [84]. In the present case study overall down accumulation of ADKs (Figure 3, Additional files 5 and 7) probably indicates pathogen mediated chloroplastic damage and hormonal alteration. Eukaryotic translation initiation factor (eIF5alpha) (sp 106) protein was uniquely accumulated at 48 h in resistant plants while susceptible plants showed no accumulation. Studies conducted on Arabidopsis showed the involvement of eIF5alpha in controlling resistance by preventing pathogen growth and development of Pseudomonas syringae [85]. Besides, eIF5alpha was also up accumulated during infection with stripe rust fungus in resistant wheat plants [54]. However, whether the accumulation of eIF5alpha protein at a specific time point post infection in resistant plants has a similar role in restricting the pathogen progression needs to be experimented. Protein disulphide isomerase (PDIL) (sp 102) was found to be up accumulated at 48 h in resistant plants which gradually declined at later time points however maintaining a moderate level compared to control plants even after 96 h of infection. In susceptible plants the accumulation level of PDIL were greater compared to control samples only at 72 h post infection and absent in other time points (Figure 3, Additional files 5 and 7). PDIL acts as chaperones of cysteine proteases, thus regulating their trafficking from endoplasmic reticulum to vacuole prior to PCD [86]. Besides, PDIL are also known to be reduced by thioredoxin reductases and actin and removing aberrant disulphides formed by oxidative stress [87]. Level of PDIL was found to be elevated in wheat following inoculation with stripe rust fungus [54]. The abundance of PDIL in resistant plants in the present case study suggests the operation of antioxidant defense machinery during incompatible interaction in chickpea against Foc1 attack.

**Role of developmental, structural, channel and storage proteins in Foc1 induced defense**

Developmental, structural, channel and storage proteins contributes to about 14% in total. Functional classification identified developmental proteins such as ripening related protein (RLP) (sp 137) and germin (sp 556, 478) to be differentially expressed in both the plants after infection (Figure 3, Additional files 5 and 7). RLP contains the conserved Bet v fold domain also present in major latex proteins (MLPs) and PR10 group of allergens. These proteins are associated with fruit and flower development as well as defense. However their role in defense is not well characterized [88]. In the present study the protein abundance of RLP at 48 h in resistant plants suggests this protein somehow modulate initial defense response which requires further characterization. Germins, known to have roles in plant development and defense, are associated with extra cellular manganese-SOD activity [89]. The up accumulation of sp 556 protein at later time points (96 h) in resistant plants and protein level undulations of sp 478 in both plants post infection suggests a differential operation of antioxidant defense mechanism in controlling pathogen invasion in both plants. However, transcriptomic based studies reported the increment of germin like transcripts in response to stripe rust fungus specifically in resistant genotypes of wheat [53]. Structural protein profilins (PRFs) are actin monomer binding proteins that regulate the assembly-disassembly of uncapped-capped actin molecules in forming cytoskeletal filaments [90]. Profilin (sp 3) protein was found to be uniquely accumulated at 48h in resistant plants post infection. Such selective accumulation probably indicated the need of cytoskeletal assembly to strengthen the cell and prevent further fungal ingress. However, such assumption needs further experimental support. Channel proteins porin (sp 267) and plasma membrane intrinsic protein (PMIP/PIP) (sp 19) belong to the aquaporin family of proteins that are known to regulate hydraulic conductance during cold and oxidative stress [91]. The present study showed differential protein accumulation profiles in both genotypes after Foc1 induction, which suggested that probably the channel proteins regulated water transport differently during incompatible and compatible interaction. The enhanced level of these proteins at 96 h post infection in resistant plants compared to susceptible ones suggested proper water conductance in resistant plants when susceptible plants succumbed to wilting symptoms. Plant albumins are known to serve as storage proteins as well as defense responsive proteins possessing insecticidal
and antimicrobial properties that are induced in response to stress [92]. The present study showed up accumulation of albumin (sp 244, 485) in resistant plants compared to susceptible ones indicating the role of storage proteins in controlling defense against fungal attack.

Unclassified proteins
This group mainly includes proteins with unknown functions (Figure 3, Additional file 5). They contribute to about 9% of total proteins identified (Figure 2). Recent availability of chickpea whole genome sequences and updating of functional annotations is believed to provide proper naming and functional designations to these unclassified proteins [93].

Probable roles of identified proteins in imparting resistance against Foc1
To understand the mechanism of resistance in plants it is important to know what are the different proteins involved and how they come into play during the pathogen attack. Pathogenesis related proteins are defense related proteins which are induced on pathogen attack and have a direct role in plant defense, but how these proteins operate or accumulate in compatible and incompatible interaction actually decides the sustainability of resistance. In the present study three important PR proteins were identified, PR1 (pathogenesis related protein 1), PR5b (Thaumatin like protein), PR2 (β-1, 3-glucanases) and their accumulation at different time points post infection were studied. All these PR proteins were found to show a stable level of accumulation in resistant plants after infection where as in susceptible interaction although the proteins appear in early time points but at later time point they either decrease or disappear (Additional file 5). More specifically PR1 which has antifungal activity showed high level accumulation in resistant chickpea plants post infection (Figure 3, Additional file 7). Both PR1 and PR2 are also known to be associated with salicylic acid and ethylene signaling indicating their probable roles in modulating defense [49,50]. In addition, uniform accumulation of TPI (trypsin protease inhibitor) in resistant plants pointed towards the role of JA in regulating defense [51]. PR5b (Thaumatin like proteins) are known to be induced exclusively in response to wounding or pathogen infection. This protein exhibit a balanced accumulation in resistant chickpea plants post infection (Figure 3, Additional file 7). Both PR1 and PR2 are also known to be associated with salicylic acid and ethylene signaling indicating their probable roles in modulating defense [49,50]. In addition, uniform accumulation of TPI (trypsin protease inhibitor) in resistant plants pointed towards the role of JA in regulating defense [51]. PR5b (Thaumatin like proteins) are known to be induced exclusively in response to wounding or pathogen infection. This protein exhibit a balanced accumulation in resistant chickpea plants post infection (Figure 3, Additional file 7). Both PR1 and PR2 are also known to be associated with salicylic acid and ethylene signaling indicating their probable roles in modulating defense [49,50]. In addition, uniform accumulation of TPI (trypsin protease inhibitor) in resistant plants pointed towards the role of JA in regulating defense [51]. PR5b (Thaumatin like proteins) are known to be induced exclusively in response to wounding or pathogen infection. This protein exhibit a balanced accumulation in resistant chickpea plants post infection (Figure 3, Additional file 7). Both PR1 and PR2 are also known to be associated with salicylic acid and ethylene signaling indicating their probable roles in modulating defense [49,50]. In addition, uniform accumulation of TPI (trypsin protease inhibitor) in resistant plants pointed towards the role of JA in regulating defense [51].

The sudden generation of ROS and lack of proper ROS scavenging machinery leads to oxidative stress in case of compatible interaction while in case of incompatible interaction they are efficiently detoxified by scavenging machinery. Hence balanced ROS generation in resistant plants act as signaling molecules and communicate downstream defense signals. Previous studies based on transcriptomic profiling indicate the significance of several ROS regulators to act as the initial trigger communicating downstream defense signals [16]. Interestingly the present case study identified similar set of ROS regulating proteins that not only provided correlation between the transcriptomic and proteomic studies, but also highlighted the conservation of ROS components in regulating host defense during Foc1 infection.

The high accumulation of signaling protein like, ABA responsive protein (RAB) only in resistant genotype in present study predicts the involvement of ABA-mediated signaling in plant defense (Additional file 5). ABA responsive protein was reported to be involved in PR-protein
induction and disease resistance in other related studies [98]. Besides, the similar accumulation pattern of AGB (Guanine nucleotide binding protein) and annexinD1 in both genotypes after infection further highlighted the role of ABA and calcium in regulating defense signals.

The accumulation of proteins related to energy metabolism, ATP synthesis and degradation, amino acid metabolism, secondary metabolism etc, in both genotypes; although of different levels suggest that in both cases the pathogen targets the primary metabolism of the host (Additional file 5). Resistant plants probably safeguard their essential metabolic elements from the fungal catastrophe while the susceptible plants fail to do so and submit to pathogenic endeavors.

Initially the events of PTI and ETI were thought to be distinct, but recent studies revealed that the components of PTI and ETI overlap [5]. In present study the initial accumulation of ROS scavengers and regulators direct towards possible responses related to PTI. However categorization of other proteins under the categories of PTI and/or ETI could prove to be erroneous without further experimentation. Even then, all these findings as a whole indicate that plant defenses are controlled by complex signaling pathways which are interconnected to each other.

**Correlation between protein and mRNA levels**

To correlate the protein levels with mRNA levels eleven representative genes corresponding to MS/MS identified proteins were selected and their transcript accumulation versus protein abundance analyzed (Figure 5). The genes of corresponding proteins selected for transcript accumulation were pathogenesis related protein 1(PR1) (sp 145), thaumatin like protein (TLP) (sp 83), glucan-endo-1,3-beta-glucosidase EC 3.2.1.39 (BGL) (sp239), elongation factor 1 (EF1) (sp 275), protein disulfide isomerase (PDI) (sp 102), guanine nucleotide binding protein (GNBP) (sp 233), triose phosphate isomerase EC 5.3.1.1 (TIM)

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**Figure 5** Comparison of mRNA and protein expression levels of eleven representative genes. Quantitative real time PCR was performed using gene specific primers (Additional file 2). The log10 transformed fold change values (infected/control) of protein spot intensities and mRNA expression level were plotted at different time intervals (48 h, 72 h and 96 h) after infection with Foc1 for both chickpea genotypes. JG62 represented by grey color bars and WR315 represented by black color bars. The proteins selected are SOD (superoxide dismutase), PMIP (Plasma membrane intrinsic protein), ICDH (Isocitrate dehydrogenase), FLP (Fructokinase like protein), TIM (Triose phosphate isomerase), GNBP (Guanine nucleotide binding protein), PDI (Protein disulfide isomerase), EF1 (Elongation factor1), BGL2 (Glucan-endo-1, 3-beta-glucosidase), TLP (Thaumatin like protein), PR1 (Pathogenesis related protein1).
dered and/or incompatible chickpea genotypes, but accumulated in response to Foc1 infection in both compatible and/or incompatible chickpea genotypes. The functional characterization of these proteins could not only yield important new findings in reevaluating the resistance mechanism of chickpea during Foc1 infection but also help in directing crop improvement programs by using breeding and genetic engineering techniques. Therefore further experiments are necessitated to strengthen the knowledge and understanding through detailed investigations.

**Conclusion**

The present study was an attempt to investigate the differential root proteome and identify defense related proteins in chickpea during Foc1 infection. Previous report based on proteome studies involving chickpea Foc5 and root knot nematode Meloidogyne artiella highlighted the presence of several defense responsive proteins [100]. But the difference in pathogenic race is expected to yield some case specific results and hence needs to be studied as an individual case study. The findings of this study suggests that albeit some common proteins are accumulated in response to Foc1 infection in both compatible and/or incompatible chickpea genotypes, but their differential temporal accumulation and regulation probably governs the net outcome of the interaction. The present study highlights the role of several important proteins like PR proteins (PR1, BGL2, TLP), Trypsin protease inhibitors (TPI), ABA responsive protein (RAB18), cysteine proteases (RD19, RD21), methyltransferases, 26S proteasome subunits, protein disulphide isomerase (PDIL), ripening related protein (RLP), profilins (PFRs) and albumins and their varied accumulation in susceptible and resistant plants. The functional characterization of these proteins could not only yield important new findings in reevaluating the resistance mechanism of chickpea during Foc1 infection but also help in directing crop improvement programs by using breeding and genetic engineering techniques. Therefore further experiments are necessitated to strengthen the knowledge and understanding through detailed investigations.

**Availability of supporting data**

The data sets supporting the results of this article are included within the article and its additional files. The protein and peptide data sets supporting the results are presented in Additional file 5.

**Additional files**

Additional file 1: Protein names, abbreviations and TAIR gene IDs. Table containing list of proteins, their abbreviations used for pathway construction and qRT-PCR and TAIR homologous IDs of the identified proteins used as input for network generation.

Additional file 2: List of primers designed for the qRT-PCR. List includes the primer pair sequences used for qRT-PCR for identified proteins with their respective spot IDs.

Additional file 3: Comparative analysis of differentially accumulated protein spots in infected chickpea genotypes. Includes details of protein yield, average number of spots, variable spots (Quantitative and qualitative) obtained in control and infected chickpea genotypes (JG62 and WR315) at different time points post Foc1 infection.

Additional file 4: Schematic representation of experimental design. A flow chart depicting the experimental design of Foc1 infected root proteome in chickpea plants. Two weeks old seedlings were infected with Foc1. Root tissues were harvested and 250 μg of proteins were extracted from pooled root tissue to run gels for each time point. The experiments were repeated three times to generate three biological replicates. The gels were stained with coomassie blue and further processed for downstream analyses (In total 72 reproducible 2DE gels were generated). Three technical replicates from three biological replicates were used for PD quest analysis. Differential spots were picked, trypsinized and processed for MALDI-TOF MS and MS/MS.

Additional file 5: Protein spots identified by MALDI-TOF MS AND MS/MS. Includes details of differential and unique proteins and their peptides identified by MS and MS/MS. The expression pattern of these proteins in control and infected chickpea genotypes (JG62 and WR315) at different time points post Foc1 infection are also illustrated.

Additional file 6: MANOVA Table. Table includes mean protein spot intensities for identified protein spots for control and infected chickpea cultivars (JG62 and WR315) at different time points upon Foc1 infection. Each value represents mean of three repeated experiments each with three replications. The means followed by the same letters within a row do not differ statistically according to Duncan’s multiple range tests at a 5% probability level.

Additional file 7: Representative cropped gel images of protein spots belonging to different functional categories. Images show quantitative changes among control and infected plants of both (JG62) and (WR315) chickpea genotypes at different time intervals of 48 h, 72 h, and 96 h after Foc1 infection. The number and name indicate the spot identity and name of the proteins mentioned in Additional file 5. Arrow represents the presence of spots. The proteins represented are Pathogenesis related Protein (PR1), Thaumatin like protein PR- 5b (TLP), Glucan-endo-1, 3-beta-glucohydrolase (BGL2), Trypsin protein inhibitor 3(Tipr-3), Superoxide dismutase (SOD; Mitochondria manganese SOD), Ascorbate peroxidase (APX), Glutathione S transferase para(GST), Monodehydro ascorbate reductase (MDAR), Annexin (ANX), ABA-responsive protein (RAB/ABARE), Auxin – induced protein PCNT1 15 (Aux ind pro), Triose phosphate isomerase(TIM), Enolase, Isocitrate dehydrogenase (NADP) chloroplastic (ICDH); ATP synthase, sub unit D chain (ATPase sub D), S-adenosyl methionine synthetase (SAM), Cysteine proteinase (Cys Pro), Chalcone isomerase(CI), Fructokinase-like protein (FLP), Cytochrome C oxidase subunit 6b-1(COX), Methyltransferase I(MER), Adenylate kinase (ADK), 20S proteasome alpha subunit D (20S Prot alpha-D), Protein disulphide-isomerase A6 (PD), Ripening related protein (RLP/IPR), Germin-like protein (GLP), Profilin-1, Outer plastidial membrane protein porin (Porin), Chain A, Crystal Structure Of A Plant Albumin (Albumin ).
Additional file 8: Network showing the total interaction of different upregulated proteins in chickpea obtained after 48 h, 72 h and 96 h post infection with Foc1. Green and pink highlighted components represent the upregulated proteins of WR315 plants and JG62 plants respectively obtained after 48h of infection with Foc1. Blue and orange highlighted components represent the upregulated proteins of WR315 plants and JG62 plants respectively obtained after 72 h of infection with Foc1. Yellow highlighted components represent the upregulated proteins of WR315 plants obtained after 96 h of infection with Foc1. Complete names of protein abbreviations are provided in Additional file 1.

Additional file 9: GO classification (biological process and cellular components). Graphical representation of differentially expressed protein spots in chickpea roots (JG62 and WR315) based on network derived (pathway studio version 7.1 software) gene ontology classification. Graphs represent upregulated and down regulated proteins at different time points under different biological processes and cellular components.

Additional file 10: GO classification (molecular function). Graphical representation of differentially expressed protein spots in chickpea roots (JG62 and WR315) based on network derived (pathway studio version 7.1 software) gene ontology classification. Graphs represent upregulated and downregulated proteins at different time points under different molecular functions.

Abbreviations
Foc1: Fusarium oxysporum f. sp. ciceri Race 1; 2D PAGE: Two-dimensional polyacrylamide gel electrophoresis; MALDI-TOF MS: Matrix-assisted laser desorption/ionization time of flight tandem mass spectrometry; GO: Gene ontology; qRT-PCR: Real-time quantitative reverse transcriptional PCR; PR: Pathogenesis related; PTI: Pattern triggered immunity; EDS4: Enhanced disease susceptibility 4; PAD2: Phytoalexin deficient 2; ETS: Effector triggered immunity; SIX1: Arabidopsis DEFICIENT IN SEXUAL DETERMINATION 1; SIX1: Arabidopsis SIX1; PR: Pathogenesis related; PTI: Pattern triggered immunity; A. thaliana: Arabidopsis thaliana; A. thaliana var. Heyi: Arabidopsis thaliana Heyi; EDS4: Enhanced disease susceptibility 4; PAD2: Phytoalexin deficient 2; ETS: Effector triggered immunity; GO: Gene ontology; JG62 and WR315: Bacterial extract and fungicide; Fusarium oxysporum f. sp. ciceri: Fusarium oxysporum f. sp. ciceri; Cicer arietinum L.: Chickpea; Foc1: Fusarium oxysporum f. sp. ciceri; Infection: Infection; Pathogens: Pathogens; Pathogenesis: Pathogenesis; DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; RNAi: RNA interference; IEF: Isoelectric focusing; MANOVA: Multivariate analysis of variance; DMRT: Duncan’s multiple range test; SA: Salicylic acid; JA: Jasmonic acid; ABA: Abscisic acid; NPR1: Non expressor of PR genes1; ACD: Accelerated cell death; MAP kinase: Mitogen activated protein kinase; EDS4: Enhanced disease susceptibility 4; PAD2: Phytoalexin deficient 2; EDR2: Enhanced disease resistance 2; EIL: Ethylene-insensitive 3 like; ROS: Reactive oxygen species; GPCRs: G-protein coupled receptors; HR: Hypersensitive response; SAR: Systemic acquired resistance.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MC and SG contributed in designing the experiments. MC carried out protein extraction, 2-DE gel analysis, and MALDI-TOF MS and MS/MS experiments. AB conducted real time experiments. DC performed the statistical analysis. MC, SG, AB, DC, DB and SD analyzed the data. MC, SG and AB drafted the manuscript. SD and DB edited the manuscript and supervised the work. All authors read and approved the final manuscript.

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Fusarium oxysporum f.sp. ciceri Race 1 Induced Redox State Alterations Are Coupled to Downstream Defense Signaling in Root Tissues of Chickpea (Cicer arietinum L.)

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Abstract
Reactive oxygen species are known to play pivotal roles in pathogen perception, recognition and downstream defense signaling. But, how these redox alarms coordinate in planta into a defensive network is still intangible. Present study illustrates the role of Fusarium oxysporum f.sp ciceri Race 1 (Foc1) induced redox responsive transcripts in regulating downstream defense signaling in chickpea. Confocal microscopic studies highlighted pathogen invasion and colonization accompanied by tissue damage and deposition of callose degraded products at the xylem vessels of infected roots of chickpea plants. Such depositions led to the clogging of xylem vessels in compatible hosts while the resistant plants were devoid of such obstructions. Lipid peroxidation assays also indicated fungal induced membrane injury. Cell shrinkage and gradual nuclear adpression appeared as interesting features marking fungal ingress. Quantitative real time polymerase chain reaction exhibited differential expression patterns of redox regulators, cellular transporters and transcription factors during Foc1 progression. Network analysis showed redox regulators, cellular transporters and transcription factors to coordinate into a well orchestrated defensive network with sugars acting as internal signal modulators. Respiratory burst oxidase homologue, cationic peroxidase, vacuolar sorting receptor, polyol transporter, sucrose synthase, and zinc finger domain containing transcription factor appeared as key molecular candidates controlling important hubs of the defense network. Functional characterization of these hub controllers may prove to be promising in understanding chickpea–Foc1 interaction and developing the case study as a model for looking into the complexities of wilt diseases of other important crop legumes.

Introduction
Plants are constantly threatened by various pathogens; however, disease manifestation seldom occurs [1]. Success of a pathogen largely depends on the irregularities of the well-coordinated host immune system [2]. Both plants and animals possessing ancient innate immunity share structural and strategic similarities [3]. In addition, animals have a specialized adaptive immune system that recruits mobile defender cells to sites of pathogen invasion, resulting in either pathogen death and/or arrest [4]. Unfortunately, plants lack this feature, and their sessile nature precludes escape from pathogens. Nevertheless, nature has equipped plants with highly specialized and orchestrated signal transduction machinery to compensate for the void of adaptive immunity [5]. Pathogen induced defense signals originating from an epicenter trigger multiple downstream effects, leading to the strengthening of the cell wall; site-specific trafficking of stored anticipins; de novo secretion of phytoalexins, small molecules, and secondary metabolites; generation of reactive oxygen and nitrogen species; and accumulation of phytohormones and pathogenesis related proteins. These features culminate in a hypersensitive response (HR)-mediated programmed cell death (PCD) at the infection site [6]. However, amidst all such signaling, the sequential events that lead to a well-coordinated defense network still appear to be blurred.

Plants use various molecular antennae and/or protein receptors to sense microbes [7]. Pathogen triggered immunity (PTI) and effector triggered immunity (ETI) are two modes of pathogen recognition utilized differently by the host [8]. The pathogen/microbe associated molecular patterns (PAMPs/MAMPs) are invariant epitopes of pathogen molecules such as flagellin, chitin, lipopolysaccharide etc that aid to virulence [9]. Often, pathogen induced host components, referred to as danger associated molecular patterns (DAMPs), such as callose, glucans, fructans etc also serve as elicitors [10]. These PAMPs/MAMPs/DAMPs are recognized by host pattern recognition receptors (PRRs), leading to the activation of PTI [11]. PTI, which is known to restrict a large number of pathogens induces a sudden imbalance in ionic concentrations, increases Ca2+ influx, activates cascades of mitogen-activated protein (MAP) kinases and protein phosphorylations, and induces altered hormone signaling [12–13]. Howev-
er, a few pathogens successfully evade PTI and secrete effector proteins that contribute to effector triggered susceptibility (ETS) [2]. For counter defense, hosts secrete effector-specific R proteins, primarily nucleotide binding-leucine rich repeat (NB-LRR) domain containing “nibblers” that either interacts directly or through decoys/guardes with patho-effectors triggering a PTI like ET1 [14–15]. ETI signaling events not only overlap with PTI but also compensate for its weakness [9]. HR mediated PCD and ethylene mediated systemic acquired resistance (SAR) are unique features of ETI. However, recent studies on Arabidopsis showed that MAMPs such as cellulose-binding elicitor lectin (CBEL), ethylene-inducing xylanase (EIX), and harpins induce SAR, whereas flg22 induces HR in plants, suggesting that the demarcation between PTI and ETI is more conceptual than factual [16–19]. Recent discoveries have indicated that quorum sensing in bacteria, siderophores of bacteria, and fungi serve as potential MAMPs and hydroxyproline containing glycopeptides (HyPSeq) and rapid alkalization factors (RALFs) serve as DAMPs. However, their specific roles in defense are not well understood [10].

PTI and/or ETI mediated pathogen perception enkindles a repertoire of overlapping signals, of which generation of reactive oxygen species (ROS) and HR assisted PCD at the infection site appear to be the most important pathogenic event controlling resistance. In contrast, ROS generation adds to host phytotoxicity and hence is often found to be tightly regulated and detoxified through efficient scavenging systems in some resistant hosts. Such hosts are known to utilize the altered redox state of the infected cell to transmit downstream defense signals [20]. ROS, acting as primary signal inducers are also connected to Ca$^{2+}$ sensors and protein phosphorylation networks through RBOH-NADPH (Respiratory burst oxidase homologue), oxidases, thiorodoxins, peroxiredoxins, glutaredoxins, and/or NADPH. They are also known to induce secondary signal inducers, such as small peptides, hormones, lipids, cell wall fragments etc, cumulatively generating ROS waves that converge into the ROS network [21]. The role of ROS in fungal pathogenesis though less studied, but has recently put forth some obvious conclusions, showing that the ROS levels in the host are often controlled by the penetrating fungus itself [22]. Soluble sugars, sucrosyl oligosaccharides, and fructans have also been reported to contribute to oxidative stress regulation and ROS detoxification [23]. Besides, small molecule hormones, like salicylic acid (SA), jasmonic acid (JA), ethylene, abscissic acid (ABA), auxins, cytokinins, gibberellins (GA), and brassinosteroids, are also considered as central players [24]. Signaling pathways involving these hormones cross communicate either in an antagonistic and/or synergistic manner, providing a fine tune balance to the host.

Among several well discussed pathosystems, detailed molecular dynamics of plant-fungal interactions are undoubtedly scarce. Moreover, reports on legume–fungus interactions are either less or antagonistic and/or synergistic manner, providing a fine tune balance to the host [20]. The present study illustrates the mode of pathogen entry inducing xylanase (EIX), and harpins induce SAR, whereas flg22 already been reported by our group and others [27–29]. Previous results highlighted some special features in chickpea–Fusarium case study in which vascular clogging and resultant HR was linked to susceptibility rather than to the classical resistance. However, it should be emphasized that resistance and PCD do not necessarily correlate [30–31].

Previous reports had suggested that wound inducing Foc1 was sensed early by the host. Besides, this host–pathogen interplay triggered transcriptomic reprogramming directed towards regulating primary host metabolism, where sugar molecules served as defense signal modulators [32]. However, recent updates in the chickpea EST database related to the chickpea–Fusarium case study have not only contributed significantly to the understanding of molecular dynamics, but have also necessitated further studies examining the details of this interaction [29]. Moreover, the knowledge of the sequential association of key nodal and intermodal molecular candidates that ultimately interconnect to form a coordinated defense-signaling network with PTI and ETI signal overlaps is still elusive and thus was the focus of this study. Earlier reports had suggested wound mediated pathogen invasion and HR at the site of infection [32]. Such wounding responses are known to generate ROS and change the redox state of the infected host [20]. The present study illustrates the role of pathogen entry and investigates the expression pattern of several redox responsive transcripts such as ROS generators and scavengers, cytochrome-dependent redox signal transducers, and intracellular ROS signal transducers during pathogen progression and establishment. These defense signals generated by ROS molecules are known to transmit downstream signals to the host interior with the help of cellular transporters. The present study also demonstrates how these altered redox signals are transmitted to host interior through several intracellular transporters. Moreover, defense networks are tightly regulated by several transcription factors (TFs) that modulate the downstream expression of defensive genes either directly or by influencing the expression of their associates. The present study explains the probable role of several transcription factors in regulating the chickpea–Fusarium defense network. Previous reports had emphasized the role of sugars as signaling molecules [27,32]. In the present study, the role of sugar metabolizers as intracellular signal transmitters and modulators are also examined.

Materials and Methods

Plant and fungal materials

Experiments were performed with wilt susceptible (JG62) and wilt resistant (WR313) chickpea (Cicer arietinum L.) seeds obtained from International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. Seeds were sown in mixture of synthetic soil and sand (1:1) and plantlets were maintained under optimum greenhouse conditions at temperatures ranging from 22–28°C, 35–40% humidity, and
16:8 hours photoperiod of day and light, respectively, as described by Haware & Nene [26].

Fungal strain of *Fusarium oxysporum* f. sp. *ciceri* (Foc1), obtained from ICRIASAT, was purified and maintained according to Summerell, Salley & Leslie [33]. Spores were harvested and stored at −80°C in 30% glycerol until further use.

**Fungal infection assay**

Sterilized seeds of both JG62 and WR315 were germinated in sterile synthetic soil. Two week old seedlings were used for the assay. Infection was induced by sick soil method as described by Gupta et al. [27]. Optimum growth conditions were offered to both control and experimental sets. Roots of infected and control plants were collected on the following days post inoculation (dpi): i.e. 1dpi, 1.5dpi, 2dpi, 3dpi, 4dpi, 7dpi and 12dpi. Root samples were weighed into 1 g aliquots, flash frozen in liquid nitrogen, and stored at −80°C until further use.

**Confocal scanning laser microscopy (CSLM)**

Root samples of both infected and uninfected JG62 and WR315 plants were collected at 4dpi, 7dpi, and 12dpi, immediately incubated in a mixture of 75% ethanol and 15% acetone for 15 minutes, and finally fixed with 50% methanol and 10% acetic acid. Serial sectioning of root samples were performed and approximately 50 μm thick sections were selected for microscopy.

Selected sections were stained individually with trypan blue, aniline blue (Himedia), propidium iodide, and SYTOX green (Invitrogen) as well as with combinatorial stain containing both trypan blue-aniline blue and propidium iodide–SYTOX green following the protocol suggested by Bhadauria et al. [34] and Truernit & Haseloff [35]. Following staining, all the sections were thoroughly washed and mounted on grease-free glass slides using Keiser’s gelatin (Merck). Imaging was performed using CLSM Model LSM-510 Meta (Carl Zeiss) with excitation and emission wavelengths as mentioned in Table S1. All images were analyzed using LSM-510 software. Three biological replicates were used for all microscopic studies.

**Lipid peroxidation assay**

**a) Biochemical assay.** Biochemical assay was carried out using enzyme extracts isolated from infected (2dpi, 3dpi, 4dpi, 7dpi, and 12dpi) and uninfected roots. Degree of lipid peroxidation in roots was measured using 2-thiobarbituric acid (TBA) assay [36]. 800 μL of TBA reagent, containing 15% w/v TBA in 0.25 N HCl was mixed with 400 μL of enzyme extract and heated in a 100°C water bath for 15 minutes. The mixture was then cooled and centrifuged at 1000 xg for 10 minutes. The supernatant was removed and absorbance measured using a Beckman and Coulter DU-520 UV/VIS spectrophotometer at 535 nm. Biological and technical replicates were performed in triplicates and error calculated (Table S2).

**b) Immunoblot Experiment.** Total protein was extracted from infected (2dpi, 4dpi, 7dpi, and 12dpi) and uninfected roots of JG62 and WR315 plants using the protocol summarized by Wang et al. [37]. Extracted proteins were used for immunoblot experiments. Lipid peroxidation was reconfirmed by immunoblot experiments using OxiSelect Malondialdehyde (MDA) Immunoblot Kit (Cell biosabs) [38–39]. Total protein (10 μg) from uninoculated and inoculated roots of 2dpi, 4dpi, 7dpi, and 12dpi were used for immunoblotting. Protein was run on 10% SDS-PAGE and blotted according to the manufacturer’s instructions. Three biological replicates were used for the assay.

**RNA and cDNA preparation**

Roots of uninfected and infected plants of both varieties were collected at 1dpi, 1.5dpi, 2dpi, 3dpi, 4dpi, and 7dpi and frozen in liquid nitrogen. Total RNA was extracted using TRI reagent kit (Sigma-Aldrich) according to the manufacturer’s instructions. First strand cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Fermentas) following the manufacturer’s guidelines, checked on 1.1% agarose–Tris acetate EDTA (TAE, pH 8.0) gel (Merck), and quantified in NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies) at 260 nm. cDNAs were stored at −80°C until further use.

**Quantitative real-time PCR (qRT-PCR)**

Quantitative transcriptome profiling of β-1,3-endo glucanase, several redox related transcripts, cellular transporters, TFs, and sugar metabolizers was conducted for infected (1dpi, 1.5dpi, 2dpi, 3dpi, 4dpi, and 7dpi) and uninfected plants of both varieties (Table 1, Table S3). qRT-PCR was performed on Bio-Rad iCycler (Bio Rad iQ5) with SyBr green. Reaction mix (20 μL) containing SyBr green qPCR Super Mix (2×) (Bio Rad), 25 ng cDNA, and 0.3 μM of forward and reverse primers was taken for PCR (Table S4). The following cycle conditions were used: 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds, 50–55°C for 30 seconds, and 72°C for 30 seconds [32]. Melt curve was analyzed to evaluate primer specificity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control [40]. The mean fold change of all the genes was calculated following 2^−ΔΔC_{m} method [41]. All experiments were conducted in triplicates. Standard deviation and standard error was calculated for each transcript. Unpaired student’s t test was performed and p value (<0.05) calculated for entire data set (Table S5, Table S6).

**Sugar assay**

Soluble sugar was extracted from the roots of induced and uninduced JG62 and WR315 plants at 1dpi, 1.5dpi, 2dpi, 3dpi, 4dpi, 3dpi, and 7dpi. Roots were immediately oven dried at 68°C for 2–3 days, crushed in mortar and pestle, and distributed in 50 mg aliquots. During extraction, freshly prepared 80% methanol was added to powdered roots, mixed by vortexing, incubated for 2 hours at room temperature, and centrifuged at 8000 rpm for 10 minutes. The supernatant was transferred to a sterile micro-centrifuge tube, vacuum dried, and dissolved in 100 μL of HPLC grade water [Merck] [42]. Sugar contents were measured using the Sucrose/d-Glucose/d-Fructose Assay Kit (R-Biopharm) according to the manufacturer’s instructions. Enzymatic conversion of sugar molecules (Glucose and Sucrose) producing equimolar amount of NADPH was detected by spectrophotometer (Beckman-Coulter, DU-520) at 340 nm. Sugar concentration was expressed as micrograms of sugar per milligram root dry weight. Experiments were conducted in triplicates and errors were calculated (Table S7).

**Network analysis**

Several redox-responsive transcripts, cellular transporters, TFs, and sugar-metabolizing ESTs identified in the chickpea–*Fusarium* case study [27,29] were subjected to BLAST analyses, their *Arabidopsis* homologous genes identified and used as inputs for network generation using Pathway Studio Software (version 7.1) (Ariadne Genomics, Table 1, Table S3). The interrelationship between redox-related transcripts, cellular transporters, TFs, sugar metabolizing components, and their regulators were analyzed by Pathway Studio Software using the neighbor joining method;
Table 1. List of transcripts, their abbreviations and their homologies used for qRT-PCR and pathway construction.

<table>
<thead>
<tr>
<th>EST names</th>
<th>Abbreviations used in the study</th>
<th>Abbreviated gene names used in pathway generation</th>
<th>Chickpea Transcriptome Database (CTDB) homologues</th>
<th>TAIR homologues</th>
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<td>(Fe-SOD)</td>
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samples with a degree of correlation of 1, i.e only neighbors having direct relationship to the gene/gene products, were used as inputs.

Results

Foc1 invasion and colonization induces callose degradation, membrane disruption, and tissue damage

Pathogenic events such as callose degradation, membrane disruption, and tissue damage were monitored using CSLM. Trypan blue, which can bind cell wall components, was used to examine fungal progression [34]. Root sections of uninduced JG62 and resistant WR315 plants served as controls (Fig. 1A–C, Fig. 2A–C). In compatible interaction, fungal pegs were initially observed in cortical root cells at 4dpi (Fig. 1D–F) which extensified at 7dpi (Fig. 1G–I). Magnified images of 7dpi showed attempted penetration of an infecting hypha arising from the cell cytosol, which pierced the adjoining cell (Fig. 1J–L). Ramification and colonization further intensified at 12dpi with visible mycelial mesh within the xylem vessels and adjoining tissue (Fig. 1M–O). The presence of microconidia both singly and in clusters inside the xylem vessel indicated stable establishment of the fungus within the susceptible host (Fig. 1P–R). Gradual loss of tissue integrity was noticed. However, in the incompatible interaction, the resistant host WR315 showed contrasting features. Although, infection pegs were visible at 7dpi (Fig. 2D–F), but showed noticeably less intensification even at 12dpi (Fig. 2G–I).

Trypan blue and aniline blue combinatorial stain was used to analyze pathogen progression and callose degradation due to Foc1 incursion. Uninduced root sections of both varieties were used as controls (Fig. 3A–H). Onset of callose degradation was marked in fungal infested cortical root cells in JG62 plants at 4dpi (Fig. 3I–L). At 12dpi, formation of mycelial network and clogging of xylem vessels with callose degraded products was noticed in susceptible plants (Fig. 3Q–T). In resistant plants, fungal pegs were observed at 4dpi, but callose degradation was absent (Fig. 3M–P). However, at 12dpi, minimal amounts of callose degradation product at root cortical cells were observed. But, the vessels remained unlogged (Fig. 3U–X). qRT-PCR of β-1,3-endo glucanase showed enhanced expression in the susceptible variety compared to resistant plants at 1dpi, 4dpi, and 7dpi. However, 2dpi showed comparable expression in both the infected varieties, while 3dpi exhibited a reversed expression pattern in resistant plants (Fig. 4).

Propidium iodide and SYTOX green effectively stain cell wall and nuclei of membrane-compromised cells [35]. However, in the present study, root sections of uninduced plants subjected to dual staining with dyes showed fluorescent signals in both the membrane and the nuclei (Fig S1. A–H). Tissue sectioning, fixation, and dehydration likely led to membrane injury resulting in abnormal stain uptake even by uninduced samples. Infected

![Figure 1. Confocal scanning laser microscopic images showing Foc1 progression and colonization in roots of JG62 plants.](https://example.com/figure1.png)

<table>
<thead>
<tr>
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<th>TAIR homologues</th>
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Table 1. Cont.
root sections of JG62 and WR315 plants also showed similar staining properties (Fig. S1. I–L, M–P). However, membrane integrity was disrupted in JG62 plants at 12dpi (Fig S1. I–L), whereas WR315 plants showed uniform cell layers as uninfected controls (Fig S1. M–P).

Pathogen induced membrane damage was confirmed using biochemical and immunoblot assays to observe lipid peroxidation-mediated membrane injury. Biochemical assay was performed using TBA as a substrate that is known to form conjugates with MDA. These MDA byproducts are formed through oxidative reactions involving reactive superoxide molecules and polyunsaturated fatty acids present in the membrane lipid bilayer. Lipid peroxidation, an indicator of membrane damage, was found to be high in JG62 plants at 2dpi, 4dpi, and 7dpi than in uninduced control plants, indicating pathogen ingress and colonization. However, samples at 12dpi showed the lowest peroxidation levels. Lipid peroxidation levels showed an initial increase at 4dpi in WR315 plants as compared to uninduced control plants, but the levels decreased at later time points (Fig. S2). However, since TBA can form conjugates with other non-MDA molecules, non-MDA–TBA byproducts often mask the absorption of MDA–TBA products during spectrophotometric detection. Hence, immunoblot experiments were conducted in which only MDA–protein adducts were detected. Immunoblot experiments conducted with anti-MDA antibody showed increased lipid peroxidation in JG62 plants from 2dpi to 12dpi, concurrent with pathogen progression.

However, an abrupt decrease was observed at 7dpi (Fig. 5). In WR315 plants, peroxidation gradually increased from 7dpi onwards (Fig. 5). Biochemical and immunoblot assays showed dissimilar results, probably due to different substrate identities for MDA–TBA and MDA–protein conjugates in biochemical and immunoblot experiments, respectively. Thus, only the results obtained through immunoblot experiments were considered to be better explanatory in later section of the study.

**Fungal attack induces nuclear adpression and cell shrinkage**

Propidium iodide is known to stain the cell wall and nuclei of membrane-compromised cells and was found to exceptionally stain uninduced root samples of JG62 plants in the present study (Fig. 6A–C). However, this experiment led to an interesting revelation of altered cell size and nuclear position of Foc1 infected cells. In uninduced root cells, the cell size and nuclear position appeared to be normal (Fig. S3A), whereas in infected JG62 cells, cell size was altered and nuclei were gradually adpressed to the cell membrane at 7dpi (Fig. 6D–F, Fig. S3B). Cell shrinkage and nuclear adpression became more prominent at 12dpi (Fig. 6G–I, Fig. S3C). In WR315, cell shrinkage and nuclear adpression were evident at 12dpi, but to a lesser extent than in infected JG62 plants (Fig. 6J–L, Fig. S3D).
Foc1 entry causes expressional alterations of redox-responsive transcripts

Expressional profiling of ROS generator such as respiratory burst oxidase homologue showed comparatively less expression in susceptible JG62 plants, while resistant WR315 plants showed expressional fluctuations throughout pathogen progression. Peroxidase expression was increased by several-fold in WR315 plants compared to JG62 plants, with a sharp expressional drop at 3dpi. In contrast, cationic peroxidase was increased in JG62 plants, while WR315 plants showed low expression throughout the study period, except at 2dpi, on which cationic peroxidase expression increased marginally. However, the expression increased sharply at 4dpi. Iron superoxide dismutase (Fe-SOD) expression was higher in WR315 plants on all days except 3dpi, on which it showed significantly decreased expression. Glutathione S transferase (TAU26) (GST-TAU26) showed a sequential rise and drop in expression in JG62 plants while WR315 plants maintained overall low expression except at 1.5dpi, which showed highest expression (Fig. 7A and B, Fig S4).

Expression of cytochrome dependent redox signal transducers when compared showed uniform upregulation of cytochrome b561 ferric reductase in WR315 plants, with both plants showing

Figure 3. Confocal scanning laser microscopic images highlighting Foc1 ramification and callose degradation products in chickpea roots. (A–D) and (E–H) represents root sections of uninduced JG62 and WR315 plants respectively. (I–L) and (Q–T) corresponds to root sections of infected JG62 at 4dpi and 12dpi. (M–P) and (U–X) corresponds to infected root sections of infected WR315 at 4dpi and 12dpi. A, E, I, M, Q, U represent fluorescent images stained with trypan blue; B, F, J, N, R, V represent fluorescent images stained with aniline blue; C, G, K, O, S, W represent DIC images; D, H, L, P, T, X represent merged images. Bars correspond to 20 μm.

doi:10.1371/journal.pone.0073163.g003

Figure 4. qRT-PCR showing expression of beta 1,3 endo glucanase in chickpea roots. Relative expression of beta 1,3 endo glucanase in susceptible JG62 and resistant WR315 roots of chickpea plants in response to Foc1 infection. Bars represent the standard errors (n = 3).
doi:10.1371/journal.pone.0073163.g004
comparable expression only at 3dpi; however, in case of FAD linked oxidase family protein expression increased at 4dpi and 7dpi in JG62 plants. NADH cytochrome b5 reductase showed an overall low expression in both plants, with only JG62 plants showing an expressional hike at 1dpi and 4dpi, while this hike was recorded at 1.5dpi in WR315 plants (Fig. 7C and D, Fig S4).

Additionally, several intracellular ROS signal transducers exhibited differential profiling during pathogen progression. Except for 3dpi and 7dpi, NADP oxidoreductase showed comparatively enhanced expression in WR315 plants, while quinone oxidoreductase showed the reverse pattern throughout pathogen progression. Both Fe (II) oxidoreductase and F-type thioredoxin showed uniformly low expression in WR315 plants.

Figure 5. Immunoblot assay showing lipid peroxidation mediated membrane damage. Lane 1; positive control showing MDA-BSA bands at 82kDa and 182kDa respectively. Lane 2 and 3; total protein profile of uninduced roots of JG62 and WR315 respectively. Lane 4, 6, 8 and 10; total protein profile of infected roots of JG62 at 2dpi, 4dpi, 7dpi and 12dpi respectively. Lane 5, 7, 9 and 11; total protein profile of infected roots of WR315 at 2dpi, 4dpi, 7dpi and 12dpi. Each lane contains 10 μg of total protein uniformly.
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Figure 6. Confocal microscopic images showing Foc1 infection induced nuclear migration in root cells of chickpea plants. (A–C) correspond to root sections of uninduced JG 62 plant; (D–F) correspond to root sections of infected JG62 plant at 7dpi; (G–I) of infected JG62 plant at 12dpi; (J–L) correspond to root sections of infected WR315 plant at 12dpi. A, D, G, J represent fluorescent images; B, E, H, K represent DIC images; C, F, I, L represent merged images. Bars correspond to 10 μm.
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compared to JG62 plants. H+ ATPase showed upregulation throughout the study in WR315 plants, with only 3dpi showing a sharp downregulation in expression (Fig. 7E and F, S4).

Amongst all the redox related transcripts seven, such as redox regulatory respiratory burst oxidase homologue F (RBOHF) (respiratory burst oxidase homologue), thioredoxin 3 (TRX3) (homologue of F-type thioredoxin), overexpressor of cationic peroxidase 3 (OCP3) (homologue of cationic peroxidase), flavodoxin-like quinone reductase 1 (FQR1) (homologue of quinone oxidoreductase), iron superoxide dismutase 1 (FSD1)

Figure 7. Relative expression of redox signalling genes in chickpea roots in response to Foc1 induction. (A) and (B) represents relative expression profile of ROS generators and scavengers like respiratory burst oxidase homologue (RBOH), peroxidase, cationic peroxidase, iron superoxide dismutase (Fe-SOD) and glutathione s transferase TAU26 (GST-TAU26) in uninduced and induced JG62 and WR315 roots respectively; (C) and (D) represents relative expression pattern of cytochrome dependent redox signal transducers like cytochrome b561 Fe reductase, FAD linked oxidase family protein and NADH cytochrome b5 reductase in uninduced and induced JG62 and WR315 roots respectively; (E) and (F) represents relative expression profile of intracellular ROS signal transducers like NADP oxido-reductase, quinone oxido-reductase, Fe(II) oxido-reductase, F type thioredoxin and H+ transporting ATPase in uninduced and induced JG62 and WR315 roots respectively. Bars represent the standard errors (n = 3). doi:10.1371/journal.pone.0073163.g007
(homologue of Fe-SOD), NADH cytochrome b5 reductase (CBR), and Fe (II) oxidoreductase 7 (FRO7) (homologue of Fe (II) oxidoreductase) showed interactions in the pathway. RBOH, indicated as a positive regulator of ROS and hypersensitive response, showed linked with TRX3 and OCP3, with hydrogen peroxide and abscisic acid acting as intermediate small molecules. FQR1, FSD1, CBR, and FRO7 formed independent nodes. OCP3 appeared as negative regulator of infection response, while FRO7 positively regulated iron homeostasis and photosynthesis. Cell fate and oxidative stress were found to be regulated by TRX3 (Fig. S5).

Cellular transporters display differential expressional profiles
Fungal penetration led to the activation of several signal transporters whose expression profiles were analyzed with pathogen migration. Both intracellular transporters, such as ABC transporter like protein and carbohydrate substrate transporters, showed enhanced expression throughout the study period in WR315 plants, with only plants of 3dpi showing a decrease in expression. However, metal transporter (FRS6), translocase (chloroplast 34), and polyol transporter showed reverse profiles at all time points in resistant WR315 plants compared to susceptible JG62 plants (Fig. 8A and B, Fig. S4).

Besides, expression analyses performed with several cellular transporters, such as vacuolar sorting receptor, clathrin coat assembly protein, secretory carrier membrane protein, nuclear pore complex protein, and intrinsic protein of tonoplast, showed upregulation in resistant WR315 plants compared to susceptible plants during nearly all infective stages. However, plants at 3dpi...
again showed an opposite expression profile (Fig. 8C and D, Fig. S4).

TRK(A–N) signaling factor showed enhanced expression in susceptible JG62 plants compared to resistant WR315 plants, while type II B calcium\(^{2+}\) ATPase showed mild expression undulations surrounding basal expression values in both plants, which peaked at 4dpi in resistant plants (Fig. 8E and F, Fig. S4).

Amongst the selected cellular transporters four, such as polyol transporter 5 (PLT5) (homologue of polyol transporter protein), vacuolar sorting receptor 1 (VSR1) (homologue of vacuolar sorting receptor), calcium ATPase 2 (ACA2) (homologue of Type II B calcium\(^{2+}\) ATPase), and intrinsic protein of tonoplast 2 (TIP2) (homologue of intrinsic protein of tonoplast) were located in the signaling pathway. PLT5 and ACA2 were found to be linked to autoinhibitory H\(^{+}\) ATPase (AHA10), while VSR1 and TIP2 served as independent nodes. PLT5 and ACA2 were linked to sugar and amino acid transport, respectively. VSR1, associated with vacuolar transport, was found to regulate plant stress. ACA2 was also found to negatively influence the expression of calcium-dependent protein kinase (CDPK), while TIP2 was known to downregulate MAP kinase (Fig. S6).

Foc1 activates expression of transcription factors

Several TFs with domains such as bZIP, homeodomain leucine zipper, MYB, helix loop helix, zinc finger (CCHC type), and heat shock family protein showed differential expression post Foc1 infection. Expression of both bZIP domain containing TF and homeodomain leucine zipper like protein showed expression changes in both plants during all time points of the assay. However, MYB domain containing protein showed enhanced expression in susceptible JG62 plants at all time points except for 2dpi, 4dpi and 7dpi where expression of resistant WR315 plants superseded that of susceptible JG62 ones. In case of helix-loop-helix motif bearing TF, enhanced expression was observed in susceptible JG62 plants immediately at 1dpi, which fell close to basal levels at later time points; whereas in resistant plants, alternate rise and drop in expression were evident. Zinc finger protein (CCHC type) showed significant changes at nearly all time points except for 2dpi and 7dpi in susceptible JG62 plants, whereas heat shock factor family protein showed nearly basal expression values for both plants (Fig. 9A and B, Fig. S4).

Figure 9. Relative expression of Foc1 induced transcription factors in chickpea roots. (A) and (B) represents relative expression profile of transcription factors containing basic domains like, bZIP domain containing transcription factor, homoeodomain leucine zipper transcription factor, MYB domain containing transcription factor, helix loop helix motif bearing transcription factor, zinc finger (CCHC type) transcription factor and heat shock transcription factor family protein in uninduced and induced JG62 and WR315 roots respectively; (C) and (D) shows comparative expression pattern of transcription factor associators like, polynucleotidyl transferase (FAR1), initiation factor (4a), prefolding helix loop helix domain containing binding factor (ILR3) and high mobility group B like protein in uninduced and induced JG62 and WR315 roots respectively. Bars represent the standard errors (n = 3).

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Transcription factor associated proteins, such as polynucleotidyl transferase (FAR1) showed comparably higher expression in susceptible JG62 plants compared to resistant plants, except at 1.5dpi and 2dpi, for which low expression was observed. Both initiation factor (4a) and the helix-loop-helix motif containing prefoldin (ILR3) showed enhanced expression in susceptible JG62 plants, while the high mobility group B like protein showed upregulation in resistant WR315 plants alternately at 1dpi, 2dpi, and 4dpi (Fig. 9C and D, Fig. S4).

Interaction network showed the location of five genes, such as zinc finger protein (CCHC type) 2 (AZF2) (homologue of zinc finger protein CCHC type), homeodomain leucine zipper REV (homologue of homeodomain leucine zipper like protein), heat shock factor 3 (HSF3) (homologue of heat shock factor family protein), high mobility group protein B3 (HMGB3) (homologue of high mobility group B like protein), and MYB (homologue of MYB domain containing protein) in the defense regulatory pathway. All TFs showed independent nodal positions. MYB negatively regulated apoptosis, while REV positively regulated lignification, auxin mediated polar transport, growth, and cell fate. REV was also found to regulate microRNAs, proteasome degradation, and other homeobox leucine zippers. AZF2 and HMGB3 showed stress mediated regulation. HSF3 downregulated peroxidases (Fig. S7).

Role of sugar metabolism in defense

Sugars are known to play crucial roles in defense and are metabolized by several enzymes, such as sucrose synthase, β-amylase, and invertase. Expression of these sugar metabolizers were found to undulate with pathogen progression. Expression of sucrose synthase increased at early time points in resistant WR315 plants while their levels increased significantly after 4dpi in susceptible JG62 plants. Both β-amylase and invertase showed increased expression in susceptible JG62 plants compared to resistant ones throughout pathogen invasion and establishment (Fig. 10A and B, Fig. S4).

Sucrose and glucose, which are known as signal generators, showed post infection metabolic undulations. Glucose and/or sucrose contents reportedly vary during different developmental stages of plant life. Hence, relative amounts of these sugars were monitored along with pathogen progression. Glucose levels showed alterations with an increase at 5dpi, which was met with an abrupt reduction at 7dpi in JG62 plants (Fig. 10C) while WR315 plants showed the same profiles for both glucose and sucrose (Fig. 10D). Sucrose content was comparable with that of uninduced controls up to 4dpi, but was found to reduce at 5dpi and 7dpi in JG62 plants (Fig. 10E). On the contrary, in WR315 plants, the pathogenic response though was obvious at as early as 1dpi but sucrose levels reached to comparable amounts with that of uninduced controls at 5dpi onwards (Fig. 10F).

Figure 10. Expression and content of sugar metabolizing genes and sugars in Foc1 infected chickpea roots. (A) and (B) represents relative expression pattern of sugar metabolizes like, sucrose synthase, beta amylase and invertase in uninduced and induced JG62 and WR315 roots; (C) shows relative content of glucose in uninduced and induced JG62 plant; (D) represents relative content of glucose in uninduced and induced WR315 plants; (E) represents relative amount of sucrose in uninduced and induced JG62 plants; (F) shows relative amount of sucrose in uninduced and induced WR315 plants. Bars represent the standard errors (n = 3).

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Foc1 induced redox regulators, cellular transporters, and transcription factors interconnect in the interaction network

Network analyses performed using redox regulators, cellular transporters, and transcription factors as inputs resulted in an integrated signaling network showing interactions between the three classes of components described above. Redox regulators, namely RBOHF and OCP3, were found to interact with the cellular transporter TIP2 and the TF AZF2, with the small molecule abscisic acid (ABA) acting as a common mediator. The TF AZF2 showed links with FSD1 through other zinc finger protein (STZ); VSR1, found as regulator of vacuolar transport, was linked to auxin responsive transcription factor (ARF). JA appeared to be a common modulator of redox responsive OCP3 and cellular transporter TIP2. RBOHF was found to be regulated by ethylene, ROS, and H$_2$O$_2$, with the latter acting as a common regulator of TRX. Iron regulated FRO7, while different sugars and H$^+$ appeared to regulate PLT5, ACA2, and AHA10. Galactose sugars as well as by lipids regulated VSR1. Apart from ABA and JA, OCP3 was also found to be positively influenced by salicylic acid and methyl jasmonates. ATP, copper, and calcium regulated ACA2, while flavin mononucleotide regulated FQR1. RBOH, a positive regulator of hypersensitive response and cell death, was also found to act as an indirect regulator of cell cycle through TRX3 connections and of defense response through OCP3 links. RBOH was also found to interact with calcium dependent serine threonine kinase (OST1) and other RBOH isoforms (RBOHD). TRX3 showed a negative regulatory relationship with glutaredoxins (GRX1and MPO 12.80) and thioredoxin reductase B (NTRB). Additionally, FRO7 was interlinked with the outer envelope membrane protein OEP7 and the photosystem II reaction center. Cytochrome b$_5$ reductase was found to negatively regulate cytochrome b$_5$. VSR1 regulated syntaxin signaling, while ACA2 influenced the sodium transporter. AZF2 showed interactions with lipoxigenases and the jasmonate domain containing protein (JAZ1). Among other TFs, REV showed interactions with other leucine zippers, while HSF3 showed negative regulatory connections with peroxidase (APX1) and was positively linked with galactinol synthase (GOLS1) (Fig. S8).

Sugar metabolizers interact with the fungal induced redox regulators, cellular transporters, and transcription factors in the interaction pathway

Sucrose synthase (SUS4), β-amylase (BAM1), serine threonine kinase (CDKB1.1), and vacuolar ATPase (TUF) were reported to play role in modulating sugar metabolism upon Foc1 invasion [27,32]. These molecular candidates also showed interactions with differentially expressed redox responsive candidates, cellular transporters, and transcription factors analyzed in the present study. RBOH was found to be directly linked with CDCKB1.1 and OCP3 through ABA and indirectly linked with TUF and BAM through CDCKB1.1. A relationship between TRX3 and the above molecular candidates was also observed through RBOH and H$_2$O$_2$ small molecules. SUS4 and BAM1 appeared as direct regulators of sugar metabolism, while TUF and CDCKB1.1 directly regulated cell differentiation and plant development. Additionally, SUS4 regulated cell wall biosynthesis, turgor pressure, and glycolysis, while CDCKB1.1 regulated cell size and mitotic entry (Fig. S9). Interaction pathways involving sugar-metabolizing genes and cellular transporters showed direct communication between SUS4, PLT5, and ACA2, with d-glucose and ATP acting as intermediate small molecules. CDCKB1.1 showed links with TIP2 through the modulator ABA (Fig. S10). CDCKB1.1 showed direct communication with AZF2, with ABA acting as a mediator. Embryonic development appeared to be the common interacting process for both TUF and REV, while plant development appeared to be common for BAM1 and AZF2. REV also showed a direct interaction with CDCKB1.1, with cytokinesis as an intermediate connecting cell process (Fig. S11).

Discussion

Previous studies conducted on chickpea–Foc1 interaction have already documented novel insights, which are considered deviations from classical concepts, where HR coupled to PCD promoted fungal colonization and establishment at the xylem vessels of compatible hosts. On the other hand, resistant plants known for early pathogen perception triggered cellular reprogramming, comprising a series of downstream defense responses, all aiding to the diversion of the oxidative burst from the main solute conducting strand, the xylem vessels [27,32]. Besides, reports of several non-canonical genes lacking a defensive history were also reported to be associated with the present pathosystem [29]. However, with all the previous reports taken together, Foc1 is known to deploy a stealthy “modus operandi” when entering compatible hosts through the breaches of root and root hairs and colonizing xylem vessels. Such diplomatic entry results in an oxidative burst, leading to vessel clogging, blocking upward translocation of essential solutes, all of which manifests into the common symptom of wilt [27]. *Fusarium oxysporum*, though primarily known for its resemblance with biotrophs, has recently been argued to hold necrotrophic features also [43]. However, a lack of information regarding functional effector toxins promoting *in planta* necrosis from Foc prevents conclusive classification. Moreover, studies reviewed by Oliver and Ipcho [44] suggest gradual erosion of such nomenclatural dogmas related to classification properties, with *Phytophthora infestans* placed in all three classes of biotrophs, necrotrophs, and the more specialized hemibiotrophs.

Successful penetration is regulated by the ability of the pathogen to breach the initial barriers of cell wall appositions and cell membrane [45]. Recent report showed the progression and differential colonization pattern of *Fusarium oxysporum* f. sp *ciceris* race 0 and race 5 both in compatible and in incompatible hosts [46]. However, it should be noted that, significant variability between the pathogenic races of *Fusarium oxysporum* f. sp *ciceris* show marked difference in their infection and colonization patterns which largely depends on inoculum densities as well as edaphic factors [47, 48]. In the present study, Foc1 was found to gradually progress and colonize the compatible host, which was marked by the presence of extensive fungal ramification and deposition of callose-degraded products at the xylem vessels. Besides, establishment of a pathogen is marked by its ability to reproduce within the host interior, thus providing a constant flush of pathogenic compounds [49]. In the present study, presence of microcomidia within the xylem vessels of susceptible hosts indicated stable establishment of Foc1. Callose, a homopolymer of linear β-1,3-glucose residues with some β-1,6 branches, is known to aid penetration resistance by forming a special permeability barrier during fungal ingress [50]. In contrast, these short-lived callose molecules are degraded by β-1, 3-glucanases, which are also known to induce pathogen invasion [51]. Previous studies have revealed induction of β-1,3-glucanases in chickpea during compatible interaction with Foc1, suggesting that callose degradation is associated with fungal penetration [28]. Present results also show callose-degraded products at the xylem vessels of...
susceptible JG62 plants along with high levels of \( \beta \)-1,3-endo glucanase at later stages of infection, corroborating the results of earlier reports. However, the observation of resistant WR315 plants with overall low \( \beta \)-1,3-glucanase levels showing no such degradations even after 7dpi, when wilting symptoms are prominently set in susceptible plants, indicates the use of a different reprogramming mechanism that prevents functional callose catabolism during incompatible interactions. However, an increase in \( \beta \)-1,3-endoglucanase expression in resistant plants at 3 dpi indicates a transient pathogenic attempt to overpower the host defense.

Pathogen invasion leads to oxidative burst and generation of active oxygen species at the site of entry, causing lipid peroxidation and membrane damage, which are believed to be key features of pathogenesis [52]. In the present study, lipid peroxidation mediated membrane damage was evident with gradual pathogen progression in susceptible plants; resistant plants showed a marginally low degree of lipid peroxidations during later stages of infection. These results were found to validate earlier reports [36]. Interestingly, in the present study, assays indicating membrane damage showed cell shrinkage and gradual nuclear adhesion in response to pathogen progression in both compatible as well as incompatible host cells, with the degree of nuclear adhesion being marginally lower in the resistant host. Previous studies involving *Vigna unguiculata* and *Uromyces vignae* showed similar trends of nuclear migration followed by the cessation of cytoplasmic streaming and alterations in plasma membrane permeability [53]. Changes in nuclear structure and organization were also reported in *Medicago truncatula* and *Daucus carota* during colonization by arbuscular mycorrhizal fungi [54]. However, in the present case study, in compatible interactions such nuclear migration was assumed to be the morphological feature indicating gradual PCD [55], but in case of incompatible interactions in which PCD was not evident, why such nuclear migration occurred was unclear. However, it opens up an exciting arena of future research where nuclear structural and organizational alterations could probably be assigned as signs of cellular reprogramming instead of being termed as signatories of PCD.

Transcript profiling is known to provide details regarding pathogen induced gene expression in hosts, particularly when complete genome datasets and their annotations are limited [56]. In the present study, attempt was made to provide logical interpretation of the defense signaling network occurring within chickpea using transcript profiling. Pathogen invasion is sensed by host cells by triggering an initial alteration in its redox state of art [57]. Previous studies performed on chickpea by our group as well as by other groups reported the involvement of several transcripts regulating the redox state during onset of Foc1 infection [27,29,32,36]. But, how their expression profiles altered with the gradual pathogen progression was elusive. The present study was focused at understanding the differential expression of selectively those transcripts that were reported to have associations with chickpea-Foc1 casestudy (Fig. 11).

Network analysis showed the location of RBOHF, OCP3, FSD1, TRX3, FRQ1, CBR and FRO7 in the interaction network. RBOHs are known to generate reactive oxygen species (ROS), promote PCD, and trigger downstream hormonal fluctuations upon pathogenic attack [58–59]. RBOH isoforms are also known to induce penetration resistance to *Blumeria graminis* f. sp. *hordei* in barley and mediate SAR in *Arabidopsis thaliana* [60–61]. In the

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**Figure 11. Schematic representation of Foc1 induced defense signalling network in chickpea.** Interaction map showing intracellular signalling involving defense responsive molecular components (redox regulators, cellular transporters, transcription factors and sugar metabolizers; marked in red colored asterisks) in chickpea upon Foc1 induction. doi:10.1371/journal.pone.0073163.g011
present study, alteration of RBOH in resistant plants suggests that it has a regulatory role during Foc1 invasion, while susceptible plants failed to show similar variations. OCP and FSD are also known to play key roles in ROS signaling. OCP, an ABA-dependent transcriptional regulator, was suppressed during necrotrophic pathogen attack in Arabidopsis thaliana [62], while cationic peroxidase itself and found to accumulate in xylem vessels of Xanthomonas axonopodis pv. axonopodis infested rice plants [63]. FSD is known to serve as the first line of defense against ROS injury [64]. In the present study the sequential upregulations of OCP and FeSOD/FSD suggests the scavenging machinery to be under constant functioning during Foc1 infection. RBOH was also found to regulate expression of TRX, which in turn regulate the redox state of other target proteins and participate in oxidative stress tolerance of plants [65]. The interaction of C19 protein with TRX was found to negatively regulate defense in tomatoes upon Cladosporium falxum attack [66]. Though results obtained in the present study also showed an overall enhanced expression of F type thioredoxin in compatible interaction compared to incompatible one, but whether it plays a similar negative regulatory role in the present case study needs to be further introspected. FQR1 acts as detoxifier along with GST [67]. CBR is known to catalyze the transfer of electrons from NADH to membrane bound cytochrome b5 reductase [68]. Besides, they also participate in the desaturation reaction during fatty acid synthesis [69]. Fatty acids are known to play important roles in basal, effector-triggered and systemic immune responses [70]. Disappearance of CBR at 3dpi in resistant plants suggests a transitory attempt of the fungus to overpower the resistant host machinery, which probably failed at later hours of infection. On the other hand, FRO7 known to play essential role in delivering iron to chloroplasts [71] showed an average increase in expression at 1dpi and 4dpi in susceptible plants. Such increment probably indicated an attempt of the susceptible host to shield its photosynthetic apparatus that was found to undergo rapid chlorosis upon pathogen progression (Fig S5, Fig 11). 

Apart from the above, in the present study transcriptomic analysis showed enhanced expression of ROS scavenger, peroxidase in resistant plants probably indicating timely elimination of ROS [72]. GSTs, known to reduce organic hydroperoxides formed during oxidative stress were induced in Nicotiana benthamiana following Colletotrichum destructivum infection [73]. Hence, low expression of GST (TAU26) in WR315 plants probably indicated an overall low concentration of oxidative stress intermediates in resistant plants. In contrast, FAD oxidase family protein known to use molecular oxygen to form disulphide bonds associated with generation of hydrogen peroxides [74], showed enhanced expression at later time points in susceptible plants. Increment of FAD oxidase family protein in susceptible plants probably indicated pathogen triggered generation of peroxides. NADP oxidoreductase helps in regulating the electron partitioning in chloroplast [75]. Sharp decline of NADP oxidoreductase in resistant plants at 3dpi was assumed to be a transient pathogenic attempt to overpower resistant host machinery. H⁺ ATPases are reported to be under dynamic spatio-temporal regulation during early stages of pathogen recognition. Besides, they are also known to control the exposure and closure of stomatal aperture [76]. In the present study, upregulation of H⁺ ATPases throughout in resistant plants suggested early pathogen perception as well as regulation of stomatal opening which is ATPases throughout in resistant plants suggested early pathogen perception as well as regulation of stomatal opening which is known to play key roles in ROS signaling. OCP, an ABA-dependent transcriptional regulator, was suppressed during necrotrophic pathogen attack in Arabidopsis thaliana [62], while cationic peroxidase itself and found to accumulate in xylem vessels of Xanthomonas axonopodis pv. axonopodis infested rice plants [63]. FSD is known to serve as the first line of defense against ROS injury [64]. In the present study the sequential upregulations of OCP and FeSOD/FSD suggests the scavenging machinery to be under constant functioning during Foc1 infection. RBOH was also found to regulate expression of TRX, which in turn regulate the redox state of other target proteins and participate in oxidative stress tolerance of plants [65]. The interaction of C19 protein with TRX was found to negatively regulate defense in tomatoes upon Cladosporium falxum attack [66]. Though results obtained in the present study also showed an overall enhanced expression of F type thioredoxin in compatible interaction compared to incompatible one, but whether it plays a similar negative regulatory role in the present case study needs to be further introspected. FQR1 acts as detoxifier along with GST [67]. CBR is known to catalyze the transfer of electrons from NADH to membrane bound cytochrome b5 reductase [68]. Besides, they also participate in the desaturation reaction during fatty acid synthesis [69]. Fatty acids are known to play important roles in basal, effector-triggered and systemic immune responses [70]. Disappearance of CBR at 3dpi in resistant plants suggests a transitory attempt of the fungus to overpower the resistant host machinery, which probably failed at later hours of infection. On the other hand, FRO7 known to play essential role in delivering iron to chloroplasts [71] showed an average increase in expression at 1dpi and 4dpi in susceptible plants. Such increment probably indicated an attempt of the susceptible host to shield its photosynthetic apparatus that was found to undergo rapid chlorosis upon pathogen progression (Fig S5, Fig 11). 

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Besides, transcriptomic profile indicated ABC transporter like protein to be upregulated in the incompatible interaction in the present study. These transporters showed enhanced expression during fungal invasion in Arabidopsis thaliana and in the presence of SA and methyl jasmonate in soybean [83–84]. Additionally, PDR-type ABC transporter was known to mediate cellular uptake of the stress-regulating hormone ABA [85]. Carbohydrate substrate transporters are known to have complex roles in plant defense. On one hand, they are believed to fight invasion, while on the other they are also known to be hijacked by the invaders [86]. In the present study, enhanced expression of carbohydrate substrate transporters in incompatible interaction probably indicated its role in preventing fungal invasion. Translocase (chloroplast 34) regulates GTPase mediated protein import of chloroplasts [87]. However, in the present study, upregulation of translocase (chloroplast 34) in susceptible plants does not describe any specific role. Clathrin coat assembly proteins and secretory carrier membrane proteins, though ill characterized in plants, are known to aid exocytosis in association with SNARE proteins and syntaxins [88–89]. Enhanced expression of these secretory proteins in resistant plants indicates that exocytosis is related to pathogenesis. Recent studies have highlighted the significance of nucleocytoplasmic transport involving nuclear pore complex proteins in plant defense [90]. Results obtained in the present study showed distinct upregulation of nuclear pore complex proteins in resistant plants, suggesting that nucleocytoplasmic transport is coupled with defense responses against Foc1 (Fig 11). 

Under stressful situations transcription factors are believed to act as prime expressional regulators of defensive genes [91]. Signaling network showed the presence of AZF2, REV, MYB, HSF3 and HMGB transcription factors. Zinc finger (CCHC type) transcription factor, which are known for their roles as repressors during drought, cold, salt, and oxidative stresses, are known to regulate jasmonate signaling, expression of lipoxygenase, and switch/sucrose non-fermentable (SWI/SNF) components, which are further known to regulate epigenetic responses [92–94].
Results of present study showing decreased expression of zinc finger protein (CCHC type) in incompatible host validated the repressive role of zinc finger protein (CCHC type) under fungal induction. REV, a homeodomain leucine zipper like protein, showed expressional changes in the present study. Similar variation was observed in sunflowers during wounding and biotic stress [95]. MYB domain containing TF, a regulator of HR, biotic stress and salt tolerance in *Arabidopsis thaliana* [96–98], was found to show fluctuations at early time points of Foc1 infection which reached stable expression levels at later time points in resistant plants. The reason behind such fluctuations needs to be investigated. However, the susceptible plants showed a steady level of expression probably indicating cellular apopotosis by Foc1 infection. HSFs function as ROS sensors by sensing hydrogen peroxides in particular [99]. Such heat shock factor family proteins were found to be downregulated in resistant plants, suggesting low levels of ROS. HMG are reported to be upregulated under salinity stress, drought or cold stresses [100]. The present study showing enhanced amounts of HMG B like protein in resistant plants probably indicated similar stress regulatory function (Fig S7, Fig 11).

Additionally, helix-loop-helix and bZIP domain bearing transcription factors, known for mediating jasmonate specific responses in *Arabidopsis thaliana* [101] also showed differential expression undulations in the present study (Fig 11). The role of poly nucleotidyl transferase that is known to have exonuclease activity needed for DNA repair [102] is unclear in the present study. Translational initiation factor 4a is reported to be the prototype of DEAD box family protein that helps in initiation of translation [103]. However, understanding the role of this factor in the present study requires additional experimentation. Prefoldin is reported to be essential for microtubule assembly [104]. Increased levels of prefoldin in susceptible plants probably indicated microtubule assembly which is thought to be an essential mechanism for cell repair.

Sugars act as signal transducing molecules that are known to integrate defense signal transduction. Previous reports have documented the role of sucrose synthase, β-amylase, vacuolar H⁺-ATPase, and serine threonine kinases in regulating defense signaling [27,32]. Network analysis showed interactions between these previously described sugar modulators and the redox responsive transcripts, cellular transporters and transcription factors discussed in the present study. These soluble sugars are known to actively participate in oxidative stress regulation [105]. Additionally, sucrose synthase levels are found to elevate during cold acclimation in *Arabidopsis thaliana*, with DREB acting as the transcriptional activator behind this elevation [106]. Sucrose synthase is also known to regulate several serine threonine kinases, which are in turn found to be under hormonal control [107]. These serine threonine kinases, along with β-amylase, directly control plant development, while both H⁺-ATPase and serine threonine kinase control cell differentiation, golgi organization, and vacuolar function [108–109]. Although, in the present study, during compatible interaction, the levels of soluble sugar metabolizing genes such as sucrose synthase, β-amylase, and invertase were found to be sharply downregulated at 1.5dpi, but showed gradual expressional increment after 2dpi, with sucrose synthase and β-amylase showing expressional peaks at 4dpi. However, a decrease in the net amount of sucrose as well as its conversion product glucose in susceptible plants observed at later time points of infection, suggested expenditure of the released hexoses to be more pathogen driven than host regulated. This result supports the previous report on *Ustilago maydis*, where the host-derived hexose sugars were utilized to fuel the primary metabolism of the invading fungus [110]. *Pseudomonas* spp. and *Xanthomonas* spp. were also known to induce SWEET, a glucose unipporter from *Arabidopsis thaliana*, to drive self metabolism [111]. In the present case study resistant plants probably reorient their primary metabolism to satisfy optimum self demands while regulating the resources needed to prevent and repair fungal induced cell injury (Fig S9, S10, S11, and Fig 11).

**Conclusion**

Considering all the illustrations and explanations, the interaction network generated from the transcriptomic profiling and pathway analyses was an attempt to delineate the probable defense signaling network found in chickpea as an outcome of Foc1 challenge (Fig 11). The challenge led to the colonization and establishment of the fungus in susceptible host, while the resistant host could judiciously reprogram its metabolism in warding off pathogenic consequences. However, a remarkable decrease in the expression of redox related transcripts such as ROS generators and scavengers as well as cytochrome dependent redox signal transducers were found at 3dpi in the incompatible host. Apart from these, intracellular transporters, basic domain containing TFs, and sugar metabolizing genes were also downregulated. These declines in expression levels suggest a pathogenic effort to overwhelm the resistant host defense, which collapsed at later time points. RBOH, OCP, VSR, PLT, SUS, serine/threonine kinase, and zinc finger protein (CCHC type) appeared to be key molecular candidates controlling important hubs of the defensive network. Functional characterization of these hub controllers may provide promising clues for further understanding of the chickpea–Foc1 interaction. Additionally, it could also help in developing this case study into a model for investigating the intricacies of vital vascular diseases such as wilt, in which the host must defend against pathogen establishment while maintaining normal metabolic homeostasis. Recent availability of the draft genome sequence of chickpea (Cicer *arietinum* L.) is not only expected to add to the knowledge of chickpea-Foc1 interaction, but also aid to the development of effective disease management strategies [112].

**Supporting Information**

**Figure S1** Confocal scanning laser microscopic images representing pathogen induced tissue damage. (a–d) corresponds to root sections of uninduced JG62 plants; (e–h) corresponds to root sections of uninduced WR315 plants. (i–l) represent root sections of infected JG62 plants at 12 dpi; (m–p) represent root sections of infected WR315 plants at 12dpi. (a,e,i,m) show fluorescent images stained with sytox green; (b,f,j,n) show fluorescent images stained with propidium iodide; (c,g,k,o) represent differential interference contrast (DIC) images; (d,h,l,p) represent merged images. Bars represent 20 μm.

(PDF)

**Figure S2** Graphical representation of biochemical assay of pathogen induced lipid peroxidation in JG62 and WR315 plants.

(PDF)

**Figure S3** Confocal Scanning Laser Microscopy images showing measurement of nuclear adpresion during pathogen progression. (a) Uninduced JG62 root cell. (b and c) Induced JG62 root cells at 7dpi and 12dpi respectively. (d) Induced WR315 root cell at 12dpi. Bars represent 20 μm.

(PDF)
Figure S4  Heat map showing differential levels of redox regulators, cellular transporters and transcription factors induced in JG62 and WR315 plants after Foc 1 infection. (PDF)

Figure S5. Network showing pathogen induced intracellular redox signaling. (PDF)

Figure S6  Network showing pathogen induced intracellular signal transportation. (PDF)

Figure S7  Network showing transcription factors and associated signaling. (PDF)

Figure S8  Network showing interaction between redox regulators, cellular transporters and transcription factors. ATCBR, Arabidopsis thaliana NADH cytochrome b5 reductase; HSF3, heat shock factor 3; FQR1, flavodoxin like quinone reductase 1; FSD1, iron superoxide dismutase; STZ, cys2/ his2 zinc finger; AZF2, zinc finger (CCHC type); VSR1, vacuolar sorting receptor 1; ATRBOH, Arabidopsis thaliana respiratory burst oxidase homologue; FRO7, ferric reduction oxidase 7; OCP3, over expression of cationic peroxidase 3; HMG3, high mobility group B protein 3; PLT5, polyol transporter protein 5; AHA10, autoinhibited H+ ATPase isoform 10; ACA2, calcium ATPase; MYB106, MYB transcription factor 106; REV, homeobox leucine zipper (REVOLUTA); TRX3, thioredoxin 3; TIP2, tonoplast intrinsic protein 2. (PDF)

Figure S9  Network showing interaction between sugar metabolizers and redox regulators. SUS4, sucrose synthase 4; BAM1, beta amylase; CDKB1.1, serine threonine kinase; TUF, vacuolar ATPase. (PDF)

Figure S10  Network showing interaction between sugar metabolizers and cellular transporters. (PDF)

Figure S11. Network showing interaction between sugar metabolizers and transcription factors. (PDF)

Table S1  Excitation and emission wave lengths of fluorescent dyes. (DOC)

Table S2  Values of error of biochemical assay indicating lipid peroxidation. (XLS)

Table S3  Database homology matches of chickpea genes used in the study. (DOC)

Table S4  Primer sequences with database references used for qRT-PCR. (DOC)

Table S5  Mean fold change, standard deviation and standard error of the transcripts used for qRT-PCR analyses. (XLS)

Table S6  Statistical t and p value calculated by Student’s t test of the transcripts used for qRT-PCR analyses. (XLS)

Table S7  Values of error calculated during sugar assay. (XLS)

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Author Contributions

Conceived and designed the experiments: SG AB SD. Performed the experiments: SG AB MC. Analyzed the data: SG AB MC SD. Contributed reagents/materials/analysis tools: SD. Wrote the paper: SG AB SD.

References

Combating photooxidative stress in green hairy roots of *Daucus carota* cultivated under light irradiation

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ABSTRACT

The light-dependent generation of active oxygen species, which can disrupt normal metabolic process of plant, is termed as photo–oxidative stress. Plants are equipped with enzymatic and non-enzymatic antioxidative defence systems to reduce the effect of such stress. Hairy root culture of *Daucus carota* when cultivated under continuous illumination (250 μmol m−2 s−1) turned green. To know the reason behind that and photo-oxidative stress response in green hairy roots, activities of several antioxidant enzymes were measured. When compared with normal hairy roots, green hairy roots showed an enhanced superoxide dismutase (SOD) activity. Treatment with a SOD inhibitor diethyldithiocarbamate led to suppression of SOD activity in a concentration-dependent manner in green hairy roots. Interestingly, SOD-suppressed root showed three-fold enhanced caffeic acid glucoside accumulation in the soluble fraction as compared to untreated ones. While ascorbate peroxidase activity showed marginal increase in green hairy roots, a decrease in the activities of guaiacol peroxidase and catalase were observed. SDS-PAGE of crude protein profile from green hairy roots showed a distinct band, which was absent in normal hairy roots. MALDI-TOF-MS/MS analysis of the extracted protein confirmed it as the large subunit of Rubisco. RT-PCR based expression analysis of betaine aldehyde dehydrogenase showed enhanced transcript levels in green hairy roots as compared to normal hairy roots, whereas reverse trends were observed with the transcripts accumulation for phenylalanine ammonia-lyase and chalcone synthase. These findings corroborate with the *in vitro* BADH activities in hairy roots, and thus indicate an important role of this stress enzyme in combating photo-oxidative stress in green hairy roots upon continuous light exposure.

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INTRODUCTION

Beside their physiological roles in plants, benzoic acid and its derivatives constitute important components of many plant-based foods (Tomas-Barberan and Clifford, 2000). Plant hydroxybenzoates are generally synthesized from side-chain degradation of hydroxycinnamic acids by CoA dependent β-oxidative and non-β-oxidative pathways (Gaid et al., 2012; Qualley et al., 2012). Among these hydroxybenzoates, 4-hydroxybenzoic acid (4-HBA) is one of the major phenolic acids that are deposited in cell wall, mainly as a defense response. Hairy root culture of *Daucus carota* (carrot) was shown to accumulate 4-HBA as the principal wall-bound phenolic compound (Sircar et al., 2007). Further studies subsequently demonstrated that a CoA independent non-β-oxidative C2 side chain cleavage reaction contributes to 4-HBA biosynthesis from 4-hydroxycinnamic acid (Sircar and Mitra, 2008). The synthesis of this compound is stimulated also as a result of plant–environment interactions. Though the biosynthetic route for 4-HBA formation was reported in recent years (Sircar and Mitra, 2009; Sircar et al., 2011), the regulation of this pathway under different environmental stimuli has not yet been studied.

Light is usually an important factor affecting growth and the formation of plant products including phenolic metabolites. The stimulatory effect of light on the formation of compounds, including anthocyanins, was shown in *Perilla frutescens* cultures (Zhang et al., 1991). Light appeared to be a positive regulator in inducing shikimate and phenylpropanoid pathways leading to formation of phenolic metabolites in *Petroselinum crispum* cultures.
(Havkin-Frenkel et al., 1996). Light also induced phenylpropanoid metabolism in Arabidopsis thaliana roots (Hemmi et al., 2004). Light-induced green hairy roots of different plant species have been reported under CO2-enriched conditions (Flores et al., 1993; Jacob and Malpathak, 2004). In these green photautotrophic or photomixotrophic hairy roots, altered production levels of different secondary metabolites were found. As an example to this is the photosynthetic hairy root cultures of Datura stramonium, where enhanced hyoscyamine and scopolamine synthesis was occurred (Flores et al., 1993). Under continuous light, heterotrophic hairy roots of Ipomoea aquatic turned green while maintaining their branched morphology (Taya et al., 1994). Although enhanced activities of antioxidant enzymes were observed, such heterotrophic green hairy root cultures of any plant species have not been examined yet on the profile of phenolic metabolites. This justifies a need for examining the effect of light irradiation on phenylpropanoid metabolism in general, and on the contents of soluble and wall-bound phenolics, in particular, in green hairy roots of D. carota.

In this paper, we report in surprise, a marked reduction of wall-bound p-hydroxybenzoic acid accumulation in green hairy roots of D. carota as compared to normal hairy roots. Reduced accumulation of PAL and CHS transcripts in green hairy roots supported the above observation. To investigate the physiological reasons behind this observation we carried out comparative studies of protein profiles on SDS-PAGE, and also on the activities of antioxidant enzymes in green and normal hairy roots. Further, transcript accumulation of betaine aldehyde dehydrogenase (BADH) was analyzed on semi-quantitative mode in both green and normal hairy roots. In addition to protect system II from photoxidative damage by BADH, we comment on a new role of BADH in green hairy roots in detoxifying or reducing reactive aldehydes generated in green hairy roots upon continuous light exposure, to non-reactive compounds.

Materials and methods

Plant material

Hairy root cultures of Daucus carota L. were established by infecting carrot disc with Agrobacterium rhizogenes as described before (Sircar and Mitra, 2008). During sub culturing on regular intervals, several cultures were kept under continuous light and these hairy roots turned green. Green hairy roots were grown under continuous light, whereas normal hairy roots were kept in dark. These green as well as normal hairy roots served as materials for this study.

Extraction and analysis of soluble and wall-bound phenolic acids

Cell wall-bound 4-HBA and methanol soluble phenolic acids were extracted from both green and normal hairy roots as previously described (Sircar et al., 2007). Cell wall-bound phenolic acids were separated on RP-Hydro (Phenomenex™) C18 column (4 μm, 250 × 4.6 mm), as described by Sircar et al. (2007). Methanol soluble phenolic acids were separated on Luna (Phenomenex™) C18 column (5 μm, 250 × 4.6 mm) using a HPLC system (Waters, USA). The mobile phase was composed according to Torres-Claveria et al. (2012), where solvent A was 1 mM aqueous trifluoroacetic acid and solvent B was methanol. Time dependent mobile phase composition was as follows. t = 0 min: 100% A and 0% B; t = 25 min: 68% A and 32% B; t = 30 min: 50% A and 50% B; t = 35 min: 40% A and 60% B; t = 40 min: 0% A and 100% B; t = 45 min: 0% A and 100% B. Chromatograms were analyzed on a Windows XP platform with a BREEZETM software ver. 3.2 (Waters). Soluble phenolic acids were monitored at 254 nm and 310 nm. Wall-bound 4-HBA content was monitored at 254 nm and quantified by co-chromatographic method using 4-HBA as standard.

Preparation of protein extracts for enzyme assays

For protein extraction, 1 g of both green and normal hairy root tissues were ground in liquid nitrogen in presence of Polyclear AT™ (20%, w/w) to minimize the phenolic oxidation (Sircar et al., 2011). For ascorbate peroxidase and catalase assays, proteins were extracted at 4 °C with 2 mL of 50 mM potassium phosphate buffer (pH 7.0) (Chance and Maehly, 1955; Nakano and Asada, 1987). For guaiacol peroxidase and superoxide dismutase assays, proteins were extracted at 4 °C with 2 mL of 0.1 M potassium phosphate buffer (pH 7.0) and 0.1 M potassium phosphate buffer (pH 7.5) (Kumar et al., 2009; Mäkinen and Tenovuo, 1982), respectively. Extraction of protein for BADH assay was carried out according to a published protocol (Kumar et al., 2004). Briefly, 1 g of green and normal hairy root tissues were ground in presence of liquid nitrogen and extracted at 4 °C with 2 mL of extraction buffer consisting of 50 mM HEPES-KOH (pH 8.0), 1 mM EDTA, 20 mM sodium metabisulfite, 10 mM sodium borate, 5 mM ascorbic acid, and 5 mM dithiothreitol. Crude extracts were centrifuged at 16,000 × g for 30 min and supernatants were collected for assay. The total protein contents were measured using standard method (Bradford, 1976).

Electrophoretic analysis of protein

Hairy root tissues (0.5 g) were homogenized in a mortar and pestle with 1 mL of ice-cold 0.1 M potassium phosphate buffer (pH 7.5). The homogenates were loaded into Amicon® Ultra Filter device (Milipore, USA) and centrifuged at 14,000 × g for 15 min. Total protein content was determined by Bradford’s method (Bradford, 1976). Equal amount of crude protein mixtures (50 μg) from both types of roots were separated by SDS-PAGE (Laemmli, 1970). Protein samples were mixed with equal volume of loading dye, and were heated at 90 °C for 2 min before separated on 10% polyacrylamide at constant current of 25 mA, and visualized by staining with Coomassie Brilliant Blue.

MALDI-TOF MS/MS analysis and database search

Protein bands were excised from Coomassie stained gel, were processed for MALDI-TOF MS/MS analysis for identification of proteins from SDS-PAGE gel (Fernandez et al., 1998). Mass spectra were obtained on an Autoflex II MALDI TOF/TOF (Bruker Daltonics, Germany) mass spectrometer equipped with a pulsed Nd laser (λ = 337 nm, 100 Hz). Peptide monoisotopic signals were analyzed in FlexAnalysis software (version 2.4, Bruker Daltonics). The processed peaks were transferred through the MS BioToolsTM (version 3.0) program as input to MASCOT search engine version 2.2 (Matrix Science, Boston, MA, USA) for plant protein identification as described before (Chatterjee et al., 2012; Kundu et al., 2011).

Superoxide dismutase assay

Superoxide dismutase (SOD, EC 1.15.1.1) activity was carried out according to a recently published method with suitable modification (Kumar et al., 2009). Here, the reaction mixture was kept in dark for 1 h after adding riboflavin. Light exposure was given for 1 h for reaction and stopped by switching off the light.

Guaiacol peroxidase assay

Peroxidase enzyme (GPX, EC 1.11.1.7) activities were determined by measuring the increase of absorbance at 436 nm for 5 min relative to guaiacol oxidation to tetraguaiacol
(ε = 25.5 × 10³ L mol⁻¹ cm⁻¹) as originally described in the literature (Mäkinen and Tenvu, 1982). The assay (1 mL) contained 24 μg protein, 0.3 mM guaiacol and 0.1 mM H₂O₂ in 0.1 M potassium phosphate buffer (pH 7.0).

Ascorbate peroxidase assay

Ascorbate peroxidase activities (APX, EC 1.11.1.11) were measured according to a published method (Nakano and Asada, 1987). Enzyme activity was determined by monitoring the decrease of absorbance at 290 nm for 5 min relative to H₂O₂-dependent oxidation of ascorbic acid (ε = 2.8 mmol⁻¹ cm⁻¹). The 1 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H₂O₂ and 50 μg protein.

Catalase assay

Catalase enzyme activities (CAT, EC 1.11.1.6) were assayed as originally described by Chance and Maehly (1955) by measuring the decrease of absorbance at 240 nm for 5 min relative to degradation of H₂O₂ (ε = 4.01 L mmol⁻¹ cm⁻¹) in 1 mL reaction mixture containing 40 μg protein, 9.8 mM H₂O₂ in 50 mM potassium phosphate buffer (pH 7.0).

![Fig. 1](image-url)

**B**

**Catalase assay**

BADH activity was determined by measuring the reduction of NAD⁺ by BADH at 340 nm for 1 min (Kumar et al., 2004). The reaction mixture (1 mL) contained 50 mM HEPES-KOH buffer (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol, 1 mM NAD⁺, 1 mM betaine aldehyde and 100 μg protein.

**Isolation of core cDNA fragment of BADH**

Total RNA pools from D. carota hairy roots were isolated using the RNeasy Mini Plant Kit (Qiagen, USA). Oligo (dT)-primed reverse transcription was carried out at 42 °C with RevertAid™ M-MuLV reverse transcriptase (Fermentas GmbH). Core cDNA fragment was amplified by PCR using Taq DNA polymerase (Fermentas GmbH) and degenerate primer 1 and primer 2 (Table 1) derived from conserved regions of plant soluble aldehyde dehydrogenase. The PCR cycle with degenerate primer was conducted at 94 °C for 3 min, followed by 10 cycles of touch down PCR consisted in: denaturation, 94 °C for 45 s; annealing starting at 55 °C and ending at 45 °C for 45 s and extension, 72 °C for 1 min; and after the touch down, 30 cycles of PCR were used with an annealing temperature of 51.3 °C and with final extension of 72 °C for 10 min. The PCR products were separated by electrophoresis in a 1% agarose gel. The bands were stained by
ethidium bromide; expected band was extracted out from the gel and purified by using QIAquick gel extraction kit (Qiagen). The purified DNA was cloned in pDrive vector using Qiagen PCR cloning kit (Qiagen) following manufacturer’s instruction. The cloned product was sequenced at Eurofins Genomic India Pvt Ltd (Bangalore, India).

**BADH, PAL and CHS expression study**

Total RNA were isolated from 14 d old normal and green hairy roots using RNeasy Plant mini kit (Qiagen) and following manufacturer’s instruction. An aliquot of 2 μg total RNA was used for reverse transcription reactions, carried out as described above under “Isolation of core cDNA fragment of BADH”. Gene-specific primer 3 and primer 4 were designed from the core BADH cDNA sequence isolated earlier and used for amplifying 200 bp fragment of coding sequence to know the relative density of transcript accumulation. To analyze PAL and CHS transcript levels, forward and reverse primers were designed based on sequence information from NCBI database (Table 1). Actin served to normalize RT-PCR

**Table 1**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1</td>
<td>5′-GARTTRGGGIAARAGCC-3′</td>
</tr>
<tr>
<td>2</td>
<td>5′-AGGTCGGGAAACAATC-3′</td>
</tr>
<tr>
<td>3</td>
<td>5′-GCAATAGTTGCTGCAAT-3′</td>
</tr>
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<td>4</td>
<td>5′-TGCATGAGTCCCTC-3′</td>
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<td>5</td>
<td>5′-CCGAAGTCCAAAATCACCC-3′</td>
</tr>
<tr>
<td>6</td>
<td>5′-CAGATTTGCAGTGCAACCTC-3′</td>
</tr>
<tr>
<td>7</td>
<td>5′-GATCAGCCGCGCCAGGAT-3′</td>
</tr>
</tbody>
</table>

**Fig. 2.** In vitro activity of SOD in green and normal hairy roots. SOD activity was determined spectrophotometrically by measuring the inhibition of photo-reduction of nitro-blue tetrazolium (NBT), at 560 nm. SOD activity is expressed in percentage of maximum for making a simplified overview. Green and normal hairy roots used for this assay were of same age. Each value is the mean ± SD from at least three independent experiments.

**Fig. 3.** In vitro activities of APX, GPX, CAT and BADH in green and normal hairy roots. (A) APX activity was measured by monitoring decreased absorbance at 290 nm relative to H2O2-dependent oxidation of ascorbic acid. (B) GPX activity was quantified by monitoring the increased absorbance at 436 nm relative to oxidation of guaiacol to tetraguaiacol. (C) CAT activity was determined by measuring the decreased absorbance at 240 nm relative to degradation of H2O2. (D) BADH activity was determined by measuring the reduction of NAD+ by enzyme at 340 nm. Enzyme activity is expressed in nkcat/mg protein. Green and normal hairy roots used for all assays were of same age. Enzyme activities are expressed in nkcat/mg protein. Each value is the mean ± SD from at least three independent experiments.
results and was amplified with forward primer 10 (Table 1). PCR reactions for BADH were performed under the following conditions: 94 °C for 3 min followed by 25 cycles of amplification (94 °C for 45 s, 49.5 °C for 45 s, 72 °C for 1 min) and by final extension 72 °C for 10 min. Similar PCR conditions were used for amplification of PAL, CHS and actin, except that the annealing temperatures were 52.3 °C, 58.0 °C and 53.2 °C respectively. PCR amplified products were separated on 1% (w/v) agarose gel, stained with ethidium bromide, and photographed using MicroBis (DNR Bio-Imaging System) gel-imaging system. ImageJ software, which analyzes the pixel intensity of PCR bands, was used to compare the BADH, PAL and CHS expression levels relative to that of actin.

Results

Study of phenolic acids in both types of hairy roots

Several lines of hairy root cultures established earlier by transformation of D. carota with wild-type Agrobacterium rhizogenes were used for this study (Sircar and Mitra, 2009). All of these lines showed a profusion of root hairs and a high degree of lateral branching with a doubling time of ca. 5 d. These hairy roots turned green, when incubated under continuous illumination (250 μmol m−2 s−1). These greening remained stable in hairy roots for more than a year, when maintained under continuous illumination under shaking with a subculturing interval of 28 d. Time course experiments were performed to monitor the growth of root biomass. It was observed that the root biomass increased almost linearly up to first 35 d, followed by a decrease in the rate of biomass formation, which finally became more or less constant after 42 d of incubation. Time-course (7–28 d) studies on accumulation of 4-HBA in cell wall of both green and normal roots showed gradual increase in cell wall-bound 4-HBA accumulation (Fig. 1A). In normal hairy roots, the 4-HBA content recorded after 7 and 28 d were 1.2 ± 0.1 mg/g dry mass and 4.3 ± 0.19 mg/g dry mass, respectively, whereas in green hairy roots these were 0.7 ± 0.12 mg/g dry mass and 3.2 ± 0.19 mg/g dry mass, respectively. It was interesting to note that in every time-course points, a reduced amount of 4-HBA contents were observed in green hairy roots, as compared to normal hairy roots. In each subculturing cycle, the difference in 4-HBA content between normal and green roots was highest (48% reduction in green roots) after 14 d. Therefore these hairy roots (14 d of subculture) from both green and normal lines were used for all subsequent biochemical and molecular analyses. In soluble extracts of normal hairy roots, analysis revealed the presence of 4-hydroxybenzoic acid glucoside together with 4-hydroxybenzoic acid glucose ester and in much smaller amount of monomeric ferulic acid glucoside. The identity of these compounds including the position of glucose conjugation was confirmed by diode-array UV spectral analysis in comparison with the spectra of authentic standard reported in the literature (Mitra et al., 2002). The levels of these compounds were reduced by 50% in green hairy roots as compared to normal ones (Fig. 1B).

Superoxide dismutase activities of green and normal hairy roots

In both types of roots SOD activity was determined by the formation of formazan by superoxide-nitro blue tetrazolium complex which leads to the decrease in absorbance by the enzymes present in crude extract. It was found that total SOD activity was enhanced by 30% in green hairy roots as compared to normal ones (Fig. 2). Treatment with a SOD inhibitor diethyldithiocarbamate led to suppression of SOD activity in a concentration-dependent manner in green hairy roots (in Supplementary Fig. 1). Interestingly, SOD-suppressed root showed three-fold enhanced caffeic acid glucoside accumulation in the soluble fraction as compared to untreated ones (data not shown).

Peroxidase and catalase activities of green and normal hairy roots

Crude protein extracts from green and normal hairy root tissues were used for spectrophotometric analyses of peroxidase activities (Fig. 3A and B). Two different substrates, guaiacol and ascorbic acid were used. In green hairy roots, ascorbate peroxidase activity was 5–10% higher than normal hairy roots. Ascorbate unlike guaiacol, often oxidized during in vitro assay even in the absence of enzyme. This background ascorbate oxidation was taking into account during calculation of ascorbate peroxidase activity. However, normal hairy roots showed around 60% higher guaiacol peroxidase activity as compared to green hairy roots. Catalase is a light sensitive enzyme, and as expected, a reduced catalase activity was observed in green hairy roots as compared to normal ones (Fig. 3C).

Difference in protein profile in SDS-PAGE analysis

In SDS-PAGE, equal amount (50 μg) of protein from both types of roots was loaded onto gel. The CBB stained protein gel revealed the presence of a distinct new band in the crude protein profile of green roots, which were absent in the protein profile of normal hairy roots (Fig. 4). This band was identified as RuBiSCo large subunit through MALDI-TOF-MS/MS analysis and explained in detail in the Supplementary Fig. 2.
BADDH activity in green and normal hairy roots

Glycine betaine is produced from choline by a two-step oxidation. In the second step, betaine aldehyde is converted to the end product glycine betaine by BADH enzyme. Glycine betaine stabilizes photosystem II and Rubisco during photosynthesis under stress conditions (Holmström et al., 2000). The activity of BADH was spectrophotometrically measured from both green and normal crude protein extracts. Activity of BADH was found to be 8-fold higher in green hairy roots as compared to normal ones (Fig. 3D).

Isolation of a core BADH cDNA

Core cDNA fragment of BADH gene was isolated using degenerate primers from conserved regions in soluble aldehyde dehydrogenases from other plants. The resulting sequence of 420 bp was cloned and sequenced. Amplified sequence showed strong homology with the amino acid sequence of plant betaine aldehyde dehydrogenase (Fig. 5) available in the public database with a percentage identity of 91%. Therefore the resulting sequence can be defined as core cDNA of BADH, and appeared to be suitable for expression studies.

BADDH, PAL, and CHS expression in green and normal hairy roots

Accumulation of BADH, PAL and CHS transcripts in both green and normal hairy roots were studied by RT-PCR after normalization of cycle numbers (Supplementary Fig. 3). Gene-specific primers resulted in amplification of a 200 bp, 765 bp and 700 bp fragments of BADH, PAL and CHS, respectively. The level of actin which was detected as 70 bp PCR product, which served as control for normalization of other transcripts level. In green hairy roots, BADH transcript accumulation was found to be 4.8 fold higher than that of normal roots whereas, PAL and CHS transcript accumulation was 3.9 and 113.6 fold higher in normal roots respectively (Fig. 6).

Discussion

Roots are typically covered by soil and thus limiting the exposure to light. However, even in soil, small amount of light can penetrate to ample depths and trigger biological responses including root photomorphogenesis and seed germination. Surface irregularities or cracks formed in drying soil can also increase soil light penetration (Mandoli et al., 1990). Protochlorophyll, chlorophylls and phytochromes absorbing in the range of 600–700 nm have been reported from different roots (Björn, 1996; McEwen et al., 1991; Salisbury et al., 2007). Even light levels lower than 10 μmol m−2 s−1 were sufficient to cause significant changes to root pigmentation and morphology by increasing root pigment content and root biomass (Vollines et al., 2012). Therefore greening of D. carota hairy roots at a continuous light exposure of 250 μmol m−2 s−1 appears to be a physiological process.

Green hairy roots of D. carota showed reduced accumulation of both soluble and wall-bound phenolic acid including 4-HBA, when grown under continuous illumination. This appears to be in sharp contrast with the earlier findings that in general, light played a positive role in upregulating phenylpropanoid metabolism even in roots (Hemm et al., 2004). It was anticipated that continuous light may produce some photooxidative stress on hairy root tissues,
Fig. 6. Expression analysis of BADH, PAL and CHS genes in green (G) and normal (N) hairy roots by semi-quantitative RT-PCR (A). Total RNA (2 μg) was reverse transcribed and used as template for RT-PCR analysis. Both type of hairy roots used in this study were of same age group. BADH, PAL and CHS transcript levels were normalized on the basis of the actin transcript levels (B, C and D).

and therefore, in order to combat photooxidative stress, a shift in metabolic functions appeared inevitable. Accordingly, a range of comparative investigations including studies on the activities of antioxidant enzymes were carried out in green and normal hairy roots of same age.

Increased SOD activity in plant tissue is a sign of amelioration from oxidative stress (Alsher et al., 2002). Catalase convert H₂O₂ to H₂O and O₂, and thus indispensable for ROS detoxification in plant. In some plants, it was observed that catalase activity increased significantly under metal stresses (Gill and Tuteja, 2010), whereas in other species, decline in catalase activities was observed (Gill and Tuteja, 2010). Induction of catalase activity in plants under abiotic stress, such as salt stress, was well documented (Gill and Tuteja, 2010). In Nicotiana plumbaginifolia, different isoforms of catalase were induced at transcript level under light irradiation (Willekens et al., 1994). However, no information was available about the fate of catalase enzyme proteins under continuous light. In our study we expected an increase in catalase activity in green hairy roots, however, a decline in catalase activity was noted in as compared to normal hairy roots. Catalase is a photosensitive enzyme and light dependent inactivation and degradation of catalase was well known (Hertwig et al., 1992). This explained the probable reason for getting reduced activity of catalase in green hairy roots.

Ascorbate peroxidase (APX) reduces H₂O₂ to water using ascorbate as an electron donor (Asada, 1999). APXs are found in almost all plant cells in four different forms – chloroplastic, mitochondrial, peroxisomal and cytosolic. In plants, APX responded to diverse ways in different abiotic stresses. In our study, an increased APX activity was found in green hairy roots. While chloroplastic APX showed little response against external environmental influences, appreciable increase in cytosolic APX activity was reported during abiotic stresses (Davletova et al., 2005; Murgia et al., 2004). Thus, an increased APX activity in green hairy roots was probably one of the strategies responsible for protection of tissues during photo-oxidative stress. Guaiacol peroxidase (GPX) reduced H₂O₂ by using aromatic electro donor such as guaiacol. GPX mainly plays an important role in lignin biosynthesis and in biotic stress (Gill and Tuteja, 2010). Salinity stress also enhanced GPX activities in different plants (Gill and Tuteja, 2010). In our study, GPX activity was found to be suppressed in green hairy roots as compared to normal hairy roots. This observation appeared consistent with the reduced 4-HBA accumulation in green hairy roots. As 4-HBA originates via phenylpropanoid pathway, a reduced GPX activity in turn point towards the diversion of carbon pool from phenylpropanoid/lignin biosynthesis in green roots leading to less 4-HBA accumulation.

Protein profiles on SDS-PAGE showed the presence of a distinct band in green hairy roots that was clearly absent in normal hairy roots. MALDI-TOF-MS and subsequent MS/MS analyses confirmed it as a large subunit of RuBisCO. This is conceivable, as numerous chloroplasts were observed in green hairy roots, but absent in normal roots as observed through light microscopy (figures not shown). However, attempts to measure Hill reaction remained unsuccessful with the crude chloroplast preparations from green hairy roots (results not shown). The likely reason could be the development of non-functional chloroplast under continuous illumination in green hairy roots (Taya et al., 1994). This also explains why only a large subunit of RuBisCO was only identified from the crude protein extracts of green roots in SDS-PAGE. Such experimental attempt has not been made in green hairy roots of other species.

Continuous light increased the amount of phenylpropanoid derivatives in plant tissues (Hemm et al., 2004). PAL and CHS are key regulatory enzymes of phenylpropanoid biosynthesis (Liang et al., 1989; Martin, 1993) and slight fluctuation in their gene expression may cause the change in phenylpropanoid accumulation. In presence of light, up-regulation of PAL transcript was observed in Phaseolus vulgaris (Liang et al., 1989), but in our case reduced PAL transcript accumulation was observed in green hairy roots. This result is consistent with reduced phenolic acids accumulation in green hairy roots. A similar situation occurred in Lithospermum erythrorhizon hairy roots, where shikonin production which involves phenylpropanoid pathway, was inhibited in presence of light (Yazaki et al., 1999). Where anthocyanin production is the main concern, light induces CHS transcript accumulation in a complex manner involving different photoreceptors (Jenkins, 1997). However, in D. carota, treatment with UV light was shown to suppress 4-HBA accumulation in cell cultures, but enhanced anthocyanin accumulation. Strikingly, in light-grown green hairy roots, the relative density of CHS transcript accumulation was much lower than that of normal hairy roots D. carota. These observations appeared consistent with transcript accumulation PAL, where a five-fold reduction of transcript level was observed in green hairy roots.

Glycine betaine, a quaternary ammonium compound is known to accumulate in higher amount in plant cells upon response to salt and drought stress (Rhodes and Hanson, 1993). It was also reported that glycine betaine can protect photosystem II (PSII) by
stabilizing protein components during high light and salt stress (Holmström et al., 2000). Transgenic tobacco, expressing a BADH showed increased tolerance against high light and salt stresses (Holmström et al., 2000). In our study with green hairy roots, 8-fold higher BADH activity was recorded. This enhanced BADH activity in green hairy roots was also preceded by a higher level of BADH transcript accumulation as compared to normal hairy roots. Similar observation was also reported in Indica rice Oriza sativa where transcript accumulation of BADH1 was found to increase upon high light stress (Hashtanasombut et al., 2011). Continuous light irradiation probably generated photo-oxidative stress in newly synthesized chloroplast of green hairy roots, and to combat this effect, the level of BADH expression was enhanced.

Greening of hairy roots appears to be a rare but physiological process. The reduced 4-HBA contents in green roots apparently raised question of the possibility of metabolic shift to combat any stress, in particular photooxidative stress that might have occurred in green hairy roots! In plants, stress conditions are associated with production of reactive oxygen species (ROS) leading to cellular damage. Therefore, critical balance is required between normal growth and stress response to sustain under in vitro condition. Hairy root cultures in reality, have limited resources to support their growth and metabolic processes, and therefore it was anticipated that all requirements cannot be met simultaneously and trade-offs might happen between usual stress and responses.

Pyruvate and glyceraldehyde-3-phosphate, the products of primary metabolism is the starting point of MEP pathway, primarily to synthesize the phytol chain of chlorophyll in green hairy roots. However, in normal hairy roots, such pathway may not be active as in green ones. Shikimate/phenylpropanoid metabolisms that utilize two primary metabolites erythrose-4-phosphate and glyceraldehydes-3-phosphate are more active in normal hairy roots than the green ones. Therefore, it is plausible that during greening more glyceraldehydes-3-phosphate has been used up by the MEP pathway, and thus limited the carbon flow towards shikimic/phenylpropanoid pathways. Future work will investigate if as a result of metabolic shift, activates MEP pathway in green hairy roots and thus diverting carbon pool from phenolic metabolism towards isoprenoid biosynthesis.

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Appendix A: Supplementary data

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References


