Label-free quantitative proteomics of *Sorghum bicolor* reveals the proteins strengthening plant defense against insect pest *Chilo partellus*

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Abstract

**Background:** Spotted stem borer- *Chilo partellus* - a Lepidopteran insect pest of *Sorghum bicolor* is responsible for major economic losses. It is an oligophagous pest, which bores through the plant stem, causing 'deadheart' and hampering the development of the main cob. We applied a label-free quantitative proteomics approach on three genotypes of *S. bicolor* with differential resistance/susceptibility to insect pests, intending to identify the *S. bicolor*'s systemic protein complement contributing to *C. partellus* tolerance.

**Methods:** The proteomes of *S. bicolor* with variable resistance to insect pests, ICSV700, IS2205 (resistant) and Swarna (susceptible) were investigated and compared using label-free quantitative proteomics to identify putative leaf proteins contributing to resistance to *C. partellus*.

**Results:** The multivariate analysis on a total of 967 proteins led to the identification of proteins correlating with insect resistance/susceptibility of *S. bicolor*. Upon *C. partellus* infestation *S. bicolor* responded by suppression of protein and amino acid biosynthesis, and induction of proteins involved in maintaining photosynthesis and responding to stresses. The gene ontology analysis revealed that *C. partellus*-responsive proteins in resistant *S. bicolor* genotypes were mainly involved in stress and defense, small molecule biosynthesis, amino acid metabolism, catalytic and translation regulation activities. At steady-state, the resistant *S. bicolor* genotypes displayed at least two-fold higher numbers of unique proteins than the susceptible genotype Swarna, mostly involved in catalytic activities. Gene expression analysis of selected candidates was performed on *S. bicolor* by artificial induction to mimic *C. partellus* infestation.

**Conclusion:** The collection of identified proteins differentially expressed in resistant *S. bicolor*, are interesting candidates for further elucidation of their role in defense against insect pests.

**Keywords:** *Chilo partellus*, Insect pests, in-solution proteomics, Plant defense, Label-free quantitative proteomics

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Background

*S. bicolor* (L.) Moench is an important food, forage and biofuel Saccharinae crop cultivated world over, and recognized for its high yield and stress tolerance. It is the fifth most important cereal crop in the world after rice, wheat, maize and barley and it is the third important cereal crop after rice and wheat in India [1]. The molecular, biochemical and biotechnological investigations in *S. bicolor* are vital for its sustainable supply and it has been recognized as a model plant system for stress proteomics and genomics research [2, 3]. Over 150 insect species are known to cause damage to *S. bicolor* crops, of which, shoot fly (*Atherigona soccata*), spotted stem borer (*Chilo partellus*), midge (*Contarinia sorghicola*) and head bugs (*Calocoris angustatus, Eurtystylus* spp.) are the major pests. The lepidopteran insect pest *C. partellus* is an oligophagous pest, which feeds on cereals like maize, *S. bicolor*, or other wild grasses and is predominant in the warmer regions of the tropics [4]. Of the 58 species in the *Chilo* genus, *C. partellus* is recognized as a major pest causing estimated global losses of over $300 million annually [5, 6]. *C. partellus* neonates feed on tender leaves, causing leaf-scarification, shot-holes and later bore into the stem, causing deadheart [7], destruction of the meristem, and disruption of flowering/seed set [8, 9].

Crop plants have lost the evolutionarily acquired defense mechanisms, due to domestication and repeated selections for agronomic traits [10]; while insects have expanded their geographical horizons to emerge as pests [11]. In *S. bicolor* breeding programs, studies have emphasized the importance of wild germplasm and host plant resistance as a source of insect defense traits for selection breeding [12, 13]. ‘Omnics’ approaches have accelerated the elucidation of regulatory processes, novel molecular mechanisms and adaptations in plant-insect interactions, the findings from which have great potential to steer biotic and abiotic stress tolerance in crop plants [14]. Proteome regulates plant phenotype, its responses to stresses and is intricately linked to its transcriptome and metabolome [15]. Proteomics, with the advances in mass spectrometry, has the promise to provide a snapshot into the molecular and functional networks operating within plants and displays a ‘plant molecular phenotype’ [16].

Proteomic studies in *S. bicolor* are swiftly increasing and are focused mainly on osmotic stress [17], grain development and nutritional quality [18], seed storage protein kafirin accumulation [19], salt tolerance [20], heavy metal tolerance [21, 22], albino mutant [23, 24] and drought tolerance [25, 26]. However, the global proteome analysis of *S. bicolor* insect-resistant genotypes and the genetic, biochemical and molecular mechanisms involved in plant defense against pests is not well elucidated. *S. bicolor* like many cereal crops is heavily sprayed with pesticides during its growth to maintain yields /grain quality [27]. Insights from plant-insect interaction studies will be valuable to envisage and employ the much desired sustainable and environmentally gracious cultivation of *S. bicolor*. *S. bicolor* is known to induce cyanogenic glucoside- dhurrin, toxic cyanides and other secondary metabolites such as triterpenols upon insect infestation [28]. Genes like NBS LRR and disease resistance phloem protein 2 were identified as contributors of defense against the sugarcane aphid *Melanaphis sacchari* [29], however, omics and molecular studies on lepidopteran pests of *S. bicolor* are scarce.

* S. bicolor— lepidopteran insect pest interaction proteomics has been attempted in this study to identify the proteins contributing to insect defense in three sorghum genotypes with varied susceptibility to the spotted stem borer infestation. *S. bicolor* genotypes ICSV700 and IS2205 are known to have variable degree of resistance to *C. partellus* respectively [1, 30] while the cultivated variety (Swarna) is susceptible. The genotypes were evaluated for insect resistance based on percentage of a ‘deadheart’ formation, the extent of leaf damage, stem tunneling, panicle damage and recovery [30].

The proteomics of leaves of *S. bicolor* genotypes at steady-state and upon infestation by the stem borer *C. partellus* has been performed with an objective to (i) elucidate the important proteins contributing to *S. bicolor* insect resistance/susceptibility (ii) proteome complement specific to *S. bicolor* genotype and *C. partellus* treatment. Thorough multivariate statistical analyses for simultaneous comparisons across more than two groups were performed on the proteomics data using the opensource statistical software R. The identified proteins need to be evaluated for potential to enhance plant defense against insect pests and will be useful to engineer these traits to improve sustainable insect tolerance in *S. bicolor*.

Materials and methods

Plant material and treatments

Three *S. bicolor* genotypes, two resistant (ICSV700, IS2205) and one cultivated, susceptible (Swarna) to infestation by insect pest *C. partellus* were grown in the fields at the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India (Table 1). Plants were grown in a randomized complete block design (RBD) (Fig. 1) containing 4-row plots of 2 m length, with ridges 75 cm apart. The seedlings were thinned and the planting was maintained at 20 seedlings per 2 m row. The infestation with *C. partellus* was carried out in fields 18 days after germination with the help of the Bazooka applicator [5]. Un-infested rows were maintained as a control. Young leaves (5–8 g)
Fig. 1 (See legend on next page.)
from insect-infested and the un-infested (control) plants were collected 5 days post infestation and flash-frozen in liquid nitrogen. It has been reported that plants signal defense against insect pests at a local level, in plant tissue damaged by the insect as well as at the systemic level, in an undamaged part of the plant [31–33]. Leaves represent a systemic tissue of *C. partellus* infested *S. bicolor* plants as the actual feeding by insect happens at the leaf bases and in the stem. Leaves collected from five plants were pooled and considered as a biological replicate, and two such replicates were collected per treatment. This was done for all the three *S. bicolor* genotypes with *C. partellus* infestation (A, C, E) and control (steady-state) (B, D, F) treatments as abbreviated and detailed in Table 2.

**Insect rearing and artificial infestation**

*C. partellus* larvae were obtained from the insect rearing laboratory at the ICRISAT, Patancheru, India. The insects were reared on *S. bicolor*-based semi-synthetic artificial diet under controlled conditions (16:8 h L: D at 25 ± 1 °C and 65 ± 5% RH) as reported [5]. Newly emerged larvae were mixed with poppy seeds and released onto the leaf whorls of 18–20 days old plants by the Bazooka applicator [5]. About 10 larvae were released on each plant using two strokes of the Bazooka.

**Protein extraction, LC-MS/MS and data analysis**

Total protein extraction was done using a phenol extraction method as described earlier [34]. In short, *S. bicolor* leaf tissues stored at −80 °C were ground to a fine powder in liquid nitrogen with mortar and pestle. The total proteins were extracted from the frozen leaf powder (~1.5 g) using the phenol extraction method and they were isolated from leaves and subjected to *in-solution* digestion. The MS-MS analysis was performed with SYNAPT HDMS™ and *S. bicolor* proteome was used for protein identification. Proteins were analyzed using non-parametric multivariate tests using R. Further, gene ontology and gene expression analysis of proteins were performed to obtain three technical replicates corresponding to each biological replicate (Table 1). The instrument was operated and controlled by MassLynx4.1 SCN781 software. The peptide resolution conditions were as detailed by Sharan et al [34]. SYNAPT™ G2 High Definition MS System (HDMS™ System) (Waters Corporation, Milford, USA) was used to carry out mass spectrometry analysis of eluting peptides with instrument settings as; nano-ESI capillary voltage − 3.4 kV, sample cone - 40 V, extraction cone - 4 V, IMS gas (N₂) flow - 90 (ml/min). All analyses were performed using positive mode ESI using a Nano-LockSpray™ source as detailed in [34]. Protein identification and label-free relative protein quantification were done by analyzing LC-MS/MS data using ProteinLynx Global Server™ v2.5.3 (PLGS, Waters Corporation) for each technical replicate. Noise reduction thresholds for low energy scan ion, high-energy scan ion, and peptide intensity were set at 150, 50 and 500 counts, respectively as suggested by the manufacturer. A peptide was required to have at least two assigned fragments, and a protein was required to have at least 2 assigned peptides and 3 assigned fragments for identification. *S. bicolor* database downloaded from the UniProt database (http://www.uniprot.org/proteomes/UP000000768; the number of sequences 41,380) was searched for protein identification and the protein false positive rate was set to 4%. A ratio of > 1.5 represented over-represented proteins and < 0.65 represents under-represented proteins (Fig. 3, Supplementary Data 1). The number of proteins identified in each of the biological and technical replicates of the *S. bicolor* genotypes is reported in Table 1.

**In house statistical analysis of the proteomics data**

Proteomics data from the *S. bicolor* genotypes at steady state and upon in field *C. partellus* infestation (consisting of two biological replicates per treatment with three technical replicates each) was analyzed using multiple non-parametric statistical tests. The pipeline used for analysis was developed in-house using R (https://www.R-project.org/) for comparing multiple treatments simultaneously. Considering the biological and technical runs samples (A-F) was represented by six replicates each. Proteins found in at least two technical replicates were considered as truly present and were used for further analysis. The protein data along with the intensity values were log-transformed with base 2 and median
normalization was carried out to remove the effect of outliers. Kruskal-Wallis test (for multiple groups) was used instead of ANOVA to compare the results among the samples as it is more robust, can handle an unequal number of observations and non-parametric method that works better for small sample sizes. The p-values were adjusted to control the false discovery rate at 5%. Multivariate statistical techniques viz. Cluster Analysis, Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were used to study the similarities and differences among protein expression patterns from different samples (Fig. 2). An average of all technical and biological replicates was used to avoid the problem of missing values during cluster analysis. As a result, for each protein, we had only six readings, one corresponding to each treatment.

In PCA, proteins identified from each technical replicate were used independently. The missing values were replaced by zeros. Proteins showing significantly different abundance from both ends of the S-plot were identified (in all 68 proteins) and studied separately to examine their behavior in each of the six groups (Table 3). The proteins commonly found in all treatments were subjected to pair-wise comparisons using the Mann-Whitney test (a non-parametric equivalent of the t-test, which can handle an unequal number of observations), to identify the proteins which were differentially expressed in either susceptible/resistant or induced/un-induced samples. Proteins not commonly found across samples (A-F) were further studied in the following ways: (i) proteins uniquely present in an individual sample, (ii) proteins common in Chilo partellus induced S. bicolor were studied as ACE comparison group and (iii) proteins common in the steady-state samples were studied as (iii) BDF comparison group represented in (Fig. 4). In the case of the infested group (ACE) and un-infested group (BDF), averaged out log-transformed data for each protein from all technical replicates was used to generate a normalized (across comparison groups) heat map using MeV 4.9.0 Multiple Experiment Viewer [36].

GO classification, pathway enrichment analysis
The functional classification of identified proteins was carried out using the UniProt database [37]. Further, gene ontology (GO) analysis of identified differentially expressed proteins was carried out using the PANTHER tool [38]. Common proteins, unique proteins, proteins from infested and un-infested samples were analyzed for molecular function, biological process and cellular component using accession number as an ID and S. bicolor as an organism in the PANTHER tool. Analysis type was selected as functional classification viewed in a pie chart. The pathway enrichment analysis of differentially expressed proteins identified from ProteinLynx Global Server™ v2.5.3 (PLGS, Waters Corporation), was done using g:Profiler web server (Fig. 3C) [39].

Relative expression profiles of candidates from proteomics data
Poly-house grown, 3 weeks old S. bicolor seedlings of - Swarna (susceptible) & ICSV700, IS2205 (resistant) were used for gene expression analysis. C. partellus extract prepared in water was applied to mechanically wounded leaves to mimic the insect infestation (W + E). In control samples, wounding was followed by the application of water (W + W) to the leaf. Leaf samples were collected 3 h and 24 h post-treatment. Total RNA was extracted using the Macherey-Nagel NucleoSpin Plant II kit (Macherey Nagel Co., Duren, Germany) according to the manufacturer’s instructions. The concentration of RNA was measured using Nano-Drop (Eppendorf, Biophotometer plus,

### Table 1 Characteristics of S. bicolor genotypes used in the proteomics study

| Characteristics | S. bicolor genotypes | ICSV700 | IS2205 | Swarna |
|-----------------|----------------------|---------|--------|--------|
| Panicle         | Fully exerted, compact, elliptic and presence of awns. | Semi-compact and elliptic. Panicle weight of 53 g. | Fully exerted, loose, erect and absence of awns. |
| Flowering       | It takes 80–85 days to flower and matures in 120–125 days. | Takes about 80 days to flowering, and matures in about 90–100 days. | Flowering takes place after 65 days. |
| Grains          | Lustrous, small-sized grains and 55% grain covered with glumes. 100 seeds weigh around 2.3 g. | White, lustrous. 100 seed weight of 2.6 g. | Lustrous and around 25% grains are covered with glumes. Mass of 100 seeds is around 3.5 g. |
| Plant height    | 250 cm | 250 cm | up to 166 cm |
| Insect Resistant/Susceptible | Moderately Resistant | Resistant | Susceptible |

Morphological, growth, seed features and Chilo partellus susceptibility of the three S. bicolor genotypes used [30]
Fig. 2 (See legend on next page.)
Hamburg, Germany). The integrity of RNA samples was checked by agarose gel electrophoresis and 2 μg RNA was used for cDNA synthesis using a cDNA synthesis kit (High capacity cDNA Reverse Transcription kit, Applied Biosystems, Foster City, California, United States) as per the manufacturer’s guidelines. Real-time quantitative PCR (7500 Fast real-time PCR systems, Applied Biosystems, Foster City, California, United States) was used to check expression levels of the candidates identified from proteomics analysis using gene-specific primers synthesized at IDT (Coralville, Iowa, United States) (Supplementary Table 3), with the help of GoTaq® qPCR Master Mix (Promega Corporation, Madison, USA). Tubulin was used as a reference house-keeping gene for analysis. The data from 3 biological replicates of leaves were analyzed with 4 technical replicates each. Threshold cycle values (Ct) were used to calculate ΔCt = CtGene of interest - CtTubulin and represented as fold change 2ΔΔCt in the graphs (Fig. 5). The uninduced control sets for all the 3 genotypes were compared and analyzed using Tukey’s HSD test and indicated by different letters showing significant difference in expression values (Fig. 5). The water treatment (W + W) and insect extract-treated samples (W + E) were compared to the respective controls with the help of a two-tailed Student’s t-test with unequal variance with the threshold of p < 0.05.

Results

C. partellus infestation induces differential shifts in leaf proteomes of three different S. bicolor genotypes

The selected S. bicolor genotypes namely ICSV700, IS2205 and Swarna varied for their insect susceptibility/resistance and other agronomic traits like plant height, panicle, flowering time, grain characters and grain mass (Table 3). The earlier studies had indicated that ICSV700 and IS2205 were having moderate to good resistance to insect pests respectively, while Swarna was insect susceptible, but displayed desirable agronomic traits namely early flowering, lower plant height and higher seed mass [30]. The leaf proteomics of these three S. bicolor genotypes at steady-state (uninduced) and induced with the insect pest C. partellus was carried out to identify the S. bicolor proteins responsible for insect resistance (Fig. 1). The proteome data consisted of 967 characterized proteins, of which 232 were commonly detected in all treatments, 93 were differentially abundant across treatments, proteins common to a subset of treatments namely -induced A, C, E and steady-state B, D, F were 72 and 80 respectively, while the sum of proteins uniquely detected in each treatment (A-F) were 617. Multivariate analysis of the proteomics data in the form of PCA (Supplementary Fig. 1) and OPLS-DA was performed on all proteins identified in the study. The results indicated the overall distribution of the samples (A-F) and closeness of the biological and technical replicates (except C, D of the S. bicolor IS2205) (Fig. 2A). Based on their separation along the X-axis of OPLS-DA (T score) the resistant S. bicolor genotype ICSV700 in the uninduced state (B) was strikingly different from the rest of the two. Moreover, upon C. partellus induction both the resistant genotypes ICSV700 (A) and IS2205 (C) showed a remarkable proteomic alteration as compared to their corresponding uninduced states (B, D) as indicated by the difference in the T score (Fig. 2A).

The S-plot helped demarcate the overall significantly differential proteins from the S. bicolor proteome (Fig. 2B) as detailed in (Table 2). Twenty two proteins from the upper end and 46 from the lower end of the S-plot were identified as significantly differential. Their gene ontology indicated that they were involved in defense and immunity, calcium-binding and signaling, cell wall modifications and catalytic activities; whereas the proteins with less abundance were mostly involved in translation, signaling, and different catalytic activities (Table 2). These proteins may positively or negatively regulate S. bicolor’s interaction with C. partellus through their involvement in defense, biotic and abiotic stress tolerance, detoxification, enzyme inhibition, hydrolysis activities and signaling.

Cluster analysis was performed on the proteins commonly detected in all the treatments (A-F) (Fig. 2C). The
analysis indicated that the proteins from the uninduced *S. bicolor* samples (B, D, F) clustered separately from the *C. partellus* induced samples (A, C, E). Moreover, the insect-resistant *S. bicolor* genotypes namely ICSV700 and IS2205 (represented by A, B and C, D) clustered separately from the insect susceptible *S. bicolor* Swarna (E, F).

Ninety three proteins were found to be differentially expressed in the *S. bicolor* genotypes (A-F), of which 57 proteins displayed similar abundance patterns in the three *S. bicolor* genotypes (Supplementary Fig. 2), representing a fraction of defense response commonly induced by the genotypes upon *C. partellus* infestation. These protein species were further categorized into two

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**Table 2** Summary of *in solution* proteomics study of leaves of three *S. bicolor* genotypes at steady-state & upon *C. partellus* infestation

| Genotype               | Sample code | Treatments | Tech. replicates | No. of proteins |
|------------------------|-------------|------------|------------------|-----------------|
| ICSV700 (Resistant)    | A           | Infested   | 1                | 384             |
|                        |             |            | 2                | 291             |
|                        |             |            | 3                | 347             |
|                        |             | Infested   | 1                | 396             |
|                        |             |            | 2                | 392             |
|                        |             |            | 3                | 388             |
|                        | B           | Steady-state| 1                | 538             |
|                        |             |            | 2                | 450             |
|                        |             |            | 3                | 448             |
|                        |             | Steady-state| 1                | 367             |
|                        |             |            | 2                | 313             |
|                        |             |            | 3                | 355             |
| IS2205 (Resistant)     | C           | Infested   | 1                | 426             |
|                        |             |            | 2                | 368             |
|                        |             |            | 3                | 378             |
|                        |             | Infested   | 1                | 380             |
|                        |             |            | 2                | 359             |
|                        |             |            | 3                | 338             |
|                        | D           | Steady-state| 1                | 483             |
|                        |             |            | 2                | 421             |
|                        |             |            | 3                | 425             |
|                        |             | Steady-state| 1                | 440             |
|                        |             |            | 2                | 364             |
|                        |             |            | 3                | 312             |
| Swarna (Susceptible)  | E           | Infested   | 1                | 324             |
|                        |             |            | 2                | 290             |
|                        |             |            | 3                | 298             |
|                        |             | Infested   | 1                | 370             |
|                        |             |            | 2                | 306             |
|                        |             |            | 3                | 257             |
|                        | F           | Steady-state| 1                | 313             |
|                        |             |            | 2                | 332             |
|                        |             |            | 3                | 340             |
|                        |             | Steady-state| 1                | 347             |
|                        |             |            | 2                | 327             |
|                        |             |            | 3                | 289             |
patterns- Pattern1 with 38 proteins downregulated upon *C. partellus* infestation and Pattern2 with 19 proteins upregulated upon *C. partellus* infestation in *S. bicolor* genotypes compared to the steady-state (Supplementary Fig. 2). The remaining 36 proteins were important as they were differentially abundant in the resistant and susceptible *S. bicolor* genotypes. They were further grouped into Pattern3 (11 proteins) and Pattern4 (25 proteins) representing under-represented and over-represented proteins in *C. partellus* induced *S. bicolor* respectively, with contrast in protein expression displayed by one of the *S. bicolor* genotypes (Fig. 2D; Supplementary Fig. 3). Pattern3 proteins indicated that the biological process of translation was contrastingly upregulated in resistant *S. bicolor* genotypes. Proteins like Photosystem II subunit, germin-like protein, serine hydroxyl methyltransferase and ATPase alpha subunit were prominent in *C. partellus* induced susceptible Swarna (E) whereas they were under-represented in corresponding treatments of resistant genotypes, ICSV700 (A) and IS2205 (C). In the Pattern4 insect susceptible *S. bicolor* Swarna displayed an under-representation of the proteins which were involved in the biosynthetic process, cellular nitrogen compound process and cellular amino acid metabolism, represented by proteins like glycine-rich protein 2, NAD(P)H-quinone oxidoreductase subunit, profilin-4, Co-chaperone CGE1 isoform b, cysteine synthase, non-specific lipid transfer protein and superoxide dismutase. Ribulose bisphosphate carboxylase, ATP synthase subunit beta, extracellular calcium-sensing receptor and elongation factor 1- delta were upregulated in the *C. partellus* induced resistant *S. bicolor* genotype IS2205 (C) whereas they were under-represented in the other genotypes.

**Analysis of differential proteins identified in a pairwise comparison of *S. bicolor* genotypes upon *C. partellus* infestation and at steady-state using ProteinLynx global server™ v2.5.3 (PLGS, waters corporation)**

Leaf proteomes of *C. partellus* induced and steady states of genotypes of *S. bicolor* were compared with the help of ProteinLynx Global Server™ v2.5.3 (PLGS, Waters Corporation) to identify over-represented (fold change > 1.5) and under-represented (fold change < 0.65) proteins. These proteins were compared to identify proteome similarities/differences amongst the genotypes (Fig. 3, Supplementary Data 1). Most of the differential proteins identified in the pair-wise comparisons were not shared...
| Status | Key | Accession No. | Name of Protein/Similar Protein | Function/GO |
|--------|-----|---------------|---------------------------------|-------------|
| Up     | 372 | CSX1U2        | Calmodulin                      | Calcium ion binding (GO:0005509), calcium-mediated signaling (GO:0019722) |
| Up     | 656 | CSYSK7        | similar to Pathogenesis related protein S | Defense response (GO:0006952) |
| Up     | 1292 | CSYBE9        | Chitin-binding type-1 domain-containing protein | Chitinase activity (GO:0004568) |
| Up     | 1510 | CSYSK6        | similar to Thaumatin like pathogenesis related protein 1 | Defense response (GO:0006952) |
| Up     | 5767 | CSZON8        | Peroxidase                      | 2 phenolic donor + H2O2 = 2 phenolic radical donor + 2 H2 |
| Up     | 6674 | CSXHS1        | similar to β-1,3-glucanase      | Hydrolysis of O-glycosyl compounds, Carbohydrate metabolic process |
| Up     | 9254 | CSXCE2        | similar to Zeamatin-like protein | Inhibition of trypsin and α-amylases, Defense response (GO:0006952) |
| Up     | 9604 | CSZ469        | Peroxidase                      | 2 phenolic donor + H2O2 = 2 phenolic radical donor + 2 H2O |
| Up     | 13,645 | CSZ3A0     | SCP domain-containing protein   | similar to pathogenesis-related protein |
| Up     | 14,437 | CSWWX5     | similar to Histone2A            | Photosystem II repair (GO:0010206) |
| Up     | 17,199 | CSZ9A2       | similar to Thylakoid luminal 16.5 kDa protein | |
| Up     | 19,206 | CSWT31       | similar to DPP6 N-terminal domain-like protein | |
| Up     | 23,877 | CSYLY5     | similar to Ribosome-recycling factor | Peptidase activity |
| Up     | 26,193 | CSY817      | similar to Carboxyl terminal peptidase precursor | |
| Up     | 26,971 | CSX8S2      | SCP domain-containing protein   | Cysteine rich secretory protein, allergen V5/Tpx-1 |
| Up     | 28,788 | CSWQE1      | similar to α-amylase/trypsin inhibitor | |
| Up     | 30,151 | CSZ8N5       | Expansin-like EG45 domain-containing protein | Chitinase activity |
| Up     | 31,567 | CSYE3       | similar to Abscisic acid stress ripening 3 | |
| Up     | 31,569 | CSYSD6      | Barwin domain-containing protein | Defense response to bacterium (GO:0042742) or fungus (GO:0050832) |
| Down   | 34 | A1E9V4        | Cytochrome b6                   | Component of the cytochrome b6-f complex |
| Down   | 102 | A1E9W6        | 30S ribosomal protein L2, chloroplastic | Mitochondrial translation (GO:0032543) |
| Down   | 121 | A1E9W0        | 30S ribosomal protein S8, chloroplastic | Translation (GO:0006412) |
| Down   | 260 | CSYH12        | Caffeic acid O-methyltransferase | Flavonol biosynthetic process (GO:0051555) |
| Down   | 353 | CSXYX5        | similar to 60S ribosomal protein L11-1 | Translation (GO:0006412) |
| Down   | 1163 | CSX1Q1        | similar to Hydroxyproline-rich glycoprotein family protein | |
| Down   | 1442 | CSY065        | Lipase_3 domain-containing protein | Lipid metabolic process (GO:0006629) |
| Down   | 1979 | CSYIF8        | Obg-like ATPase 1               | ATPase activity (GO:00016887), Negative regulation of response to salt stress (GO:1901001)& defense response to bacterium (GO:1900425) |
| Down   | 3699 | CSYRK9        | similar to Pentatricopeptide repeat-containing protein | RNA modification (GO:0009451) |
| Status Key | Protein Accession No. | Name of protein/similar protein | Function/ GO |
|------------|----------------------|-------------------------------|-------------|
| Down 4242  | C5XW30               | similar to Phorphobilinogen deaminase | It catalyzes head to tail condensation of four porphobilinogen molecules releasing 4 ammonia molecules |
| Down 5841  | C5YRL0               | Non-specific lipid transfer protein | Bifunctional protease and alpha amylase inhibitor inhibitor, lipid binding (GO:0008289), lipid transfer (GO:0006869) protein |
| Down 6172  | C5XYT6               | FAD_binding_3 domain-containing protein | FAD binding (GO:00719494), Geranylgeranyl reductase activity (GO:0045550) |
| Down 10,362| C5YL07               | Aldehyde domain-containing protein | Beteain-aldehyde dehydrogenase activity (GO:0008802), Response to anoxia (GO:0071454) |
| Down 11,647| C5WTC9               | Ribosomal_L16 domain-containing protein | Translation (GO:0006412) |
| Down 12,657| C5Z267               | similar to 60S ribosomal protein L9 | Cytoplasmic translation (GO:0002181) |
| Down 14,425| C5YAD0               | similar to 60S ribosomal protein L6 | Cytoplasmic translation (GO:0002181) |
| Down 15,418| C5SEA1               | similar to Fructose-bisphosphate aldolase 1, chloroplastic isoform X1 | |
| Down 15,466| C5YHF2               | similar to Rubredoxin family protein | |
| Down 15,661| C5X284               | 40S ribosomal protein S8 | Translation (GO:0006412) |
| Down 15,716| C5WZ25               | Tubulin beta chain | GTPase activity (GO:0003924), microtubule cytoskeletal organization (GO:000226) |
| Down 16,668| C5YA8                | Pyruvate kinase | ATP + pyruvate = ADP + H+ + phosphoenolpyruvate, Glycolytic process (GO:0006036) |
| Down 17,564| C5YCD5               | PhKB domain-containing protein | Adenosine kinase activity (GO:0004001), Purine ribonucleoside salvage (GO:0006166) |
| Down 18,075| C5YXW7               | Guanosine nucleotide diphosphate dissociation inhibitor | Rab GTPase binding (GO:0017137), small GTPase mediated signal transduction (GO:0007264) |
| Down 19,332| C5X6V0               | similar to Extracellular ribonuclease LE | RNA catabolic process (GO:0006401) |
| Down 19,346| C5YG66               | Aminomethyltransferase | Aminomethyltransferase activity (GO:0004047), Glycine decarboxylation via glycine cleavage system (GO:0019464) |
| Down 21,133| C5YG29               | similar to 60S ribosomal protein | Translation (GO:0006412) |
| Down 22,396| C5YCD6               | Phenylalanine ammonia-lyase | L-phenylalanine = NH4+ + trans-cinnamate, Cinnamic acid biosynthetic process (GO:0009800), L-phenylalanine catabolic process (GO:0006559) |
| Down 22,977| C5WTZ6               | 40S ribosomal protein S4 | Translation (GO:0006412) |
| Down 23,733| C5YXS7               | 40S ribosomal protein S4 | Translation (GO:0006412) |
| Down 23,995| C5YU66               | similar to Heat shock 70 kDa protein 4 | Stress response |
| Down 24,630| C5YJP1               | HATPase_c domain-containing protein | Unfolded protein binding (GO:0051082), Response to chlorate (GO:0010157), heat (GO:0009408), salt stress (GO:0009651), water deprivation (GO:0009414) |
| Down 25,743| C5XZ55               | similar to Formate tetrahydrofolate ligase | |
| Down 25,986| C5WXD2               | similar to Protein TIC110, chloroplastic | |
| Down 26,465| C5XTT8               | Phenylalanine ammonia-lyase | L-phenylalanine = NH4+ + trans-cinnamate, Cinnamic acid biosynthetic process (GO:0009800), L-phenylalanine catabolic process (GO:0006559) |
| Down 28,031| C5XIT6               | Pectinesterase | [(1 \rightarrow 4)-α-D-galacturonic methyl ester(n) + n H2O = [(1 \rightarrow 4)-α-D-galacturonic acid](n) + n H+ + n methanol, cell wall modification (GO:0042545) |
| Down 28,874| C5YMU8               | similar to Puromycin-sensitive aminopeptidase | |
| Down 29,216| C5YPW8               | similar to ATP-citrate synthase | ATP binding (GO:0005524) |
| Down 30,018| C5WZ87               | similar to Ribosomal protein S9 | Translation (GO:0006412) |
| Down 30,990| C5X118               | S-adenosylmethionine synthase | ATP + H2O + L-methionine = diphosphate + phosphate + S-adenosyl-L-
between the 3 genotypes, signifying unique ways of each genotype to deal with the *C. partellus* induction (Fig. 3A & 3B). The enrichment analysis of over-represented proteins from Swarna and ICSV700 is involved in photosynthesis or carbon fixation. Under-represented proteins were enriched for the ribosome, protein processing in the endoplasmic reticulum, biosynthesis of amino acids (Fig. 3C). The gene ontology analysis of these proteins indicated that the majority of them were involved in cellular and metabolic processes related to binding and catalytic activities. It is important to note that *S. bicolor* upon *C. partellus* infestation suppresses the accumulation of several proteins from these GO categories and initiates the accumulation of other proteins representing the same categories (Fig. 3D). Under-representation of proteins related to response to stimulus in Swarna was one interesting find from this analysis. To maximize the useful information derived from the data, the induced and un-induced states were compared separately in further analysis.

**GO analysis of differential proteins in *C. partellus* induced *S. bicolor* (A, C, E) and *S. bicolor* at steady state (B, D, F)**

Comparing the insect-induced (A, C, E) or steady-state (B, D, F) treatments across *S. bicolor* genotypes helped to widen the analysis by maximizing the information obtained (Fig. 4). The comparison amongst the three treatments led to the identification of a higher number of differential proteins and also account for the intrinsic differences amongst the varieties. The analysis was done on 72 and 80 proteins differentially abundant in *C. partellus* induced *S. bicolor* genotypes (A, C, E) or at steady state (B, D, F) respectively (Supplementary Table 2 and Fig. 4). Of the set, a large number of protein species were significantly differentially abundant in the susceptible genotype Swarna than resistant genotypes. It represented the protein species through which both the resistant *S. bicolor* genotypes responded similarly to the *C. partellus* infestation. Intriguingly, protein species that were found to be differentially abundant in both the resistant *S. bicolor* genotypes either at steady state or upon *C. partellus* infestation were found to be involved in cellular metabolic processes, organic substance metabolic process, nitrogen compound and small molecule metabolic process, oxidation-reduction and response to abiotic stimuli (Fig. 4C). These proteins had the molecular function (MF) of binding and catalytic activity though these were represented by different proteins in A, C, E or B, D, F comparisons (Supplementary Table 2).

**The *S. bicolor* resistant genotypes are rich in unique proteins**

The resistant genotype ICSV700 was found to contain the highest number of unique proteins at steady-state - (B) (180) followed by the other resistant *S. bicolor* IS2205 - (D) (135) while the *C. partellus* induced ICSV700 (A) also displayed around 105 unique proteins (Fig. 5). The GO analysis of the unique proteins identified in each indicated that the molecular functions such as catalytic activity, binding, structural molecular activity were represented predominantly from un-induced resistant genotypes, ICSV700 (B) and IS2205 (D) whereas these functions were very low in the susceptible variety, Swarna. The biological processes like cellular process, metabolic process, cellular component, localization, response to stimulus and cellular components like membrane, macromolecular complex, cell part, organelle
Fig. 4 (See legend on next page.)

(A) 

(B) 

(C) 

Signal transduction 
Cell communication 
Regulation of biological process 
Cellular comp. organization 
Regulation of biological quality 
Response to chemical 
ATP metabolic process 
Cellular component biogenesis 
Catabolic process 
Response to stress 
Cellular response to stimulus 
Protein folding 
Response to abiotic stimulus 
Oxidation-reduction process 
Biosynthetic process 
Small mol. metabolic process 
Nitrogen comp. met. process 
Primary metabolic process 
Organic substance met. proc. 
Cellular metabolic process 
Guanyl-nucleo. ex. wt. activity 
Transferase activity 
Ligase activity 
Cofactor binding 
Amide binding 
Translation regulator activity 
Lyase activity 
Small molecular binding 
Drug binding 
Carbohydrate deriv. binding 
Oxidoreductase activity 
Hydrolyase activity 
Protein binding 
Ion binding 
Structural const. of ribosome 
Organic cyclic comp. binding 
Heterocyclic comp. binding

Biological processes

Molecular function

No. of proteins
were also higher in unique proteins found in un-induced S. bicolor resistant genotypes, ICSV700 and IS2205.

The top 10 most abundant unique proteins from each sample (A-F) are listed in Table 4. The C. partellus induced ICSV700 (A) showed the presence of proteins like β-caryophyllene synthase involved in indirect defense; RPP-13 like protein, Ankyrin repeat domain-containing protein 2, adenylyl cyclase associated protein which plays an important defense role in plants; proteins involved in protein turnover DNA repair, wound healing was also detected. Some interesting proteins like ATP synthase CF1 alpha subunit involved in inducing changes in plant surface structures like spines were also seen. The other resistant genotype of S. bicolor IS2205 (C) upon C. partellus infestation showed the unique presence of plant defense proteins like chitinase, RPP-13 like; biotic and abiotic stress-related proteins like monogalactosyldiacyl glycerol synthase, zinc finger CCH domain-containing protein 55, thiazole synthase; and proteins involved in protein turnover over. The susceptible S. bicolor upon C. partellus induction (E), however, showed the expression of proteins like kinases, proteins involved in growth, turnover and homeostasis like adenylate isopentyl transferase, ubiquitin E3-protein ligase, triacylglycerol lipase and UDP-d-glucuronate decarboxylase.

The resistant S. bicolor genotypes ICSV700 and IS2205, at the steady-state level (B, D) and upon C. partellus infestation (A, C) had a far high number of unique proteins while susceptible S. bicolor Swarna displayed strikingly smaller number of unique proteins. The susceptible S. bicolor variety Swarna lacks the proteins involved in metabolic processes related to nitrogenous compounds, sulfur compounds, secondary metabolites and biosynthetic processes and after infestation by C. partellus, it is inefficient in the upregulation of nitrogen compound biosynthesis.

Relative expression profiles of genes corresponding to protein candidates identified in S. bicolor-C. partellus interaction proteomics
Serine hydroxymethyltransferase, germins, cyanate hydratase, β-glucanases, lipid transfer proteins (LTP), zeamatin like proteins, endochitinases, superoxide dismutase (SOD), chaperonins and 14–3-3 like proteins were selected for gene expression analysis based on their protein expression pattern in non-targeted S. bicolor proteomics study as well as their function. We set up an independent experiment (methods section 2.6) to study the candidate gene expression kinetics at early time points (3 h, 24 h) after mimicking insect infestation.

The gene expression studies were carried out in the three genotypes of S. bicolor (ICSV700, IS2205 and Swarna) under treatments namely (i) steady-state, (ii) wounding + C. partellus extract application (W + E) and (iii) wounding + water application (W + W) at 3 h and 24 h post-treatment. Distinct gene expression patterns were noted amongst the S. bicolor genotypes at steady state. Additionally, the W + E and W + W treatments also displayed differential gene expression patterns at 3 h and 24 h post-treatment across the S. bicolor genotypes. ICSV700 showed over-expression of germins, cyanate hydratase, LTP, zeamatin, endochitinase, chaperonins in W + E; whereas serine hydroxymethyltransferases, β- glucanase, SOD, 14–3-3 like proteins were under-expressed in W + E. In W + E, IS2205 genotype showed over-expression of serine hydroxymethyltransferases, germins, SOD, chaperonins and downregulation of cyanate hydratase, β- glucanase, 14–3-3 like proteins. While the susceptible genotype showed over-expression of LTP, chaperonins, and downregulation of cyanate hydratase, endochitinase, zeamatin and 14–3-3 like protein in W + E.

Over-expression of LTP and chaperonins and under-expression of 14–3-3 like proteins upon insect extract treatment were commonly observed across resistant and susceptible genotypes in W + E. Germins were differentially over-expressed in resistant genotypes in W + E treatment. Over-expression of zeamatin, endochitinase, cyanate hydratase was observed in ICSV700 while serine hydroxymethyltransferases, SOD were abundant in IS2205 in W + E treatment. The differences in over-expressed proteins in W + E in resistant genotypes suggest that they have different mechanisms to confer the resistance to the insect pest. Except for LTP and chaperonins, the susceptible genotype Swarna is not able to overexpress the genes which have a putative role in defense against the insect. The relative expression
Fig. 5 (See legend on next page.)
pattern of genes in early time points (3 h and 24 h) post treatment was correlated to the late (20 days after initiating *C. partellus* infestation) expression profile of proteins identified from non-targeted proteomic studies. Proteins like zeamatin, endochitinase showed a correlation in early gene expression and late protein expression pattern whereas, serine hydroxymethyltransferase, SOD, chaperonins, 14–3–3 like proteins showed a partial correlation across timepoints and genotypes. LTP and β-glucanase showed no correlation between the early gene expression and the late protein expression profile.

**Discussion**

Our study originated from the observations that the two genotypes of *S. bicolor*, ICSV700 and IS2205 are resistant to insect pests while the genotype Swarna is susceptible [1]. Proteins being one of the direct effector molecules against the insects, proteomic study on these genotypes would reveal many secrets about the plant defense [41]. We carried out a comparative proteomic analysis of *S. bicolor* – *C. partellus* interaction to identify the major protein components from *S. bicolor* genotypes responsible for resistance to *C. partellus* (Fig. 1). The study was focused on 967 characterized proteins from the *S. bicolor* proteome, their analysis which allowed us to investigate the intrinsic differences in the three genotypes of *S. bicolor* and analyze their proteomic response when induced by the pest *C. partellus*. This led to the identification of several proteins that strongly supported the insect resistance traits in *S. bicolor* genotypes, and will be important for further studies.

The study revealed that the three *S. bicolor* genotypes differentially responded to the induced infestation by *C. partellus* and also had intrinsically different proteomes at steady state levels (Fig. 2A, C). Plant domestication has led to changes in the crop plant defense pathways leading to their susceptibility (as seen in the genotype Swarna) to pests and pathogens [42], while their wild relatives and improved lines (like *S. bicolor* genotypes - ICSV700, IS2205) possess the molecular components contributing to their defense [43, 44]; the proteomic analysis of these genotypes helped in discovering the protein networks involved in strengthening plant defense to insect pests.

The differential protein complements from *S. bicolor* genotypes in response to *C. partellus*

Sixty eight proteins with differential abundance across *S. bicolor* genotypes at steady state and upon *C. partellus* infestation were identified and they were classified as significantly high or low abundance proteins (Fig. 2B; Table 2). The catalytic activities of abundant proteins were endochitinases, peroxidases and glutathione S-transferase like, all involved in promoting defense against insect pests; whereas the catalytic activities of less abundant proteins were flavone/caffeic acid 3-O-methyltransferase, ATP citrate synthase and betaine aldehyde dehydrogenase involved in the biosynthesis of a multitude of small molecules and methylated flavonoids useful in herbivore deterrence and abiotic stress [45, 46].

Cellular signaling machinery like Calmodulin-related proteins or G-protein and G protein modulators, various kinases, heat shock proteins, phenylalanine ammonia-lyase, were identified and need functional characterization to determine their contribution to *S. bicolor* pest resistance [47, 48]. Additionally, the known defense proteins like PR-5, alpha-amylase/trypsin inhibitor, osmotin, non-specific lipid transfer protein were also amongst the candidates identified, reinforcing their role in plant defense against insect pests [49, 50].

Enrichment analysis of over-represented and under-represented proteins have helped to gain a bird’s eye view of the proteome remodeling upon *C. partellus* infestation in *S. bicolor* genotypes (Fig. 3). Overall, there is more protein suppression; and selective protein accumulation as represented by the higher number of proteins in ‘response to stress’ category. The under-representation of proteins involved in translation and amino acid biosynthesis was conspicuous and as expected; but the accumulation of proteins involved in the protection and maintenance of photosynthesis upon *C. partellus* infestation, is a feature that contrasts other reports [51].

*C. partellus* resistant *S. bicolor* genotypes have commonalities in their proteome which are not detected in the susceptible *S. bicolor* Swarna

*S. bicolor* Swarna had less abundance of proteins involved in defense, signaling and protein remodeling which might negatively influence its defense against the invading lepidopteran pest (Fig. 2D; Supplementary Fig. 3; Supplementary Table 1). Swarna was seen to have high levels of PR proteins which are generally directed to deter pathogen attack, while the resistant *S. bicolor* genotypes are seen respond by signaling the activation of certain proteins having broad-spectrum activity against
Table 4 Top 10 of the uniquely represented proteins from *S. bicolor* genotypes at steady-state and upon *C. partellus* infestation

| Key | Protein Accession No. | Protein Name | Function |
|-----|-----------------------|--------------|----------|
| **A** - *S. bicolor* ICSV700 infested by *C. partellus** |
| 23, 819 | C5Y8S3 | similar to ATP synthase CF1 alpha subunit | Chloroplastic, correlation with spiny-ness |
| 1056 | C5WWL7 | similar to Beta-caryophyllene synthase | Indirect defense against Lepidoptera by attracting predators |
| 38 | C5YUK3 | Flap endonuclease 1-A | Catalysis of the cleavage of a 5' flap structure in DNA, but not other DNA structures; processes the 5' ends of Okazaki fragments in lagging strand DNA synthesis, Acts as a genome stabilization factor |
| 107 | A1E9R4 | DNA-directed RNA polymerase subunit beta | DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates, Nucleoside triphosphate + RNA(n) = diphosphate + RNA(n + 1) |
| 9314 | C5XSB2 | similar to ADP-ribosylation factor GTPase-activating protein AGD3 | Binds to and increases the activity of a GTPase, plasma membrane remodeling |
| 19, 695 | C5Y746 | similar to disease resistance RPP13-like protein 3 isoform X3 | Disease resistance against pathogens |
| 28, 942 | C5YHK1 | similar to Ankyrin repeat domain-containing protein 2 | Chloroplast targeting sequence binding |
| 1890 | C5XAM0 | similar to ubiquitin-like | Protein turnover |
| 20, 222 | C5X7K7 | similar to RNA polymerase beta subunit | RNA polymerization |
| 4393 | C5YLQ0 | Adenylyl cyclase-associated protein | Cyclase-associated protein 1-like, cytoskeleton organization, response to pathogen |

| **B** - *S. bicolor* ICSV700 at steady state |
| 3162 | C5YWCS | similar to Proliferation-associated protein 2G4 | Change in state or activity of a cell or an organism as a result of a cytokinin stimulus |
| 28, 629 | C5Z4X4 | similar to reverse transcriptase, Brassinosteroid insensitive-1 like | Plant architecture |
| 7943 | C5XJS0 | similar to Retrotransposon protein | Probable member of endonuclease, exonuclease, phosphatase family |
| 13, 397 | C5WSY0 | similar to Arginine decarboxylase | Drought tolerance, defense |
| 27, 809 | C5XAT9 | Histone H2A | DNA binding, chromatin silencing |
| 2862 | C5XCT6 | Nitrate reductase | Cell signaling & survival under stress |
| 11, 807 | C5WU06 | similar to FACT complex subunit SPT16 | Histone binding and remodeling outside the context of DNA replication |
| 25, 101 | C5X9S7 | Ribosomal protein L15 | Structural constituent of ribosome, Cytoplasmic translation |
| 14, 173 | C5WQ44 | similar to enolase | Phosphopyruvate hydratase activity |
| 16, 161 | C5YDVS | similar to putative quinone oxidoreductase | Oxidoreductase activity, chloroplastic |

| **C** - *S. bicolor* IS2205 infested by *C. partellus** |
| 29, 614 | C5YI1 | similar to Monogalactosyldiacyl glycerol synthase 2 | Thylakoid membrane biogenesis under stress |
| 13, 788 | C5YMZ5 | similar to Zinc finger CCCH domain-containing protein 5S-like | ABA biosynthesis, drought, post-transcriptional regulation of gene expression |
| 9243 | C5WNH3 | similar to ATP binding protein | Protein kinase activity, Serine/Threonine protein kinase STY46 like |
| 5008 | C5GY94 | similar to thiazole synthase | ADP binding, Cell wall integrity, and stress response component 1-like |
| 25, | C5YJ73 | similar to Ubiquitin and WLM domain-containing | Ubiquitin and WLM domain-containing metalloprotease |
Table 4 Top 10 of the uniquely represented proteins from *S. bicolor* genotypes at steady-state and upon *C. partellus* infestation (Continued)

| Key | Protein Accession No. | Protein Name | Function |
|-----|----------------------|--------------|----------|
| 363 | protein              |              |          |
| 5014 | CSXXC0 similar to Protein kinase domain-containing protein | Triggered in response to the presence of a foreign body or the occurrence of an injury, Introducing a phosphate group on to a protein, ATP binding, Cysteine-rich receptor-like protein kinase 26 |
| 702 | CSX8K4 similar to disease-resistant protein RPP-13 like 1 | Disease resistance protein against pathogen |
| 17 | CSZ5B4 similar to 26S protease regulatory subunit 6A-like protein | ATP binding, Interacting selectively and non-covalently with a member of the class of TATA-binding proteins (TBP), including any of the TBP-related factors (TRFs), 26S protease regulatory subunit 6A homolog |
| 19 | CSYVH3 60S acidic ribosomal protein P0 | Ribosomal subunit tRNA binding, Cytoplasmic translation |
| 21 | C6JSV0 similar to Chitinase Catalysis of the hydrolysis of (1->4)-beta linkages of N-acetyl-D-glucosamine (GlcNAc) polymers of chitin and chitodextrins |
| 125 | C5Y227 similar to Indole-3-acetic acid-amido synthetase GH3.3 | Synthesis of IAA-conjugates, a mechanism to cope up with excess auxin |
| 22 | CSX8X8 similar to AT-hook motif-containing protein, Helicase | NTP + H2O = NDP + phosphate, to drive the unwinding of a DNA helix, Process of restoring DNA after damage, Telomere maintenance, ATP-dependent DNA helicase PIF1-like |
| 125 | CSXNN6 Thiamine thiazole synthase 1, chloroplastic | Involved in the biosynthesis of the thiamine precursor thiazole, Suicide enzyme, Additional roles in adaptation to various stress conditions and DNA damage tolerance |
| 6474 | C5WWV5 similar to Inactive ubiquitin carboxyl-terminal hydrolase S3 | Thiol-dependent ubiquitinyl hydrolase activity, protein deubiquitination, inactive ubiquitin carboxyl-terminal hydrolase S3 |
| 16 | CSYS29 similar to Diaminopimelate decarboxylase | Diaminopimelate decarboxylase activity, meso-2,6-diaminopimelate + H(+) = L-lysine + CO2, systemic acquires resistance |
| 31 | CSXSW5 Glutaredoxin-like protein | Photooxidative stress, antioxidant activity |
| 19 | CSZ949 similar to RING zinc finger domain superfamily protein | Ubiquitin specific protease binding, ERAD-associated E3 ubiquitin-protein ligase HRD1-like isoform X1 |
| 2715 | CSX0X0 similar to NEFA-interacting nuclear protein NIP30 | Protein FAM192A isoform X1 |
| 23 | CSY1Y1 Peroxidase | 2 phenolic donor + H2O2 = 2 phenoxyl radical of the donor + 2 H2O |
| 29 | CSZ7K8 Pyruvate dehydrogenase E1 component subunit alpha | Catalyzes the overall conversion of pyruvate to acetyl-CoA and CO2 |

**D - *S. bicolor* IS2205 at steady state**

| Key | Protein Accession No. | Protein Name | Function |
|-----|----------------------|--------------|----------|
| 28 | CSY227 similar to Indole-3-acetic acid-amido synthetase GH3.3 | Synthesis of IAA-conjugates, a mechanism to cope up with excess auxin |
| 22 | CSX8X8 similar to AT-hook motif-containing protein, Helicase | NTP + H2O = NDP + phosphate, to drive the unwinding of a DNA helix, Process of restoring DNA after damage, Telomere maintenance, ATP-dependent DNA helicase PIF1-like |
| 125 | CSXNN6 Thiamine thiazole synthase 1, chloroplastic | Involved in the biosynthesis of the thiamine precursor thiazole, Suicide enzyme, Additional roles in adaptation to various stress conditions and DNA damage tolerance |
| 6474 | C5WWV5 similar to Inactive ubiquitin carboxyl-terminal hydrolase S3 | Thiol-dependent ubiquitinyl hydrolase activity, protein deubiquitination, inactive ubiquitin carboxyl-terminal hydrolase S3 |
| 16 | CSYS29 similar to Diaminopimelate decarboxylase | Diaminopimelate decarboxylase activity, meso-2,6-diaminopimelate + H(+) = L-lysine + CO2, systemic acquires resistance |
| 31 | CSXSW5 Glutaredoxin-like protein | Photooxidative stress, antioxidant activity |
| 19 | CSZ949 similar to RING zinc finger domain superfamily protein | Ubiquitin specific protease binding, ERAD-associated E3 ubiquitin-protein ligase HRD1-like isoform X1 |
| 2715 | CSX0X0 similar to NEFA-interacting nuclear protein NIP30 | Protein FAM192A isoform X1 |
| 23 | CSY1Y1 Peroxidase | 2 phenolic donor + H2O2 = 2 phenoxyl radical of the donor + 2 H2O |
| 29 | CSZ7K8 Pyruvate dehydrogenase E1 component subunit alpha | Catalyzes the overall conversion of pyruvate to acetyl-CoA and CO2 |

**E - *S. bicolor* Swarna infested by *C. partellus***

| Key | Protein Accession No. | Protein Name | Function |
|-----|----------------------|--------------|----------|
| 2587 | CSXAW9 Serine/threonine-protein kinase | ATP + a protein = ADP + a phosphoprotein, reactions triggered in prevention/recovery from the infection caused by the attack |
| 21 | CSXLE9 similar to Photosystem II CP47 reaction center protein | Chlorophyll-binding, Photosynthetic ETS, Similar to Photosystem II CP47 chlorophyll apoprotein |
| 21 | CSXXY1 similar to Serine-threonine kinase receptor-associated protein | Inolved in defense |
| 13 | CSYV23 similar to Adenylyl isopentenyl transferase-like | Cytokinin biosynthesis |
| 23 | CSWW05 similar to Triacylglycerol lipase SDP1 | Hydrolase activity, Catalysis of the reaction: triacylglycerol + H2O = diacylglycerol + a carboxylate, membrane protein homeostasis |
| 17 | CSYTB0 similar to Inosine-5′-monophosphate dehydrogenase, Serine/Threonine Kinase activity | |
pathogens and pests or specifically directed against the pest. These are represented by proteins like chitinases, polyphenol oxidases and zeamatin.

The analysis of Pattern3 and Pattern4 proteins led to commonly expressed yet differentially abundant proteins across treatments. Serine hydroxymethyltransferase, from Pattern3, known for constitutive expression of salicylic acid-inducible genes and H$_2$O$_2$ detoxification genes [52] responsible for reducing the endogenous oxidative stress, was over-represented in the susceptible $S. \text{bicolor}$ unlike resistant ICSV700 & IS2205 genotypes (Fig. 2D; Supplementary Table 1). It was observed in previous studies that conditions favoring oxidative stress lead to redox signaling and hormonal crosstalk responsible for fine-tuning, enhancing the defense responses in plants [53]. Further, Swarna could not accumulate proteins involved in maintaining photosynthesis upon infestation by $C. \text{partellus}$ like the resistant genotypes of $S. \text{bicolor}$ as represented by Pattern4. In the pair wise comparison of proteins expressed before and after infestation by $C. \text{partellus}$ in the $S. \text{bicolor}$ genotypes, a number of distinct proteins were identified (Fig. 3, Supplementary Data 1). Photosynthesis related proteins were strongly upregulated in ICSV700 and Swarna upon $C. \text{partellus}$ infestation, however IS2205 was seen to show least perturbations as indicated by the pathway analysis (Fig. 3C). Susceptible Swarna genotype may lack networks for fine-tuning of defense responses manifested by the absence or less abundance of several proteins detected in resistant genotypes.

The insect-resistant $S. \text{bicolor}$ genotypes were enriched with elongation factors and chaperons, represented by proteins 14–3-3 like proteins, calmodulins, heat shock proteins and glutamine synthetase signifying an accelerated protein synthesis, downstream signaling and refolding activity upon infestation (Fig. 4A, C; Supplementary Table 2). Similar proteomic turnover has been demonstrated recently in wheat plants as a response to the pest infestation [54].

### Table 4: Top 10 of the uniquely represented proteins from $S. \text{bicolor}$ genotypes at steady-state and upon $C. \text{partellus}$ infestation (Continued)

| Key | Protein Accession No. | Protein Name | Function |
|-----|----------------------|--------------|----------|
| 550 | CSYW3 | similar to UDP-D-glucuronate decarboxylase | Oxidoreductase activity, sorbitol metabolism, development |
| 6258 | CSX3 | NADP-dependent D-sorbitol-6-phosphate dehydrogenase | Oxidoreductase activity, sorbitol metabolism, development |
| 349 | CSX3U1 | similar to BOI-related E3 ubiquitin-protein ligase 1 | Abiotic stress tolerance, protein turnover |
| 28,758 | CSYH55 | similar to 5'-methylthioadenosine/ S-adenosylhomocysteine nucleosidase 2 | Catalytic activity, nucleoside metabolic process |

Many proteins were found to be uniquely accumulated in specific genotypes and treatments. The top 10 of these unique proteins were selected based on their intensity values obtained from the in solution proteomics. The table provides the details of the proteins and their functional significance.
Fig. 6 (See legend on next page.)

(A) Serine hydroxymethyl transferase

(B) Germin

(C) Cyanate hydratase

(D) β-glucanases

(E) Lipid transfer protein

(F) Zeamatin

(G) Endochitinase

(H) Superoxide dismutase

(I) Chaperonin

(J) 14-3-3 like protein

Treatments
wheat stem sawfly [54]. 14–3-3 isoforms are differentially regulated by hormonal treatments, biotic and abiotic stress [55]; and in turn signal defense response to stresses in plants. Another protein specifically accumulated in resistant genotypes of S. bicolor was the superoxide dismutase (SOD), a radical quenching enzyme. High SOD activity has been noted in aphid-infested wheat plants [56], upon mite infestation in cassava [57] and has been strongly correlated to enhanced resistance to the invading pest. Differential SOD levels and isoform diversity are found to play a role in maintaining the cytosolic redox state which in turn regulates response to a variety of pathogens [58] and is probably important in mediating defense against Lepidopteran pests as well. Further, our proteomic analysis on insect-resistant S. bicolor indicated abundance of polyphenol oxidases (PPO) upon C. partellus infestation, unlike that in the susceptible genotype Swarna. Apart from its role in defense against pests and pathogens, our data supports the co-upregulation/co-expression of PPO with PSII and other photosynthesis proteins, signifying its function in protecting the photosynthetic apparatus and eventually in maintaining plant viability and growth [59]. Both the resistant genotypes at steady state (B, D) were rich in proteins involved in primary metabolic processes, efficient protein synthesis, regulation and nitrogen compound biosynthesis contributing to the insect resistance characters.

At steady-state both of the resistant S. bicolor genotypes were found to have a higher abundance of more than 50 proteins as compared to the susceptible genotype Swarna (Fig. 4B; C; Supplementary Table 2). These proteins were involved in maintaining a strong primary metabolism, efficient generation of energy, proficient cell communication and cell cycle in the resistant genotypes. These were represented by proteins like malate dehydrogenase which performs a key role in plant metabolism, chlorophyll a-b binding protein in photosynthesis, magnesium chelatases to regulate ascorbic acid (ABA) signaling [60, 61], Glutathione S-transferases (GST) involved abiotic stress tolerance [62]. An interesting protein namely the F-box associated LRR protein was also detected only in the resistant S. bicolor genotypes at steady-state and may be looked upon as an important contributor to defense against insects. Recent studies have highlighted the importance of rice LRR protein as a component of plant exocyst, majorly contributing resistance to the insect pest - brown planthopper (BPH) [63].

At steady-state, ICSV700 was found to have higher levels of S-adenosyl methionine synthase (SAM synthase), subtilisin, pectinesterase, PPO, ascorbate peroxidase. Enhanced plant defense against insect pests has been demonstrated by SAM synthase through its role in polyamine synthesis [64], subtilisin, pectin esterases [65], polyphenol oxidases [66] and ascorbate peroxidase [67] showing them to be interesting candidates for reverse genetic studies and further elucidation of their mechanisms in defense (Fig. 4B and Supplementary Table 2).

Distinctive proteomic features of S. bicolor genotypes

A high number of unique proteins in resistant S. bicolor, even at steady-state, indicated that they may act synergistically to maintain the resistance against pests, thereby, reducing the chances of infestation (Fig. 5; Table 4). Some of the high expressing unique proteins from S. bicolor ICSV700 at steady-state are involved in the development, maintenance of plant architecture, defense and drought tolerance represented by proliferation-associated protein 2G4, FACT complex subunit SPT16, brassinosteroid insensitive-1 like protein [68], arginine decarboxylase and nitrate reductase [69] respectively. While upon infestation by C. partellus, S. bicolor ICSV700 uniquely expressed several transcription factors and enzymes which were involved in defense against pathogens, indirect defense to herbivorous pests, development of defensive structures, wound healing /cell proliferation and showed high protein remodeling and turnover. Notable amongst them were the ATP synthase CFI alpha subunit, β-caryophyllene synthase, and Ankyrin repeats domain-containing protein. β-Caryophyllene synthase is known to enhance the volatile emission from S. bicolor attracting C. partellus’s larval parasitoid, Cotesia sesamiae Cameron (Hymenoptera: Braconidae) [70]. It is exciting to detect it in infested resistant variety ICSV700 and it also explains different strategies taken by the genotypes to deter the pest. When cultivated maize varieties were not able to express β-Caryophyllene synthase upon C. partellus infestation, it rendered them susceptible to insect pests [71, 72]. Ankyrin repeat
domain-containing proteins are involved in growth, development, protein-protein interactions and have a potential role in plant defense [73].

The other resistant variety IS2205 at steady-state uniquely expressed proteins involved in mediating stress tolerance, conferring antioxidant property and plant resistance represented by peroxidases, thiamine thiazole synthase 1, glutaredoxin and IAA amido synthase GH3, diaminopimelate decarboxylase respectively (Fig. 5; Table 4). While upon C. partellus infestation it uniquely expressed proteins involved in signaling stress tolerance like monogalactosyldiacyl glycerol synthase, zinc finger CCCH domain-containing protein, thiazole synthase; and proteins involved in direct defense signaling like RPP-13 like and chitinase. Maintaining thylakoid membrane biogenesis and stomata opening for retention of photosynthetic capacities in plants under stress is a prominently noted process in IS2205 S. bicolor genotype mediated by monogalactosyldiacyl glycerol synthase and thiazole synthase [74, 75]. Further, NBS-LRR family protein RPP-13 is an important contributor to disease, insect herbivore resistance and also abiotic stress tolerance in plants [63, 76].

In contrast to the S. bicolor resistant varieties the susceptible variety Swarna at steady-state uniquely expressed proteins involved in development and homeostasis and upon C. partellus infestation proteins for development, stress management/defense and homeostasis represented by adenylate isopentenyltransferase, sorbitol-6-phosphate dehydrogenase, serine-threonine kinases, BOI related E3 ubiquitin-protein ligase and triacylglycerol lipase SDP1 respectively were expressed (Fig. 5 and Table 4). Serine/threonine kinases are involved in a wide array of processes ranging from signal transduction, disease resistance, developmental regulation to self- versus non-self-recognition [77] and plant defense response signaling against the pathogen [78, 79]. Ubiquitin/proteasome system (UPS) plays an important role in proteome remodeling in plant-virus interactions, defense against pathogens and survival during environmental stress [80, 81].

The dynamics of gene expression and protein accumulation lead to differences in the correlation of gene vs proteomics profiles in S. bicolor

The gene expression profiles of selected genes thought to be involved in insect defense were studied in S. bicolor upon wounding and/or insect extract-treatment. The analysis confirmed that S. bicolor genotypes responded differently to the insect extract and wounding treatments. The analysis indicated that early gene expression profiles of only some gene candidates correlate with the late proteomic profiles. The differences in proteomic vs gene expression studies in S. bicolor can be attributed to the variation in age of plants used; field-grown vs polyhouse grow plants; actual C. partellus infestation vs mimicking of the infestation and prolonged infestation vs early hours after mimicking infestation in the S. bicolor genotypes respectively. The differences in the proteomic and mRNA expression patterns are noted in many studies and have been attributed to the existence of gene isoforms [82]; feedback regulatory circuits [83] and can be indicative of varied rates of protein translation or post-translational regulations [84].

Conclusions

In conclusion, the proteomic analysis of 967 proteins from S. bicolor genotypes at steady-state and upon infestation by C. partellus was performed. The different statistical comparisons amongst the genotypes and treatments revealed the proteins which would be important for insect defense in S. bicolor. Due to the intrinsic limitations associated with protein annotations, there is a possibility of missing out on some very interesting proteins which are yet to be functionally annotated. However, the present analysis has revealed several proteins that are probably individually or synergistically used by undomesticated S. bicolor genotypes to strengthen its resistance to insect pests. The differentially expressed proteins in resistant vs susceptible S. bicolor genotypes and the uniquely expressed proteins identified, potentially contribute to the build-up of defense against C. partellus using different mechanisms. Further analysis of the protein-protein interactions, pathways and reverse genetic approach would help to identify the different strategies plants may adopt simultaneously to fight against insect pests and to develop agronomically beneficial yet insect-resistant crop plants.

Abbreviations

JA: Jasmonic acid; SA: Salicylic acid; ROS: Reactive oxygen species; RBD: Randomized complete block design; GO: Gene ontology

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12953-021-01173-z.

Additional file 1: Supplementary Figure 1. PCA plot across different treatment groups in S. bicolor - C. partellus interaction proteomic]. The PCA score plot shows the variation amongst different treatments and biological and technical replicates. Supplementary Figure 2. GO analysis of proteins commonly expressed across resistant & susceptible S. bicolor upon C. partellus infestation] Proteins down-regulated in infested samples (highlighted in blue) and up-regulated in control samples are included in Pattern1 whereas Pattern2 indicates proteins that are up-regulated in infested samples (highlighted in red) and down-regulated in control samples. GO of proteins displaying Pattern1 (38) and Pattern2 (19) are indicated molecular function (A) biological processes (B) cellular component (C). Supplementary Figure 3. GO biological process analysis of differentially expressed proteins across treatments in S. bicolor genotypes. Commonly present yet differential abundance proteins were classified into patterns based on their expression across S. bicolor genotypes.
Acknowledgments
This work was supported by the Young Scientist research grant to VT from the Science and Engineering Research Board, Department of Science and Technology, Government of India, India. Grant Number SB/YS/S-132/2013. The authors express great thanks to Dr. Vandana Mhaske for her valuable suggestions and editing of the manuscript. The funding support from the Departmental Research and Development Program (DRDP), Institute of Bioinformatics and Biotechnology, Savitribai Phule Pune University, Pune is acknowledged.

Conflict of interest
The authors declare no competing financial or non-financial conflict of interest.

Authors’ contributions
VT conceived, planned, supervised and procured funding for the project; AJ performed the field experiment and AJ, SS performed the laboratory experiments; AJ, SS, VT collected and analyzed the data; AW and HS suggested the plant genotypes be used and provided the field experimentation; AJ carried out the MS experiments and analysis; AK and SS carried out the statistical analysis of the data; SS, AK, AJ and VT prepared the figures and illustrations; VT and SS wrote the manuscript with inputs from the other authors. The authors read and approved the final manuscript.

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SS and AJ contributed equally to this work.

Funding
This work was supported by the Young Scientist research grant to VT from the Science and Engineering Research Board, Department of Science and Technology, Government of India, India. Grant Number SB/YS/S-132/2013.

Availability of data and materials
The data in excel sheets has been attached with the manuscript as supplementary files. Any other data set generated in the study will be made available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Ethical approval was not needed for this work. All authors and participating institutes willing participated in the study.

Consent for publication
Not applicable.

Competing interests
Authors declare no competing interests.

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Received: 14 September 2020 Accepted: 11 March 2021
Published online: 02 April 2021

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