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Metabolic profiling of chickpea-*Fusarium* interaction identifies differential modulation of disease resistance pathways

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ABSTRACT

Chickpea is the third most widely grown legume in the world and mainly used as a vegetarian source of human dietary protein. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceri* (Foc), is one of the major threats to global chickpea production. Host resistance is the best way to protect crops from diseases; however, in spite of using various approaches, the mechanism of Foc resistance in chickpea remains largely obscure. In the present study, non-targeted metabolic profiling at several time points of resistant and susceptible chickpea cultivars using high-resolution liquid chromatography–mass spectrometry was applied to better understand the mechanistic basis of wilt resistance or susceptibility. Multivariate analysis of the data (OPLS-DA) revealed discriminating metabolites in chickpea root tissue after Foc inoculation such as flavonoids, isoflavonoids, alkaloids, amino acids and sugars. Foc inoculated resistant plants had more flavonoids and isoflavonoids along with their malonyl conjugates. Many antifungal metabolites that were induced after Foc infection *viz.*, aurantion-obstine β-glucosides and querecitin were elevated in resistant cultivar. Overall, diverse genetic and biochemical mechanisms were operational in the resistant cultivar for Foc defense as compared to the susceptible plant. The resistant chickpea plants employed the above-mentioned metabolic pathways as potential defense strategy against Foc.

1. Introduction

Apart from the large number of harmless soil-dwelling saprobes, numerous economically important agricultural pathogens are covered under the genus *Fusarium*. Some of these notorious pathogens include *Fusarium graminearum*, *Fusarium verticillioides* and *Fusarium oxysporum* causing wheat scab; root and stalk rot in maize and vascular wilt disease in chickpea, respectively. Fusarium wilt threatens global chickpea production with occurrence of eight races (0, 1, 1B/C, 2–6) of *F. oxysporum* f. sp. *ciceri* (Foc) in all major chickpea growing regions of the world. Based on their disease phenotypes in chickpea, these races can be classified as yellowing types (races 0 and 1B/C) and wilting types (races 1, 2–6). At least three Foc races are predominant in India (Jimenez-Gasco et al., 2004; Gurjar et al., 2009; Gupta et al., 2013). It has been a serious challenge for disease management as the fungus can survive in soil even without its host for many years. After penetrating the roots, Foc hampers nutrient supply to the plant shoot by blocking the xylem vessels, which eventually results in wilting and plant death. The annual chickpea production losses in infected fields due to wilt alone have been estimated to be up to 90% (Haware et al., 1995). Various non-chemical and chemical control measures have been applied to control Foc; however, the use of resistant cultivars is one of the best ways in disease management.

Biochemical and molecular analyses after Foc inoculation have indicated that some unconventional defense mechanisms are active in chickpea rather than the salicylic acid mediated classical response found during other plant–biotrophic interactions (Giri et al., 1998; Gupta et al., 2010). Molecular basis of resistance or susceptibility to Foc in chickpea has been investigated through candidate gene identification and differential gene expression approaches (Nimbalkar et al., 2006; Ashraf et al., 2009). Marker-assisted gene mapping and quantitative trait loci (QTL) identification studies have also been reported for wilt resistance in chickpea (Gowda et al., 2009; Gupta et al., 2013). Often these QTL harbor several functional genes; and due to the difficulties in detecting such genes by QTL analysis approach, there are large gaps in the knowledge of biochemical/molecular basis of Foc resistance. Recent advancements in the state-of-the-art analytical approaches like metabolomics have enabled deeper understanding of the molecular mechanisms modulated by plant-pathogen interactions.

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Metabolomics is a powerful method for comprehensive investigation of metabolite variations in biological systems. It generally consists of total metabolic profiling and multivariate statistical analysis, thereby providing efficient visualization, identification and quantification of the metabolites generated under specific physiological conditions (Allwood et al., 2008). In recent years, non-targeted metabolomics has been applied to reveal the complex mechanisms of plant defense against pathogens, such as resistance against *F. graminearum* in wheat and barley (Gunnaiah et al., 2012). However, such comprehensive investigation of metabolic changes induced by Foc in chickpea has not been performed till now. Here, we employed the non-targeted metabolomics approach using high-resolution mass spectrometer to systematically identify metabolic modulations during various time-points in roots of susceptible and resistant chickpea cultivars upon Foc inoculation. Further, we employed the multivariate data analysis approach to select the features that were differentially expressed after Foc inoculation. This approach enabled us to understand the Foc induced mechanism for resistance as well as susceptibility in chickpea and the metabolic network modulated during disease development.

2. Results

2.1. Phenotypic evaluations of chickpea cultivars upon fungal inoculations

The Fusarium wilt susceptible (JG62, here onwards JG) and resistant (Digvijay, here onwards DV) chickpea cultivars were inoculated with Foc race 1 (Foc1). JG showed typical wilting phenotype (>95%) with yellowing on 2 days after inoculation (DAI), followed by drooping of leaves that finally caused complete wilting by 12 DAI. Whereas, the mock-inoculated resistant or susceptible cultivar and Foc1 inoculated resistant cultivar remained healthy. The establishment of the pathogen within host vascular tissue, characterized by colonization in the xylem vessels, was observed after 2 DAI in chickpea (Gupta et al., 2010; Jimenez-Fernandez et al., 2013). Therefore, root tissues from these Foc1 and mock-inoculated chickpea plants were collected at 2, 4, 8 and 12 DAI (Supplementary Fig. S1). These root tissues were used to identify the total metabolic changes during chickpea-Foc interactions with high-throughput non-targeted quantitative metabolomics approach.

2.2. Global metabolic profiling after Foc1 inoculation in chickpea roots

The stability and reproducibility of the liquid chromatography–mass spectrometry (LC–MS) system were validated using several parameters before the samples were run. The stability of the system was confirmed based on the retention times (RT), mass accuracies and peak areas of the two selected extracted ion chromatograms (EICs) in the quality control (QC) samples. Intensity coefficient variation of leucine (Leu) and isoleucine (Ile) was 0.148 and 0.145, respectively, in all the QC runs. Alignment of the EIC of Leu and Ile revealed the least deviation in RT shift (Fig. 1A). Mass accuracy was ~2 ppm during data acquisition (Fig. 1B). These results demonstrated that the reliability and stability of the system were qualified for running the samples and data acquisition.

The non-targeted metabolomics investigation revealed a total of 5018 and 6508 compounds from DV and JG, respectively, in the electrospray ionization-positive [ESI(+)] mode with significance of P <0.005, false detection rate (FDR) <1% and more than two fold change. Similarly, electrospray ionization-negative [ESI(−)] analysis revealed total 4909 and 5058 compounds in DV and JG, respectively. In ESI(+) mode, 1297 metabolites were shared among the four time-points (2–12 DAI) in DV, whereas in JG, 1112 metabolites were common among them (Fig. 2). Similarly in ESI(−), 968 metabolites were common in DV as compared to 890 in JG (Supplementary Fig. S2). However, stage specific metabolites were also detected in both the chickpea varieties. Total 1229, 213, 168 and 495 stage specific metabolites were detected in DV roots at 2, 4, 8 and 12 DAI, respectively (Fig. 2A) while JG had 324, 147, 409 and 2157 such metabolites specifically at 2, 4, 8 and 12 DAI, respectively (Fig. 2B) in ESI(+) mode. With ESI(−) mode, 169, 124, 1752 and 335 stage specific metabolites were identified in DV plants at 2, 4, 8 and 12 DAI, respectively (Supplementary Fig. S2A); whereas 215, 248, 218 and 1665 metabolites were specific to 2, 4, 8 and 12 DAI stages, respectively, in JG (Supplementary Fig. S2B).

An unsupervised pattern recognition technique was used to observe a potential clustering behavior and pattern in the metabolite data. For this purpose, principal component analysis (PCA) was employed on the whole data set to establish the presence of any Foc1 induced metabolic change. UV-scaled PCA score plots showed fairly clear differences between control and infected chickpea root tissue in the resistant and the susceptible cultivars, indicating significant changes in metabolite profile after Foc1 inoculation (Fig. 3). Further, PCA analysis of metabolites from each time point (2, 4, 8 and 12 DAI) separately also revealed clear separation between control and Foc1 inoculated samples with good R^2X and Q^2 values (Supplementary Fig. S3). Moreover, to ascertain the time course of metabolic variations that were induced in the resistant and the susceptible plants after Foc1 inoculation, a PCA model was built for LC–MS considering all the metabolomics data for resistant and susceptible plants separately using trajectory plot. The average PCA scores for the control and the Foc1 inoculated resistant and susceptible plants were calculated for the first two PCs. The PCA trajectory plots illustrated the time dependence of alterations in the metabolic profiles of Foc1 inoculated resistant (DVI) and susceptible (JGI) plants (Supplementary Fig. S4). This revealed similar metabolite accumulation patterns for both the control chickpea cultivars in the trajectory plots; however, significant differences were observed in trajectory plots of the Foc1 inoculated DVI and JGI plants. Total metabolic profile of JGI samples showed rapid upward metabolic shift after 8 DAI, in contrast to that in DVI. The PCA trajectory plots clearly illustrated time and infection progression dependent pattern with significant metabolic deviations.

2.3. Metabolic alteration in chickpea roots after Foc1 inoculation

First, we constructed the orthogonal partial least squares-discriminant analysis (OPLS-DA) model of three groups, control of resistant (DVC) and susceptible (JGC) plants together as one group and Foc1 inoculated resistant (DVI) and susceptible (JGI) plants as two separate groups to uncover any metabolic changes associated with fungal infection. OPLS-DA score plot (Fig. 4A) revealed complete separation between the control and the infected plants with three distinguished clusters. Similarly, OPLS-DA model of these three comparison groups, separately at each time point (2, 4, 8 and 12 DAI) also indicated clear separation between the control and the infected chickpea plants (Fig. 4B–E).

Further, we performed OPLS-DA analysis between Foc1 inoculated resistant-DVI and susceptible-JGI plants of all time points together to identify important metabolites responsible for the variation in phenotypic response between the resistant and the susceptible genotypes (Fig. 5). OPLS-DA score plots showed clear separation between all the DVI and JGI plants. Moreover, CV-ANOVA validated model parameters in the permutation test for the explained variation (R^2X = 0.588) and the predictive capability (Q^2 = 0.812) were significantly high (Fig. 5A). S-plot from the above generated model between all the Foc1 inoculated resistant-DVI and susceptible-JGI genotypes

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