Indian Journal of Biochemistry & Biophysics
Vol. 63, March 2026, pp. 288-296
DOI: 10.56042/ijbb.v63i3.18676

A novel *in silico* approach for identifying defense-related biomarkers from *Neurospora crassa* during plant associations to understand pathogenicity mechanisms

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Received 09 April 2025; *received 30 May 2025*

Neurospora crassa, an ideal eukaryotic model organism, offers key advantages for studying various biological processes. While it is predominantly known for its saprotrophic lifestyle, thriving on dead, decaying organic matter and burnt vegetation, investigations on their pathogenicity potential and the nature of its association with living plants remain largely unexplored and limited. A novel gene analysis pipeline has been proposed for the identification of defense-related biomarkers through an *in silico* biocomputational approach. GSE34098, a gene expression dataset from the GEO database, has been utilized as a source for our study. Deployment of a customized Python script in this pipeline has enabled gene refinement of 10,926 DEGs and in protein enrichment processes. Comprehensive parametric gene and interactome analyses identified five key proteins as potential biomarkers. These findings are substantiated by scientific literature, which contribute to validating the outcomes of the current study. The implementation of the proposed workflow will help in the identification of key biomolecules of elicitor and effector potential involved in pathogenicity mechanisms. Importantly, insights regarding the potential of the host and the corresponding pathogen's ability to impact each other can be understood. The knowledge of the same can pave way to improve crop resilience.

Keywords: CentiScaPe 2.2, Defense related biomarkers, GEO2R, Interactome analysis, *Neurospora crassa*

A diverse array of secreted proteins is synthesized during interactions between microbes and their plant host. Secreted proteins greatly influence the lifestyle of an organism and play a key role in triggering and activating the defense responses in the host immune system. Several microbial derived molecules can modify the host cell structure and function, influencing pathogenicity and defense responses[1,2]. Investigations of these secreted proteins provide insights regarding pathogen reinforced strategies, for their successful establishment within the host system. Pathogen synthesized biomolecules, secreted during interactions with plant hosts, have the potential to serve as valuable biomarkers.

Neurospora crassa is a well-known saprotroph and resides in certain plants as an endophyte[3]. When the balanced interaction within the host is disrupted, *N. crassa* has been shown to switch from an endophytic lifestyle to a pathogenic lifestyle. This was experimentally demonstrated by Kuo *et al.*[4], suggesting that, the genetic makeup of this fungus harbour genes capable of transitioning between endophytic, pathogenic, and saprotrophic lifestyles. Present study focuses on investigating the secreted proteins of *N. crassa* to assess their potential role as defense biomarkers. The pipeline employed in this study helps in the identification of key biomarkers among the secreted proteins of *N. crassa* during its plant association, unveiling its pathogenic lifestyle.

NCBI-GEO derived microarray based differential gene expression dataset (GSE34098) of *N. crassa* has been utilized. Adaptation of *N. crassa* to different carbon sources and their coordinated gene expression to efficiently utilize complex substrates is the context of the data source selected. This gene expression dataset included four distinct experimental conditions. Comparisons between the wild-type and mutant xlr-1 gene deletion strains of *N. crassa* grown on distinct media, were investigated to decipher the regulatory mechanisms associated with hemicellulose

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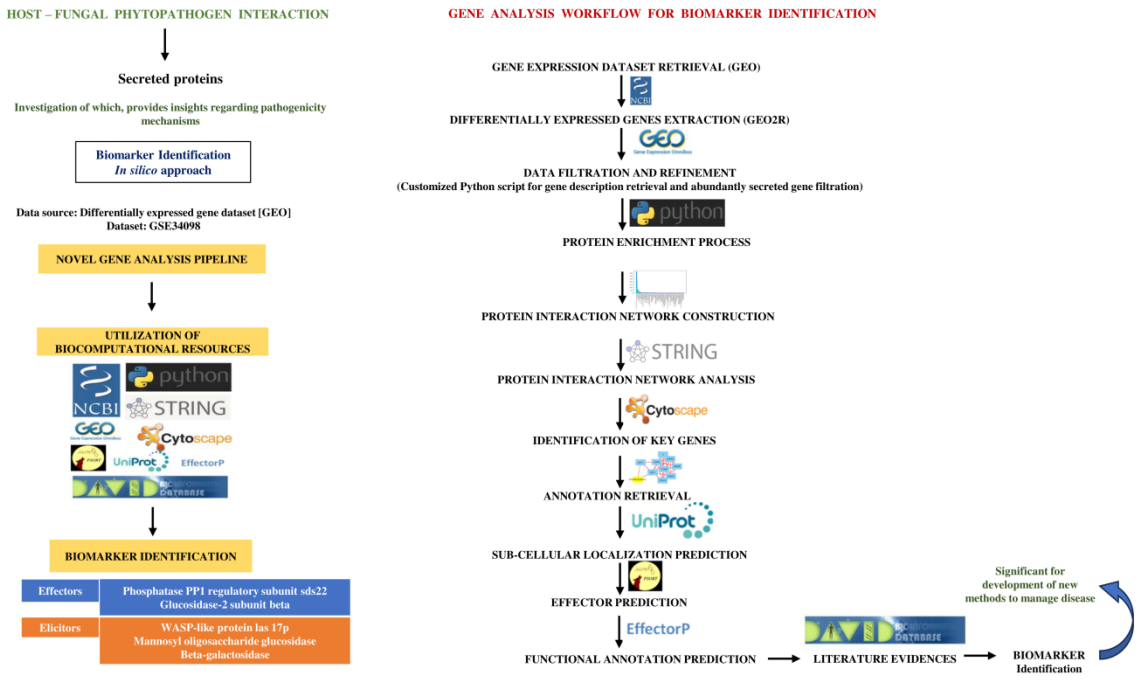
Suppl. data available on respective page of NOPR



Graphical abstract

degradation[5]. A novel gene analysis pipeline deploying in-house Python script has been proposed for the identification of biomarkers from this dataset. The current study has incorporated various biocomputational resources for the analysis of differentially expressed genes (DEGs) and the identification of a comprehensive set of biomarkers. The analysis of this gene expression dataset using parametric gene analysis and interactome analysis, revealed the key proteins synthesized by the *N. crassa* during host associated interactions.

Identification of biomarkers, contributes for effective disease management and understanding ecological roles of an organism to adapt to distinct lifestyle. The current study has identified key genes, including WASP-like protein Las17p, mannosyl oligosaccharide glucosidase, phosphatase PP1 regulatory subunit Sds22, β-galactosidase, and glucosidase-2-subunit beta, as potential biomarkers. The functional relevance of key genes identified as biomarkers, has been strengthened through multiple scientific studies. These secreted biomolecules of *N. crassa* were found to be integral to key biological processes that facilitate its establishment, growth, and pathogenicity. Their synthesis is inherently connected to biochemical functions that contribute to *N. crassa*'s adaptability.

Materials and Methods

Microarray data collection

The microarray gene expression dataset GSE34098 associated with array based platform GPL14949 GlassLab-Ncrassa 10k-V1 was obtained from gene expression omnibus (GEO) (https://www.ncbi.nlm.nih.gov/)[5,6]. The dataset comprised of 23 samples of *N. crassa* (wild type and mutant) grown on 4 different media (minimal, xylan, xylose, and avicel media). The 23 experimental samples were grouped into two sets: 11 wild-type samples and 12 xlr-1 gene deletion mutants vs. wild-type samples. GEO2R analysis was performed using Benjamini and Hochberg p-value adjustment with a significance level cutoff value of 0.05 and differential gene expression dataset was extracted (https://www.ncbi.nlm.nih.gov/geo/geo2r/).

Differential gene expression analysis

Up-regulated genes bearing positive logFC values were subjected to gene refinement process. Using Python (version 3.6.15) in-house script and selenium web automation engine, gene descriptions were retrieved from the corresponding gene IDs[7]. Deployment of this script enabled efficient data extraction in a considerably shorter amount of time, eliminating the possibility of errors that might have occurred during the manual gene description retrieval

process. This step further helped in the exclusion of uncharacterized and hypothetical proteins from the dataset for refinement process.

Protein enrichment process

Filtration of abundantly secreted proteins

The abundance plot was generated using a custom Python script. The top 10% of genes bearing highest log-fold change values were considered for further analysis.

Construction and analysis of protein interaction network (PIN)

The PIN was constructed using top 10% genes as query input in the STRING database v.12.0 (https://string-db.org/) and clustered using the K-means clustering algorithm and the number of clusters was set to three[8]. The clustered network data were then exported as a TSV file to the Cytoscape 3.9.1 software (https://cytoscape.org/) for visualization and analysis[9]. An edge-weighted force-directed biolayout format was applied to the clustered protein interaction network data, followed by application of grid layout format to the network. Network construction of these parametric-based refined proteins was conducted according to Kumar *et al.*[10]. Parametric-based analysis and identification of key genes within the protein network was conducted using the network analyzer tool and the CentiScaPe 2.2 plugin. The centrality metrics considered within CentiScaPe 2.2 plugin were stress, betweenness, and centroid value[11]. The annotations of the key genes obtained from Cytoscape analysis were retrieved from the UniProt databank (https://www.uniprot.org/)[12].

Subcellular localization, effector and functional annotation prediction

The subcellular localization of key genes was predicted using DeepLoc2.0 (https://services.healthtech.dtu.dk/services/DeepLoc-2.0/) and WoLFPSORT (https://wolfpsort.hgc.jp/)[13,14].

EffectorP 3.0 was used for effector prediction of key genes (https://effectorp.csiro.au/)[15]. Further, the gene ontological data of the identified proteins were retrieved from DAVID functional annotation server (https://davidbioinformatics.nih.gov/)[16,17].

Results

Differential gene expression data extraction

10,926 DEGs were obtained from GEO2R analysis.

Filtration and refinement of differentially expressed genes

The deployment of an in-house Python script accurately retrieved gene descriptions for 4,720 genes out of 10,926 genes. Among these 4,720 genes, 3,560 genes were down-regulated, 74 genes had logfold change values of zero, and 1,086 genes were up-regulated. A total of 1,086 up-regulated genes were considered for further analysis.

Protein enrichment

Filtration of abundantly secreted proteins

Abundance plot of secreted proteins generated using an in-house Python script facilitated the quantification of the frequency of each gene expressed. Genes exhibiting significant changes in expression level bearing highest log fold values were considered (top 10%) to minimize protein interaction network complexity. This approach highlights the most influential genes (Suppl. Tables 1-10).

Interactome analysis

Using the K-means clustering algorithm, a protein interaction network was built, for the top 10% of abundantly expressed proteins, leading to the network categorization into three distinct clusters. The edge-weighted force-directed biolayout tool organizes the network on the basis of edge weights, integrating closely interacting proteins into fewer and more significant nodes. The grid layout further arranges these nodes in a structured manner excluding redundant and less crucial nodes. 48 consolidated genes were identified in a grid layout (Suppl. Tables 1-10). The number of genes got streamlined with fewer, but more significant genes than the number of input genes.

PIN constructed using these 48 genes as query, comprised 42 nodes and 140 edges (Fig. 1). The

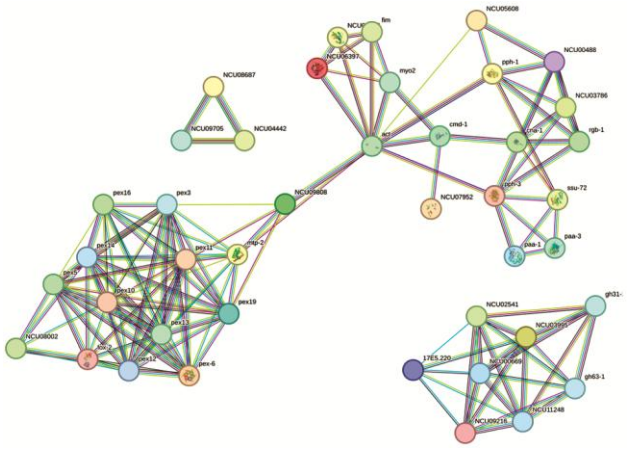


Fig. 1 — Protein interaction network reconstruction *via* k-means clustering

average node degree, which represents the average number of links per node, was found to be 6.67. This highlights the network's degree with aspect to connectivity. The average local clustering coefficient was found to be 0.792, which is high, indicating that, this network is characterized by closely interlinked group of nodes. The TSV file of this STRING interaction network exported to Cytoscape software, resulted in visualization of overall network construct categorized into 2 sub networks. Sub network 1 comprised of 51 nodes and sub network 2 comprised 10 nodes (Fig. 2).

Network analyzer output

Within sub-networks 1 and 2, the nodes with the highest betweenness centrality values identified were WASP-like protein las17p (EFNCRP00000007408) and mannosyl-oligosaccharide glucosidase (gh63-1), respectively (Table 1). Betweeness centrality value of a specific node indicates the frequency of that node, lying on shortest path between other nodes within a network. These 2 nodes with higher betweenness centrality values lie on many shorter paths between other nodes serving as a critical point (bridge) for network's connectivity.

CentiScaPe 2.2 output

CentiScaPe 2.2 analysis revealed phosphatase PP1 regulatory subunit Sds22 (EFNCRP00000006498) and beta-galactosidase (gh2-2) in subnetwork 1 (Fig. 3) along with glucosidase 2 subunit beta (EFNCRP00000005613) in subnetwork 2 (Fig. 4) as significant hubs. They constitute high betweeness centrality and stress values, which represents the frequency of nodes appearing on shortest paths, making them important connectors within the network. Additionally, their low centroid values suggest that they are centrally located, facilitating efficient communication and interaction within their sub networks. UniProt databank helped in retrieval of

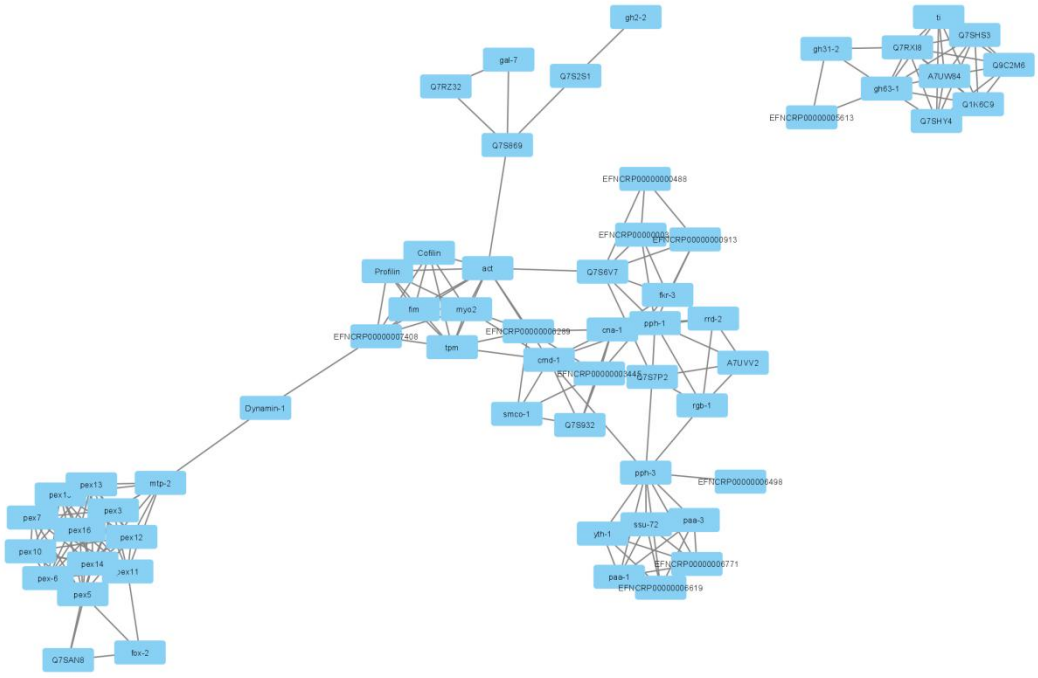


Fig. 2 — Categorization and visualization of distinct subnetworks of protein interaction network using Cytoscape

Table 1 — Network analyser output: Betweenness centrality-based identification of key nodes in subnetwork 1 and 2, emphasizing the importance of these nodes as structural and functional hubs that facilitate network communication and stability

	Subnetwork 1	Subnetwork 2
Gene	EFNCRP00000007408	gh63-1
UniProt annotation	WASP-like protein las17p	Mannosyl-oligosaccharide glucosidase
UniProt ID	Q7SF15	V5IM04_NEUCR
Betweenness centrality values	0.4285714286	0.246031746

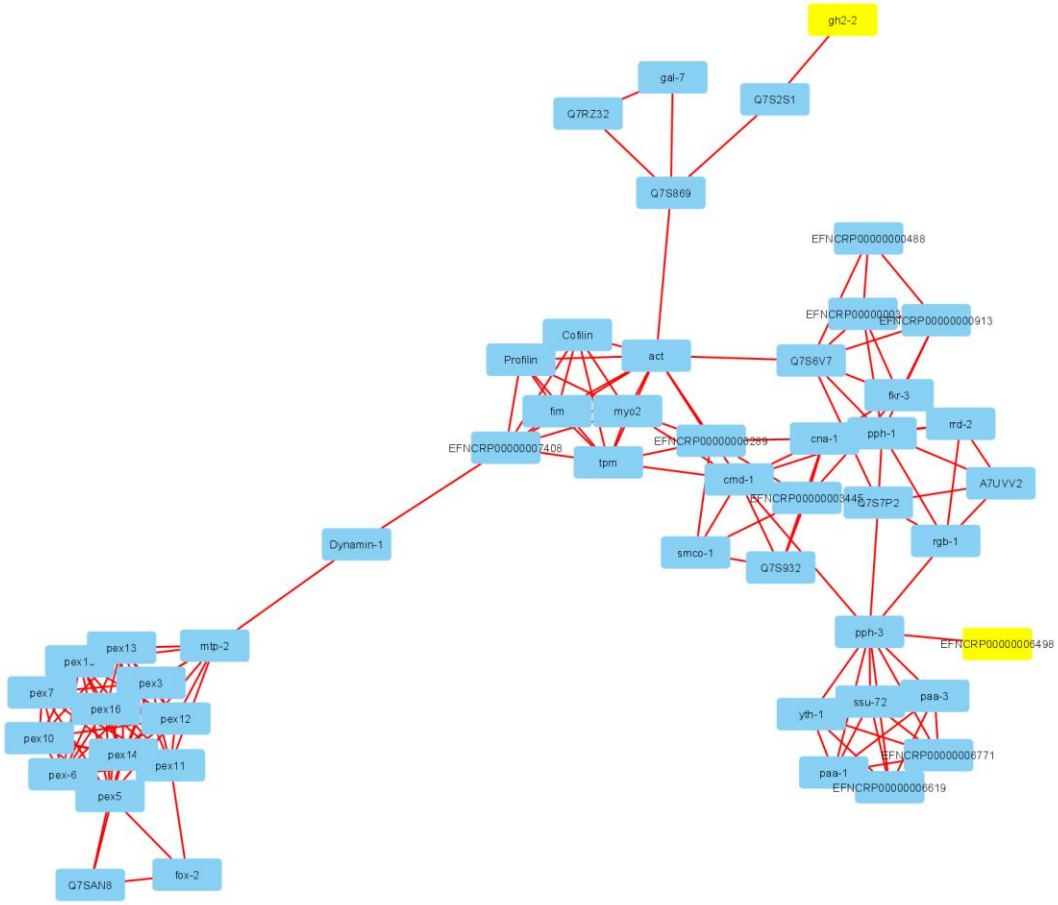


Fig. 3 — Key nodes identified within subnetwork 1 using CentiScaPe 2.2 plugin based on stress, betweenness, and centroid value metrics [EFNCRP00000006498 (Protein phosphatase PP1 regulatory subunit sds22) and gh2-2 (Beta-galactosidase)]

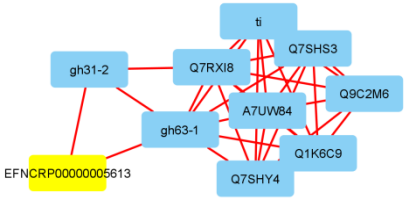


Fig. 4 — Key nodes identified within subnetwork 2 using CentiScaPe 2.2 plugin based on stress, betweenness, and centroid value metrics [EFNCRP00000005613 (glucosidase 2 beta subunit)]

annotations of key genes obtained from Cytoscape analysis, facilitating further downstream analyses (Table 2) (Suppl. Tables 1-10).

Subcellular localization, effector and functional annotation prediction

The subcellular localization prediction is critical, as it determines the precise cellular compartment in which proteins operate, thereby influencing their functional role within biological system. Table 3 highlights the predicted subcellular localizations and effector predictions of key genes identified within subnetworks 1 and 2. Phosphatase PP1 regulatory subunit sds22 and glucosidase-2 subunit beta were identified as cytoplasmic effectors. WASP-like protein las17p, mannosyl-oligosaccharide glucosidase, and beta-galactosidase were identified as non-effectors. These proteins identified as non-effectors, play important roles in signalling pathways, metabolomic processes and stress responses within the biological system. Therefore, this could be identified as elicitors. The DAVID functional annotation server enables elucidation of the intricate biological roles of specific genes within the biological system. The functional annotations of the 5 identified key genes retrieved from the DAVID functional annotation server along with scientific literature evidence regarding the

Table 2 — Comprehensive set of key genes identified from Cytoscape analysis (Network analyzer and CentiScaPe 2.2 outputs for subnetworks 1 and 2)

Network analyser output		CentiScaPe 2.2 plugin output	
Subnetwork 1	Subnetwork 2	Subnetwork 1	Subnetwork 2
EFNCRP00000007408 WASP-like protein las17p Q7SF15	gh63-1 Mannosyl-oligosaccharide glucosidase V5IM04_NEUCR	EFNCRP00000006498 Phosphatase PP1 regulatory subunit sds22 Q7SD66_NEUCR	EFNCRP00000005613 Glucosidase-2 subunit beta Q7S6V9_NEUCR
		gh2-2 Beta-galactosidase Q7S1W5_NEUCR	

Table 3 — Subcellular localization and effector prediction of key genes identified within subnetworks 1 and 2 using WoLFPSORT, DeepLoc 2.0, and EffectorP 3.0 prediction tools

Gene	UniProt ID	Annotation	Subcellular localization prediction (WoLFPSORT & DeepLoc 2.0)	Effector prediction (EffectorP 3.0)
EFNCRP00000007408 (Subnetwork 1)	Q7SF15	WASP-like protein las17p	Mitochondrial matrix, Nuclear region Cytoplasm	Non-effector
gh63-1 (Subnetwork 2)	V5IM04_NEUCR	Mannosyl-oligosaccharide glucosidase	Mitochondrial matrix, inner membrane, cytoplasmic region Endoplasmic reticulum, extracellular secreted	Non-effector
EFNCRP00000006498 (Subnetwork 1)	Q7SD66_NEUCR	Phosphatase PP1 regulatory subunit sds22	Cytoplasm, Nucleus	Cytoplasmic effector
gh2-2 (Subnetwork 1)	Q7S1W5_NEUCR	Beta-galactosidase	Mitochondrial region Cytoplasmic region	Non-effector
EFNCRP00000005613 (Subnetwork 2)	Q7S6V9_NEUCR	Glucosidase-2 subunit beta	Mitochondrial matrix, Endoplasmic reticulum	Cytoplasmic effector

involvement of these five key genes in pathogenicity contexts, have proven useful in understanding their potential as effector and elicitor candidates (Suppl. Tables 1-10).

Below are the substantiated scientific literature studies that support the effective roles of the five key proteins.

WASP-like Protein Las17p

The development of highly polarized, tip-growing tubular hyphae is a defining feature of fungal morphogenesis and lifestyle diversification[18]. Las17p, a WASP-like protein plays an important role in organization and polymerization of actin filaments in *N. crassa*. Disruption of the actin cytoskeleton leads to rapid tip swelling, emphasizing the crucial role of filamentous actin in mediating targeted secretion at the plasma membrane. This protein involves and regulates several key biological functions such as assemblage of actin filament bundles, actin nucleation, cortical patch localization and Arp2/3 complex-mediated actin nucleation processes[19-21]. Las17p protein family has also been reported in facilitating the formation of specialized infection structures, crucial for fungal penetration and host colonization[22]. Therefore, it is important to note that this protein is vital for successful invasion of *N. crassa* and their interaction with the host, serving as key regulator of cytoskeletal actin dynamics. Also, this protein further supports the synthesis and secretion of enzymes capable of degrading plant cell walls contributing to pathogenicity. These substantiations support the consideration of WASP-like protein Las17p as potent elicitor biomarker for fungal pathogenicity.

Mannosyl-oligosaccharide glucosidase (MOG)

Mannosyl-oligosaccharide glucosidases have been reported to participate in various biochemical pathways, especially in N-linked protein glycosylation, N-glycan processing and oligosaccharide metabolism[16,17]. MOGs mediated trimming of glucose is the key step in N-glycosylation process. MOGs facilitate the initial trimming of nascent N-glycans by cleaving terminal glucose residues from oligosaccharide precursor in N-glycosylation

pathway. Glycoproteins accounts for 10-20% of the cell wall of *N. crassa*. Glycosylation of these glycoprotein conjugates is very much necessary for their proper maturation and folding[23]. A structural study conducted on cell wall of *N. crassa* revealed the presence of galactomannan-conjugated glycoproteins. For these conjugates to be properly glycosylated, multiple enzymes and precursors are necessary, amongst which MOGs plays a key role[24]. One more study reported that, the hydrolase activity of cell wall glycoproteins is crucial for remodeling the cell wall architecture, thereby facilitating the formation of new hyphal tips and branches[25]. The role of MOGs in protein processing and its glycosylation function, modulates the synthesis and activity of enzymes that impacts the fungus ability to infect and colonize host tissues successfully[26]. Glycosylation defects leads to unstable cell wall functions. Therefore, MOGs may be regarded as potential elicitor biomarker, contributing to the survival of *N. crassa* during host interactions by promoting hyphal development and effective cell wall biogenesis.

Phosphatase PP1 regulatory subunit Sds22

Phosphorylation and dephosphorylation are crucial processes for cellular homeostasis. Kinases and phosphatases catalyzes the phosphorylation and desphosphorylation mechanisms respectively[27]. Phosphatases require specific regulatory subunits that modulate the enzyme's substrate selectivity and direct them to the appropriate subcellular compartments. Protein Phosphatase 1 (PP1) is one such serine/threonine phosphatase. Its regulatory interactions are crucial for various cellular activities, including carbohydrate metabolism and cell regulation[28]. Phosphorylation events predominantly occur at serine/threonine residues that lie outside the catalytic domain in eukaryotes. These residues acts as an attachment sites for regulatory proteins influencing the enzymatic activity[29]. In *N. crassa*, sixteen serine/threonine phosphatases have been identified, amongst them, the PP1 regulatory subunit Sds22, a leucine-rich protein plays a key role by binding to PP1 and facilitating efficient dephosphorylation process. Sds22 has been reported to play a key role in cell wall expansion, and its regulatory functions extend to carbohydrate metabolism and overall cell signalling. The absence of Sds22 disrupts normal signalling processes, particularly those governing hyphal tip extension in *N. crassa*[30]. Sds22 is also reported to be involved in the positive regulation of the mitotic metaphase/anaphase shift, acting as protein phosphatase activator, and accounting for hydrolase activity[16,17]. Based on its roles in promoting cell wall construction required for hyphal tip extension and branch formation, in directing PP1 activity, and its impact on key regulatory cascades, the phosphatase PP1 regulatory subunit Sds22 could be considered as potent effector biomarker.

Beta-galactosidase

Beta-galactosidase is an enzyme which facilitates the breakdown of complex carbohydrates into simpler sugars. Beta-galactosidase secreted by *N. crassa* during host interaction, plays a key role in biodegradation of plant biomass by hydrolysing hemicelluloses[16,17]. This enzyme has also been reported to function as precursors of polysaccharide synthesis in addition to the degradation processes, which assists in energy production by providing essential substrates for fungal establishment, colonization, growth and cell wall formation[31]. Beta-galactosidase involvement in lactose catabolism facilitates the utilization of metabolic products, directing them into biochemical cascades that serve as basic source for carbon and energy utilization by the fungus[32]. Ability of this enzyme to catabolise lactose, metabolize carbohydrates and their active involvement in cell wall biogenesis during interaction with host system, makes it an exceptional candidate to act as potent elicitor biomarker.

Glucosidase-2 subunit beta

Glucosidase II enzyme complex, a heterodimer is composed of 2 subunits (α and β subunits). α subunit catalyzes the hydrolysis of glucose residues from N-linked glycans, and β subunit is involved in regulation of the complex accounting for its stability. Glucosidase II subunit beta (Gls2) is actively involved in N-glycan processing, by ensuring the effective localization of the enzyme complex within the endoplasmic reticulum (ER), and facilitating the interaction with its substrates. Gls2 mainly contribute in glycosylation and maturation of glycoproteins prior to their transport into ER[16,17,33]. A study pertaining to effect of deletion of Gls2 in *Magnaporthe oryzae* resulted in delayed conidial development, impairment of mycelial growth and decreased pathogenicity. These findings emphasize the significance of Gls2 in managing the glycoprotein regulation with context to pathogenicity. It is important to highlight that, simultaneous deletion of Gls2 and other ER quality control components like glucosidase I (Gls1),

glycosyltransferase B1 (GTB1), and calnexin (Cnx1) lead to defects in mycelial development, conidial germination, and invasive hyphal tip extension[34].

In the other study, the role of MoGls2 in *M. oryzae*, specifically its involvement in glycoprotein folding, cell wall synthesis, and host adaptation suggests a conserved mechanism likely extends to *N. crassa*. This MoGls2 facilitates the trimming of terminal glucose residues and their deletion resulted in impaired mycelial growth, influenced conidial dynamics, loss of sexual reproduction, and reduced pathogenicity[35]. By considering Gls2 role in regulating glycoprotein folding and overall pathogenicity, this regulatory component serves as a potent effector biomarker.

Discussion

Microarray gene expression dataset GSE34098 constitutes a transcriptomic dataset belonging to *Neurospora crassa* in relation to its ability to utilize various carbon sources. This dataset serves as an invaluable resource for investigating enzyme functions beyond their biomass degradation capabilities. Therefore, GSE34098 has been utilized in the current work. The parametric based approach of gene analysis implied utilizing this dataset involved trimming the data by excluding uncharacterized and hypothetical proteins in the initial step. The subsequent step focused on gene filtration and refinement. The customized Python script facilitated efficient gene refinement, thorough data clean up and retrieval of gene descriptions. Further, several biocomputational resources have helped in the identification of membrane localized proteins with good interactome characteristics and their functional annotations. Interactome analysis focused on the top 10% of abundantly expressed proteins, highlighting five key nodes within the dense network based on comprehensive set of topological and statistical parameters. The functional annotations of these key genes were obtained from the DAVID functional annotation server, followed by subcellular localization and effector prediction. The key biomarkers reported from our study suggest that, all five identified proteins have potential to take part in pathogenesis development by helping the pathogen's establishment in the host. They achieve this by strengthening hyphal tip extensions, accounting for cell wall biogenesis, cell signalling and regulation. These proteins greatly influence the cellular roles that allow *N. crassa* to successfully colonize the plant host. It can be emphasized that, in addition to being a saprotroph, *N. crassa* has the genetic ability to lead distinct pathogenic lifestyles.

Conclusion

Our findings from the exploration of GSE34098 transcriptomic dataset, suggests that *Neurospora crassa* possesses a genetically regulated enzyme consortium capable of degrading plant biomass by infecting the host system through accelerated cell wall morphogenesis. Consequently, *N. crassa* can successfully damage plant cell walls by colonizing, establishing, and infecting them, potentially transitioning from a saprotrophic lifestyle to a distinct pathogenic mode. Workflow implied in this study represents an optimized pipeline for identifying potential elicitor and effector candidates from microarray gene expression datasets. This approach contributes to the identification and characterization of defense-related gene repertoires and can be applied to other commercial crops vulnerable to phytopathogens.

Acknowledgement

The authors thank Tumkur University for offering facility to carry out the research work.

Conflict of interest

All authors declare no conflict of interests.

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