Proteomic Analysis Demonstrates a Molecular Dialog Between *Trichoderma guizhouense* NJAU 4742 and Cucumber (*Cucumis sativus* L.) Roots: Role in Promoting Plant Growth

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Trichoderma is a genus of filamentous fungi that play notable roles in stimulating plant growth after colonizing the root surface. However, the key proteins and molecular mechanisms governing this stimulation have not been completely elucidated. In this study, *Trichoderma guizhouense* NJAU 4742 was investigated in a hydroponic culture system after interacting with cucumber roots. The total proteins of the fungus were characterized, and the key metabolic pathways along with related genes were analyzed through proteomic and transcriptomic analyses. The roles played by the regulated proteins during the interaction between plants and NJAU 4742 were further examined. The intracellular or extracellular proteins from NJAU 4742 and extracellular proteins from cucumber were quantified, and the high-abundance proteins were determined which were primarily involved in the shikimate pathway (tryptophan, tyrosine, and phenylalanine metabolism, auxin biosynthesis, and secondary metabolite synthesis). Moreover, ¹⁵N-KNO₃ labeling analysis indicated that NJAU 4742 had a strong ability to convert nitrogenous amino acids, nitrate, nitrile, and amines into ammonia. The auxin synthesis and ammonification metabolism pathways of NJAU 4742 significantly contributed to plant growth. The results of this study demonstrated the crucial metabolic pathways involved in the interactions between *Trichoderma* spp. and plants.

Keywords: ammonification, auxin synthesis, fungal effectors, fungus–plant interactions, genomics, metabolomics, promotion mechanism, proteome, *Trichoderma guizhouense* NJAU 4742

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Trichoderma spp. make up a group of cosmopolitan soil fungi that are beneficial to agricultural activities. These fungi can act as biofungicides, remediate soils being contaminated with metals or chemical wastes, or even elicit plant defense after interacting closely with the surroundings (Meena and Swapnil 2019; Sharma et al. 2019). Numerous researchers have observed that *Trichoderma* fungi can colonize plant roots, promote plant growth, enhance nutrient availability, and increase crop productivity by competing with other microorganisms, releasing nutrients, modifying the rhizosphere, producing antibiotics, and secreting cell-wall-degrading enzymes (Gupta and Bar 2020; Viterbo et al. 2010). Importantly, these functions help to mediate the molecular interactions between plants and microorganisms. Meanwhile, all of these beneficial associations require mutual recognition and substantial coordination of plant and microbial responses.

For plant-growth-promoting rhizobacteria, it was demonstrated that the secreted proteins were central to the bacteria–host interaction (Sayyed et al. 2019). Similarly, changes in secreted proteins from filamentous fungi might affect fungus–plant molecular interactions after association with plant roots. Small secreted proteins (SSPs) secreted by fungi always have fewer than 300 amino acids, secretion signal peptides, and cysteine-rich subgroups (Kim et al. 2016). The proportion of SSPs was observed to range from 40 to 60% of the secretomes across all lifestyles and phylogenetic groups within the fungal kingdom. SSPs can suppress host defense responses, as well as modulate their physiology (Feldman et al. 2020). Fungal secretomes are primarily composed of several protein categories, including proteases, lipases, and carbohydrate-active enzymes (CAZymes). Among the secretomes of *Trichoderma* spp., CAZymes might facilitate penetration and colonization by surmounting the first barrier by partially digesting the cell wall (Mendoza-Mendoza et al. 2018). Chitinase participates in the catalytic decomposition of chitin from the phytopathogenic fungal cell wall and releases chitooligosaccharides that have mitogenic and morphogenic effects such as cytokines and inhibitors of auxin transport. Therefore, these products indirectly contribute to increasing root formation and stimulating plant defense against phytopathogenic fungi (Hermosa et al. 2013). Cheng et al. (2018) found that small (Sm1) protein and eliciting plant response-like (Epl1) protein could serve as microbial or microbial-associated molecular patterns to stimulate plant immunity in cotton and maize. Moreover, molecular recognition involved in plant–microbe interaction depends on phytohormone communication. The high

level of 1-aminocyclopropane-1-carboxylate deaminase (ACCD) was observed to induce considerable growth promotion in tomato, which might prevent acceleration of the transformation of ethylene degradation of indole-3-acetic acid (IAA) by rhizosphere microorganisms (Vejan et al. 2016). In addition, the production of microbial phytohormone, including auxins, cytokines, gibberellins, abscisic acid, and ethylene, is also very important for improving plant physiology, regulating root architecture, and promoting plant growth (Spaepen 2015).

Studies in the past few decades have contributed to our understanding of the detailed molecular mechanisms of the communication between plants and microbes. The metabolic results of this communication, however, are only beginning to be investigated. Analysis of the proteome will provide new insight. In this study, we characterized the proteome of the biocontrol strain *Trichoderma guizhouense* NJAU 4742 interacting with cucumber in a hydroponic system.

RESULTS

Growth promotion effect of the NJAU 4742 strain on cucumber.

Coculture of the NJAU 4742 strain for 25 days with cucumber in a hydroponic system was applied to assess the growth promotion effect (Fig. 1). After cocultivation for 25 days in a 250-ml triangular flask containing 100 ml of half-strength Murashige and Skoog (MS) (Murashige and Skoog 1962) medium, a significant growth promotion effect on cucumber seedlings was observed in the cucumber roots grown with NJAU 4742 in 0.2× MS medium (TC) treatment compared with the cucumber grown without NJAU 4742 under the same conditions (C) treatment (Fig. 1A). The growth index of cucumber seedlings in the TC and NJAU 4742 grown without cucumber under the same conditions (T) treatments was also evaluated, including shoot biomass and root biomass production (Fig. 1B). The results indicated that the plant height, plant diameter,

aboveground biomass, and leaf area of cucumber seedlings in the TC treatment increased by 13.5, 19.1, 35.5, and 57.4%, respectively, compared with those in the C treatment, which demonstrated that NJAU 4742 had a considerable ability to promote plant growth. In particular, the number of lateral root tips in the TC treatment increased by 64.7% compared with that observed in the C treatment. Additionally, the root activity (tetrazolium chloride [TTC]) at 243.7 µg per gram of fresh weight per hour) in the TC treatment was higher than that in the C treatment (TTC at 95.6 µg per gram of fresh weight per hour) (Fig. 1C). All of the results described above suggested that the inoculation of NJAU 4742 could significantly increase the growth of cucumber seedlings, probably due to the presence of various compositions that were essential for increasing plant biomass and regulating root morphology.

Proteomic data quality control and quantification of the proteins.

In this study, proteomic analysis was employed to demonstrate how NJAU 4742 promoted plant growth and how plants responded to the presence of this fungus. Intracellular protein samples were extracted from the coculture system containing NJAU 4742 and cucumber roots (TCI) and pure cultured NJAU 4742 without cucumber roots (TI) under the same conditions. Also, extracellular proteins were extracted from the coculture system containing NJAU 4742 and cucumber roots (TCE) and pure cultured NJAU 4742 without cucumber roots (TE) under the same conditions. These proteins—TCI, TI, TCE, TE, and pure cultured cucumber (CE)—from various treatments were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results indicated that there were no distinct differences between the TCI and TI lanes, which indicated that the effect of microbe–plant interactions on the intracellular protein in NJAU 4742 was not significant (Fig. 2A). The SDS-PAGE results showed that extracellular proteins from the

Fig. 1. Effects of strain NJAU 4742 inoculation on the growth of cucumber roots. **A**, Growth condition of strain NJAU 4742 and cucumber seedlings in the hydroponic system. T = the pure culture of strain NJAU 4742, TC = the cultural system of cucumber inoculated with strain NJAU 4742, and C = the individual culture system for cucumber seedlings. **B**, Growth index of cucumber seedlings, including shoot length, shoot diameter, soil plant analysis development value (SPDA), leaf area, fresh shoot weight [SW(F)], root length, fresh root weight [RW(F)], surface area (in square centimeters), and root tips after 25 days. **C**, Root vigor of cucumber roots in different treatments after 25 days of cultivation. Bars indicate the standard error of five replicates and an asterisk (*) indicates significance at $P < 0.005$.

same treatment had good repeatability, and significant differences were observed among TCE, TE, and CE (Fig. 2D). Both the band diversity and intensity in TCE were more extensive than those in CE and TE, which also demonstrated that microbe–plant interactions might increase the secretomes of NJAU 4742 and cucumber. After preanalysis by SDS-PAGE, liquid chromatography tandem mass spectrometry (LC-MS/MS) data quality control was also performed, because this step might be considered one of the critical parameters for quantitative proteomic analysis. The results showed that the abundances in all samples (TCI/TI, TCE/CE, and TCE/TE) conformed to a normal distribution (Fig. 2B and E), indicating that most of the proteins were authentically detected squared. Moreover, Pearson correlation analysis was applied to reveal the relationship among different treatments, and the repeatability of different biological replicates was described by the value of R^2 . In this instance, high-correlation R^2 values ($0.78 < R^2 < 0.97$) were obtained in all biological replicates, which indicated that high-quality LC-MS/MS data were obtained in this study (Fig. 2C and F).

In total, 1,059 intracellular proteins were identified in the whole-cell lysates obtained from NJAU 4742 mycelium. The quantitative analysis results indicated that 510 proteins (48.2%) exhibited significant differences (fold-change > 2 or fold-change < 0.5 , P value < 0.05) between TCI and TI treatments; among these proteins, 247 were upregulated and 263 were downregulated in the TCI treatment (Supplementary

Dataset S1). The secretomes from NJAU 4742 and cucumber roots were also quantified, and the results indicated that 62 proteins were identified in both the TCE and TE samples. In all, 24 proteins (38.7%) in TCE exhibited significant differences from those in the TE treatments, with 18 being upregulated and 4 being downregulated (Supplementary Dataset S2). In contrast, fewer secreted proteins from cucumber were identified and quantified; in total, 36 proteins were identified in both the TCE and CE samples, including 4 upregulated and 7 down-regulated proteins (TCE/CE) (Supplementary Dataset S3). These data concerning the identified proteins enabled in-depth analysis of the responses during the plant–microbe interaction process to be performed.

Proteomic analysis of the total NJAU 4742 proteins and the secreted cucumber root proteins.

Differentially expressed proteins were annotated by using the Gene Ontology (GO) classification database to further characterize their locations, functions, and processes (Supplementary Fig. S1). In total, 1,059 intracellular proteins were identified, and most of them were classified as being involved in catalytic (42.8%) and cellular metabolic (46.74%) processes. In addition, 48 extracellular proteins from NJAU 4742 were determined to primarily belong to catalytic activity (43.18%) and cellular metabolic process (44.56%). Moreover, 36 extracellular proteins secreted by cucumber roots were identified as participating in catalytic activity (41.18%) and antioxidant activity (8.25%)

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography tandem mass spectrometry data qualification of strain NJAU 4742 total proteins and cucumber extracellular proteins in different treatments. **A**, SDS-PAGE analysis of the different treatments. M = the standard protein marker, TCI = the intercellular proteins extracted from strain NJAU 4742 in the NJAU 4742–cucumber interaction system, and TI = the intercellular proteins of strain NJAU 4742 in the pure cultured system. **B**, Distribution of the intracellular protein abundances in the TCI versus TI treatment. **C**, Correlation analysis of intracellular proteins the TCI versus TI treatment. **D**, SDS-PAGE analysis of extracellular proteins extracted from the coculture system containing NJAU 4742 and cucumber roots (TCE) versus pure cultured NJAU 4742 without cucumber roots (TE) and TCE versus pure cultured cucumber (CE). **E**, Distribution of the extracellular protein abundances in the TCE versus TE and CE versus CE treatments. **F**, Correlation analysis of extracellular proteins in the TCE versus TE and TCE versus CE treatments.

pathways. These results indicated that the identified proteins were primarily involved in different biological processes, which influenced the responses of *Trichoderma* spp. and plants to construct a positive molecular dialog.

After GO analysis, the data were filtered for a 1% false discovery rate (FDR), and the protein abundance ratios were calculated. Furthermore, statistical parameters (*P* values, coefficient of variation values, fold change, and protein abundances) were also employed to select the expression of differential proteins at the peptide and protein levels. Hierarchical clustering of significantly different intracellular and extracellular proteins obtained from NJAU 4742 showed some more abundant and specifically accumulated proteins in TCI (Fig. 3). The analysis of intracellular protein samples from TCI and TI performed by using sequential window acquisition of all of theoretical fragment ions (SWATH) quantitation indicated excellent separation of two major distinct clusters corresponding to the culture conditions, in which 263 proteins were upregulated, while the other 247 proteins were downregulated (TCI/TI) (Fig. 3A). Notably, 109 proteins in cluster I involved in metabolic pathways, biosynthesis of secondary metabolites, and some basic metabolic pathways were significantly upregulated in TCI compared with TI. Cluster II showed that 67 proteins involved in ribosome and amino acid metabolism were downregulated in TCI compared with TI, while another 99 proteins primarily involved in the biosynthesis of secondary metabolites were upregulated in cluster III. Most of the proteins involved in the biosynthesis of secondary metabolites (cluster IV) or proteasomes (cluster V) were expressed at low levels in the TCI or TI treatment compared with the TC condition.

The extracellular proteins in all treatments were also analyzed, and the results indicated that the proteins involved in the pathways of starch and sucrose metabolism ($n = 13$), biosynthesis of secondary metabolites ($n = 5$), and peroxisome- and cell-wall-degrading enzymes ($n = 13$) were significantly upregulated in NJAU4742 after interacting with cucumber roots (Fig. 3B). Notably, the secretomes from cucumber were associated with the biosynthesis of secondary metabolites and peroxisomes after interacting with NJAU 4742 (Fig. 3C), which also supported the hypothesis that the NJAU 4742 strain could activate the biosynthesis of secondary metabolites in plant roots, thereby promoting plant growth.

Kyoto Encyclopedia of Genes and Genome pathway analysis.

To further characterize the roles of the significantly different proteins in metabolic regulatory processes, Kyoto Encyclopedia of Genes and Genome (KEGG) analysis was performed in this study (Fig. 4). In total, 779 intracellular proteins were annotated by the KEGG database, and they were divided into different metabolic pathways. Based on the results of the KEGG analysis, protein–protein interactions (PPIs) were evaluated by constructing protein networks with Cytoscape (Fig. 4A). All annotated proteins were categorized into 104 categories, and 92% of these proteins were linked and formed a large interaction network. The results of the PPI analysis showed that these categories, including amino acid metabolism, the oxidative phosphorylation pathway (6.29%), pyruvate metabolism (5.39%), glycolysis/gluconeogenesis metabolism (5.13%), glycolysis/gluconeogenesis metabolism (5.13%), citrate cycle (tricarboxylic acid [TCA] cycle) (3.47%), energy production, ribosome metabolism (7.32%), RNA transport (3.59%), proteasome metabolism (3.59%), oxidoreduction, and the biosynthesis of secondary metabolites pathway (18.36%), were upregulated in TCI compared with TI. In particular, tryptophan (Trp) metabolism (3.85%), arginine metabolism (2.82%), glutamate metabolism (2.44%), tyrosine metabolism (3.34%), nitrogen metabolism (1.41%), phenylalanine metabolism (2.18%), and the biosynthesis of other amino acids were significantly affected after interaction with cucumber roots, which demonstrated that these pathways may be related to plant growth promotion in the NJAU 4742 strain. In addition, 57 ribosomal subunit proteins containing 50S and 30S ribosomal subunit complexes were determined to be highly enriched in the ribosome pathway (P value = 2.3×10^{-23}); among these proteins, most were upregulated and accelerated the entire metabolism of the NJAU 4742 strain. KEGG enrichment analysis for extracellular proteins of the NJAU 4742 strain and cucumber roots was also performed, and the 20 most critical enriched pathways were presented. All of the annotated extracellular proteins from NJAU 4742 were categorized into 38 categories, primarily starch and sucrose metabolism (10.29%), biosynthesis of secondary metabolites (10.29%), amino sugar and nucleotide sugar metabolism (7.35%), peroxisome (2.94%), Trp metabolism (2.94%), and phenylalanine metabolism (2.94%), in which Trp metabolism, phenylalanine metabolism, and starch and sucrose metabolism were significantly overexpressed in TCE compared

Fig. 3. Hierarchical cluster analysis of the identified total proteins from strain NJAU 4742 and extracellular proteins of cucumber in different treatments. **A**, Hierarchical cluster analysis of the identified intracellular proteins extracted from the coculture system containing NJAU 4742 and cucumber roots (TCI) and pure cultured NJAU 4742 without cucumber roots (TI). **B**, Hierarchical cluster analysis of the identified extracellular proteins from pure cultured NJAU 4742 without cucumber roots (TE) and the coculture system containing NJAU 4742 and cucumber roots (TCE). **C**, Hierarchical cluster analysis of the identified extracellular proteins from pure cultured cucumber (CE) and TCE.

with TE (Fig. 4B). Meanwhile, all of the annotated extracellular proteins from cucumber roots were categorized into 26 categories, primarily including biosynthesis of secondary metabolites (42.86%), phenylalanine metabolism (23.81%), amino sugar and nucleotide sugar metabolism (9.52%), plant–pathogen interaction (9.52%), peroxisome (4.76%), starch and sucrose metabolism (4.76%), the Trp metabolism pathways (3.62%), the tyrosine metabolism pathways (2.84%), and cyanoamino acid metabolism (2.38%), in which the Trp metabolism pathways, tyrosine metabolism pathways, and starch and sucrose metabolism pathways were significantly overexpressed in TCE compared with those in CE (Fig. 4C).

In this study, in total, 56 intracellular proteins from NJAU 4742 were obtained during the interaction between *Trichoderma* spp. and plants, and they were determined to be involved in cell wall degradation and the synthesis of secondary metabolites (Table 1). Additionally, the extracellular proteins from the NJAU 4742 strain and cucumber in different treatments were also quantified (Table 1), and the results showed that nine upregulated proteins, including 1,3- β -glucosidase, the glycosyl hydrolase (GH) family, expansion-like proteins, and peroxidase, were related to phenylalanine metabolites, while another six upregulated proteins were related to peroxidases. A low content of the expansion-domain protein (A1A105468.1) was obtained in TE, and the content of this protein increased dramatically in TCE (Supplementary Dataset S2). Three GH family proteins (A1A107070.1, A1A107404.1, and A1A101886.1) were strongly secreted by the NJAU 4742 strain in TCE, whereas they were not observed in TE. In addition, two peroxidases (A1A111042.1 and A1A111046.1) observed at higher abundance in TCE than in TE might play important roles in plant–microorganism interactions. Overall, these findings indicated that the proteins from NJAU 4742 and cucumber involved in different biological processes could influence the interaction process between plants and microorganisms.

Secondary metabolite-associated proteins related to auxin biosynthesis and nitrogen metabolism pathways.

As in the auxin precursor synthesis pathway, the shikimate pathway was observed to play a critical role in regulating secondary metabolism, and the proteins involved in this pathway were also selected to clarify their functions (Supplementary

Dataset S4). Based on the KEGG analysis results, 71 intracellular proteins related to the shikimate metabolism pathway were significantly up- or downregulated in TCI compared with those in TI (Fig. 5). The shikimate pathway comprises seven reactions catalyzed by six enzymes to produce chorismate. Two phospho-2-dehydro-3-deoxyheptonate aldolases (A1A105819.1 and A1A111349.1) were upregulated in TCI, which accelerated the transformation of phosphoenolpyruvate and erythrose 4-phosphate to form shikimate. Subsequently, shikimate could be hydrolyzed by chorismate synthase (CS) (A1A108794.1), which was increased by 1.55-fold in TCI compared with TI. The metabolism of chorismate was divided into two branches, and the first branch was the L-phenylalanine biosynthesis pathway, in which one chorismate mutase (A1A110052.1) and two aspartate aminotransferases (A1A107455.1 and A1A109221.1) were upregulated after interacting with cucumber roots. The second branch was related to the L- Trp metabolism pathway, in which four of seven differentially expressed enzymes (A1A103857.1, A1A102940.1, A1A108213.1, and A1A110565.1) were upregulated in TCI. IAA synthesized by microbes plays a critical role during plant–microbe interactions; thus, further research investigating biosynthesis in NJAU 4742 was strongly warranted. The proteomic analysis results showed that six differentially expressed proteins involved in auxin biosynthesis increased in TCI, and two amidase proteins (A1A101473.1 and A1A103233.1) were upregulated by 1.38- and 1.58-fold, respectively, compared with those in TI, which resulted in an increase in IAA. In addition, two important enzymes designated nitrilases (A1A107668.1 and A1A101490.1) were significantly upregulated by 9.79- and 2.52-fold, respectively, and played important roles during catalysis of the conversion of 3-indole-acetonitrile into IAA in the final step of auxin biosynthesis. As another category of enzymes involved in auxin biosynthesis, aldehyde dehydrogenases (A1A100829.1 and A1A101682.1), which were responsible for the formation of IAA biosynthesis by catalyzing 3-indole-pyruvic acid, were observed to increase by 1.73- and 2.62-fold, respectively.

Aside from the auxin biosynthesis pathway, 12 differential proteins involved in nitrogen metabolism were observed to change significantly, especially in secreting ammonium ions (Fig. 5). Nitronate monooxygenase (NMO), sulfite oxidase (SO), and monodehydroascorbate reductase (MDHAR), which

Fig. 4. Protein–protein interaction and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analysis results of different treatments. **A**, Protein–protein interaction analysis results of the intracellular proteins extracted from the coculture system containing NJAU 4742 and cucumber roots (TCI) versus pure cultured NJAU 4742 without cucumber roots (TI) treatments; nodes were annotation proteins (circles) and edges were protein interactions defined by KEGG databases. Protein node sizes indicate the $\log_{10}$ of protein expression values and node colors represented the fold-change (red = upregulated and blue = downregulated). **B**, KEGG pathway analysis results of the extracellular proteins from strain NJAU 4742 in coculture system containing NJAU 4742 and cucumber roots (TCE) versus pure cultured NJAU 4742 without cucumber (TE) treatment. **C**, KEGG pathway analysis results of the extracellular proteins from cucumber roots in TCE versus pure cultured cucumber (CE) treatment. Circles indicate the top 20 of the enrichment pathways, and the circle color indicates the expression level of different pathways (red represents upregulation and blue represents downregulation in the form of $\log_2$), while the circle size indicates the number of different proteins.

Table 1. Biological function, pathway, and location of differential proteins from *Trichoderma guizhouense* and cucumber (*Cucumis sativus*) after 25 days of interaction^a

Treatment, accession number	Protein name	PI	Molecular size (kDa)
Intracellular proteins (<i>T. guizhouense</i> NJAU 4742 with cucumber roots [TCI] and without cucumber [TI])			
A1A108890.1	β-Glucosidase	5.80	94,148
A1A108695.1	Exochitinase	5.72	64,250
A1A107668.1	Nitrilase	5.07	34,619
A1A101208.1	Putative cytochrome P450 oxidoreductase	5.64	36,626
A1A105563.1	Cytochrome P450 55A1	5.49	44,221
A1A105566.1	Lovastatin nonaketide synthase	6.18	195,485
A1A108307.1	Small GTPase mediated signal transduction	7.70	23,332
A1A108939.1	Acid trehalase	6.17	116,885
A1A103847.1	Aldo/keto reductase	5.72	38,583
A1A111753.1	Core histone H2A/H2B/H3/H4	10.12	14,845
A1A108926.1	Zinc-binding dehydrogenase	4.86	37,425
Extracellular (<i>T. guizhouense</i> NJAU 4742 with cucumber roots [TCE] and without cucumber [TE])			
A1A103792.1	Glucan 1,3-β-glucosidase	4.84	107,129
A1A108471.1	β-Glucosidase	5.10	52,950
A1A107070.1	Glycosyl hydrolases family 16	4.86	31,448
A1A107404.1	Glycosyl hydrolase family 18-1	4.57	42,108
A1A101886.1	Glycosyl hydrolase family 18	4.74	149,586
A1A105468.1	Expansin-like protein	5.20	13,247
A1A111042.1	Cytochrome c peroxidase, mitochondrial	9.14	47,104
A1A111046.1	Cytochrome c peroxidase, mitochondrial	9.14	47,104
A1A107883.1	Peroxidase; WSC domain	4.32	97,643
A1A112027.1	Peroxidase activity; response to oxidative stress	4.70	60,831
A1A105229.1	Replication factor C subunit 1	8.59	114,378
A1A102263.1	GTP binding proteins	5.05	43,245
A1A110737.1	Heterokaryon incompatibility protein	6.43	152,862
A1A106704.1	Cysteine-rich secretory protein family (SCCP)	5.28	17,458
A1A101889.1	LysM domain	6.47	59,827
A1A100181.1	Prolyl-tRNA synthetase, partial	6.10	63,096
A1A110737.1	Heterokaryon incompatibility protein	6.43	152,862
Extracellular proteins (<i>Cucumis sativus</i> , TCE/CE)			
XP_004151874.1	Blue copper protein-like	4.62	—
XP_004143988.2	Protein P21-like	4.49	—
XP_004139239.1	Pathogenesis-related protein PR-4	8.02	—
XP_004151729.2	Acidic endochitinase	4.63	—
XP_004149369.1	Peroxidase P7-like	8.5	—
XP_011658156.1	Glucan endo-1,3-β-glucosidase	6.47	—
XP_004154012.1	Peroxidase 7	6.19	—
XP_011658861.1	Basic form of pathogenesis-related protein 1-like	4.87	—
XP_011654331.1	Extensin-2-like	9.54	—
XP_011660113.1	β-Glucosidase 12-like	9.19	—
XP_004142246.1	Peroxidase 2-like	9.19	—
XP_011653567.1	Peroxidase 2-like	5.86	—
XP_004145227.1	Endochitinase PR4-like	4.88	—

(Continued on next page)

^a FC = fold change; Sig = signal peptide.

^b TRP = tryptophan metabolism, NIT = nitrogen metabolism, PHE = phenylalanine metabolism, GLY = glycolysis or gluconeogenesis, PPI = protein–protein interaction, BSM = biosynthesis of secondary metabolites, SSM = starch and sucrose metabolism, PEO = peroxisome, MME = microbial metabolism in diverse environments, MP = metabolic pathways, and GLD = glyoxylate and dicarboxylate metabolism.

are related to the transformation of nitroalkane, nitrate and nitrite into ammonia, were detected in the proteomes. NMO (A1A109247.1) stimulated the conversion of nitroalkane into nitrite and increased by 2.13-fold in TCI compared with that observed in TI. Also, SO (A1A106983.1), which could promote the conversion of NO_3^- to NO_2^- , was determined to increase by 1.03-fold in TCI. The proteomic data also showed that MDHAR (A1A105864.1) was upregulated by 4.02-fold in TCI, which could convert NO_2^- into ammonia. Moreover, nitrile could be transformed to ammonia via two nitrilases (A1A101490.1 and A1A107668.1), and the results indicated that these enzymes were also strongly upregulated by 2.49- and 9.81-fold, respectively, in TCI. In addition, another two pathways (glutamate or proline to ammonia and urea to ammonia) were also analyzed, and the results indicated that enzymes such as glutamate dehydrogenase, cystathionine γ-lyase, and

cystathionine β-lyase were all upregulated in NJAU 4742 after interacting with cucumber roots. Ammonium transporters were observed to increase by 3.24- and 3.56-fold, which indicated that the NJAU 4742 strain had a considerable ability to adjust the balance of ammonium, thereby decreasing ammonium toxicity. The results described above implied that the auxin biosynthesis and ammonification level of NJAU 4742 could be directly influenced by the interaction with cucumber roots, in which auxin biosynthesis, shikimate metabolism, nitrogen metabolism, and peroxisomes appeared to play important roles.

In situ studies of the auxin biosynthesis mechanism and intracellular ammonification process in the NJAU 4742 strain.

To verify whether auxin biosynthesis could truly reflect the condition of the NJAU 4742 strain, more thorough research,

Table 1. (Continued from previous page)

Biological function	Pathway ^b	FC	P value	Sig
TCI/TI (continued)				
Hydrolyzing O-glycosyl compounds; carbohydrate metabolic process	SSM	14.053	0.000153	No
Hydrolyzing O-glycosyl compounds; β -N-acetylhexosaminidase activity	GLD	8.981	0.009271	No
Acting on carbon-nitrogen bonds; nitrogen compound metabolic process	TRP/NIT	9.787	0.000141	No
Oxidoreductase activity, acting on paired donors; binding oxidation-reduction process	MP	13.573	0.002722	No
Oxidoreductase activity, acting on paired donors; binding oxidation-reduction process	MME	8.487	0.000005	No
Polyketide synthase dehydratase; AMP-binding enzyme	—	6.625	0.002334	No
GTP binding; small GTPase mediated signal transduction	—	6.433	0.000050	No
Catalytic activity, carbohydrate binding; carbohydrate metabolic process	SSM	8.721	0.017427	No
Oxidoreductase activity; oxidation-reduction process	SSM	8.377	0.000141	No
Nucleosome, DNA binding; protein heterodimerization activity	—	6.989	0.044956	No
Oxidoreductase activity; oxidation-reduction process	—	6.246	0.003320	No
TCE/TE (continued)				
Hydrolyze glucosidic bonds, releasing glucose	PHE/BSM	3.830	0.000006	Yes
Hydrolyzing O-glycosyl compounds; carbohydrate metabolic process	PHE/BSM	3.770	0.000007	Yes
Hydrolyzing O-glycosyl compounds; carbohydrate metabolic process	PHE/BSM	3.860	0.000308	Yes
Hydrolyzing O-glycosyl compounds; extracellular region cellulose binding	PHE/BSM	2.070	0.003347	Yes
Hydrolyzing O-glycosyl compounds, chitinase activity	PHE/BSM	0.500	0.000018	Yes
Restore cell wall expansion activity; nonenzymatic cell wall active protein	PHE/BSM	2.290	0.026323	Yes
Response to oxidative stress; heme binding, oxidation reduction	PHE/BSM	2.670	0.007990	Yes
Response to oxidative stress; heme binding, oxidation reduction	—	2.470	0.000656	Yes
Response to oxidative stress; heme binding, oxidation reduction	PPI	2.010	0.004094	Yes
Response to oxidative stress; heme binding, oxidation reduction	—	0.440	0.024212	Yes
ATP binding, DNA replication, DNA repair	—	0.490	0.000299	Yes
Control cell division	—	0.450	0.000024	Yes
Heterokaryon incompatibility protein; ankyrin repeats (three copies)	—	0.300	0.001882	Yes
Multifunctional calcium-binding glycoprotein	—	2.990	0.000867	Yes
Identify the receptor; transmit signals; binding to the cell wall chitin	SSM/PHE	8.850	0.004117	Yes
ATP binding, cytoplasm	PHE/BSM	2.650	0.000045	Yes
Protein binding	—	0.300	0.001882	Yes
TCE/CE (continued)				
Attack pathogenesis	—	0.13	0.001242	Yes
Attack pathogenesis	BSM	0.33	0.001159	Yes
Attack pathogenesis	BSM	0.38	1.41E-05	Yes
Hydrolyzing O-glycosyl compounds	PHE/BSM	0.4	7.11E-05	Yes
Response to oxidative stress	BSM	0.48	0.004295	Yes
Carbohydrate metabolic process	BSM	0.54	0.002577	Yes
Response to oxidative stress	PEO	0.58	0.002294	Yes
Attack pathogenesis	BSM	0.69	0.002805	Yes
Restore cell wall expansion activity	PHE/BSM	0.71	0.004988	Yes
Carbohydrate metabolic process	PHE/BSM	0.73	0.001055	Yes
Response to oxidative stress	PEO	1.32	0.013377	Yes
Response to oxidative stress	PEO	1.6	0.001115	Yes
Hydrolyzing O-glycosyl compounds	PHE/BSM	1.72	0.011102	Yes

including critical gene expression analysis, auxin content determination, and cucumber biomass evaluation, was performed (Fig. 6). Most of the genes involved in auxin biosynthesis in the NJAU 4742 strain were selected to determine their contribution to this pathway (Fig. 6A). The results of quantitative fluorescence detection showed that *chorismate mutase* and *tryptophan synthase* genes increased by 6.12- and 2.74-fold, respectively (Fig. 6B). In particular, the *nitrilase*, *amidase*, *aldehyde dehydrogenase1*, and *aldehyde dehydrogenase2* genes exhibited notably increased transcription levels of 53.4-, 38.1-, 4.72-, and 2.30-fold, respectively, after interacting with cucumber roots. The auxin content in cucumber roots from the hydroponic system was determined (Fig. 6C and D). Based on the result analysis of auxin contents in cucumber roots at different time points, we observed that the NJAU 4742 strain could significantly increase the auxin content, which was higher than that of the cucumber culture control (CK) and IAA treatments. Notably, the IAA treatment yielded the highest auxin content in the liquid medium compared with that in the other two treatments after 5 days. However, the auxin content in the NJAU 4742 treatment exhibited a gradually increasing trend, especially on the 30th day, and the auxin content

significantly increased by 1.15- and 0.5-fold compared with the CK and IAA treatments, respectively. All of these results clearly indicated that IAA biosynthesis in NJAU 4742 was enhanced significantly after interacting with cucumber roots, which might be an important plant-growth-promoting mechanism.

As a kind of available nitrogen, ammonia can be absorbed by various plants, and ammonium excretion by the NJAU 4742 strain could represent a possible mechanism of promoting plant growth (Fig. 7B). The fluorescence quantification results of the critical genes involved in the synthesis and secretion of ammonium indicated that all of these genes, including *cystathionine γ -lyase* (*cgl1*), *ammoniate transporter*, *cystathionine β -lyase*, *sulfite oxidase*, *monodehydroascorbate reductase*, *nitronate monooxygenase* (*nmo*), *glutamate dehydrogenase* (*gd1*), *nitrilase*, and *arginase* (*arg*), were significantly upregulated with NJAU 4742 treatment (Fig. 7B). Among these genes, *cgl1*, *arg*, *gd1*, and *nmo* exhibited higher expression levels in the TC treatment than in the C treatment, and the relative expression levels increased 197.4-, 127.3-, 203.5-, and 154.1-fold, respectively. These results were highly consistent with the results of proteomic analysis. ¹⁵N-labeled KNO₃ was employed to further investigate ammonia

synthesis and secretion of the NJAU 4742 strain, and it was shown that both the NJAU 4742 strain and cucumber roots could absorb $^{15}\text{N-KNO}_3$. In addition, $^{15}\text{N-NH}_4^+$ was detected in the C and TC treatments after 25 days at abundances of 4.47 and 5.30%, respectively. Meanwhile, the abundance of $^{15}\text{N-NH}_4^+$ in the T treatment was only 0.23%, which was almost below the detection limit (Fig. 7D). The abundance of ^{15}N -total nitrogen in the plant roots was also determined, and the results showed that there was a significant difference between the TC (23.6%) and C (11.3%) treatments after cocultivation for 25 days (Fig. 7E). In brief, these results indicated that the intracellular ammonia synthesis and secretion capacity of the NJAU 4742 strain could be improved after interacting with cucumber roots.

DISCUSSION

In this study, the interaction between cucumber roots and the NJAU 4742 fungal strain in a hydroponic system was explored. The total proteins of NJAU 4742 and secretomes of cucumber roots growing alone or in association were also identified. Furthermore, the identities and functions of these proteins were analyzed to demonstrate the molecular interaction between NJAU 4742 and cucumber roots and elucidate the roles played by various proteins during this symbiotic interaction.

Effect of *Trichoderma* spp. in promoting plant growth.

Previous reports determined that *T. virens* could colonize plant roots, and this fungus had been applied in various host plants due to its positive effect on plant growth and productivity (Nieto-Jacobo et al. 2017; Singh et al. 2016). Our results

demonstrated that NJAU 4742 could increase the plant biomass and regulate the root morphology by accelerating lateral root formation, which suggested that NJAU 4742 could promote cucumber growth by regulating different metabolism and signal substances during interactions with the host root system. Many studies have reported that ACCD, peroxidase, Epl1 protein, Sm1 protein, TVHYDIII1 protein, and mitogen-activated protein kinase 6 from *Trichoderma* spp. were upregulated after interaction with the host, which could differentially increase plant growth (Guzmán-Guzmán et al. 2017; Nogueira-Lopez et al. 2018; Salas-Marina et al. 2015). The signaling pathways or secondary metabolites identified from *Trichoderma* spp. in the microbe-plant interaction system were involved in cucumber root-system architecture alterations (Contreras-Cornejo et al. 2015; Garnica-Vergara et al. 2016). *Trichoderma* spp. might employ similar mechanisms to regulate root morphology under coculture conditions. Therefore, elucidating the molecular mechanisms governing specific metabolic pathways of *Trichoderma* spp. may facilitate the manipulation of host root morphology.

Intracellular proteins obtained from NJAU 4742 during the interaction with cucumber roots.

The identified NJAU 4742 proteins associated with protein metabolism, carbon or energy, and secondary metabolism during the interaction with cucumber roots were analyzed by the SWATH approach. These metabolic pathways were previously reported to participate in the molecular mechanisms governing microbe elicitor activation (Lombardi et al. 2020), which suggested that *Trichoderma* spp. could produce secondary metabolites with several biological activities to influence plant

Fig. 5. Metabolism schematic of various pathways in strain NJAU 4742 after interacting with cucumber roots. Differentially changed proteins involved in the vital pathways are labeled in red; CAT = catalase; NIT = nitrilase; CHIT = chitinases; PHEA = phenylalanine ammonia-lyase; GLU = β-1,3-glucanase; PO = peroxidase; and SSCPs = small, secreted, cysteine-rich proteins.

metabolism. Notably, the overexpressed intracellular proteins were primarily related to shikimate metabolism (L-tryptophan, L-phenylalanine, and L-tyrosine), auxin biosynthesis, and nitrogen metabolism, except for the energy metabolic pathways. Among these pathways, the aromatic amino acid (AAA) pathway was believed to play important roles in UV protection, electron transport, signaling, communication, plant defense, structural components, and the wound response by providing the basic building blocks for the synthesis of aromatic compounds (Tzin and Galili 2010). The AAA pathway generally participated in the synthesis of numerous natural products, including pigments, alkaloids, flavonoids, hormones, lignin, aromatic antibiotics, and cell wall components (Rodriguez et al. 2015; Tohge

et al. 2013). The chorismate mutases of the AAA pathway from *Trichoderma* spp. were detected, and they were also found in many plant-associated microbes to serve as general tools for host manipulation by changing the metabolic status (Djamei et al. 2011). Rivas-San Vicente and Plasencia (2011) noted that more salicylic acids were produced after interacting with the host, which resulted in lower chlorophyll contents and growth-stimulating effects due to its function as endogenous signal-mediating local and systemic plant defense responses to pathogens and abiotic stresses (e.g., drought, chilling, heavy metal toxicity, heat, and osmotic stress). Many proteins in the AAA pathway were identified in this study, and they were able to participate in the synthesis of different metabolites, including cinnavalininate, kynurenine,

Fig. 6. In situ study results of the auxin biosynthesis mechanism in strain NJAU 4742. **A**, Differentially regulated proteins involved in shikimate metabolism pathway. **B**, Transcriptional levels of vital genes related to shikimate pathways: 1 = tryptophan synthase (*trps*); 2 = aldehyde dehydrogenase1 (*ald1*); 3 = aldehyde dehydrogenase2 (*ald2*); 4 = amidase (*ad*); 5 = indole pyruvate dehydrogenase (*ipdc*); 6 = nitrilase1 (*nit1*); 7 = nitrilase2 (*nit2*); 8 = chorismate synthase (*chs*); 9 = chorismate mutase (*cmm*); 10 = catalase (*cat*); 11 = aminotransferase (*amt*); and 12 = phospho deoxyheptonate aldolase (*pdda*). TC = cucumber roots grown with NJAU 4742 and C = cucumber grown without NJAU 4742. **C** and **D**, Variations of indole-3-acetic acid (IAA) content of cucumber root in different treatments. CK = the pure culture of cucumber seedlings, IAA = addition of IAA at a final concentration of 200 μ M, and NJAU 4742 = the treatment inoculated with strain NJAU 4742.

Additionally, 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase was upregulated in TCI treatment, and Tzin and Galili (2010) found that DAHP primarily participated in regulating the cellular metabolism level of AAAs and was essential for microbial metabolism and energy supply. For 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS) and CS, the high expression of these two proteins would accelerate the metabolism of the shikimate pathway and might provide precursors for increasing a variety of hormones such as auxin and salicylate, or a very wide range of aromatic secondary metabolites (Tohge et al. 2013). Similar results, in which EPSPS and CS were dramatically upregulated, were also obtained in

Remarkably, nine of the identified intracellular proteins of the AAA pathway were related to Trp and auxin metabolism, which implied that the colonization of NJAU 4742 was a response to the cucumber roots during the microbe–plant interaction process. Tzin and Galili (2010) reported that indole-3-glycerol-phosphate synthase (IGPS) was the only known enzyme that formed the indole ring in Trp metabolism, which indicated that the increased IGPS in NJAU 4742 could accelerate the formation of the indole ring for auxin biosynthesis. Additionally, the upregulated 3-hydroxyanthranilic acid dioxygenase in NJAU 4742 could catalyze the conversion of the intermediate product (aminomuconate) into acetyl-CoA, which suggested that it could improve the basic energy metabolism level of NJAU 4742 to minimize the impact of nutrient competition with plants. A high content of

Fig. 7. Nitrogen metabolism pathway and ammonification characterization in strain NJAU 4742. **A**, differentially regulated proteins involved in nitrogen metabolism. **B**, Transcriptional level of the genes involved in nitrogen metabolism pathway by PCR: 1 = *cystathionine β-lyase1 (cbl1)*; 2 = *cystathionine β-lyase2 (cbl2)* 3 = *argininosuccinate lyase (arl)*; 4 = *cystathionine γ lyase1 (cgl)*; 5 = *nitrilase (nii)*; 6 = *arginase (arg)*; 7 = *sulfite oxidase (so)*; 8 = *monodehydroas reductase (mdhar)*; 9 = *glutamate dehydrogenase1 (gd1)*; 10 = *glutamate dehydrogenase2 (gd2)*; 11 = *aminotransferase (amt)*; and 12 = *nitrone monooxygenase2 (nmo)*. TC = cucumber roots grown with NJAU 4742 and C = cucumber grown without NJAU 4742. **C** and **D**, Abundance of $^{15}\text{N-NH}_4^+$ in the liquid medium after 25 days. **E**, Abundance of ^{15}N -nitrogen in cucumber after inoculating with NJAU 4742.

acetyl-CoA could promote colonization and plant growth. Therefore, it is plausible that the regulation of the Trp synthesis pathway could serve as a component of the basal mechanism governing the promotion of host growth.

Proteins involved in phytohormone signaling were also identified, and they were determined to be responsible for the biosynthesis of the precursor of the auxin biosynthesis pathway. The upregulated proteins could also enhance the accumulation of auxin in the rhizosphere after interacting with plant roots, which was in keeping with the results reported by Nieto-Jacobo et al. (2017). Concurrently, IAA could mediate root architectural changes by accelerating the formation of apical dominance, initiation of lateral and adventitious roots, regulation of root morphology, cell division, and cell expansion (Verma et al. 2016). Furthermore, proteins involved in the ammonification pathway were also significantly upregulated, enabling nitrate or nitrite reductase to enhance dissimilation or assimilation in NJAU 4742. Notably, NJAU 4742 possessed a considerable capacity for nitrate reduction, which was also observed previously in anaerobic ammonium-oxidizing microbes (anammox) based on proteomics (Kartal et al. 2007). Nevertheless, *Epsilonproteobacteria* could reduce the transformations of nitrite and hydroxylamine into ammonium (Haase et al. 2017), and certain fungi also exhibited interesting features of inhibiting dissimilatory nitrate reduction to ammonia (Suzuki 2009). As components of another ammonia-producing pathway, nitrogen-related amino acids could also be transformed into ammonia in NJAU 4742, as supported by the results that argininosuccinate synthase, argininosuccinate lyase, and arginase were also upregulated in TCI compared with their levels in TI. Arginine degradation is one of the amino acid metabolism pathways in which arginine is typically decomposed into urea by arginases and, finally, into ammonia (Fujii et al. 2019). In this study, arginases exhibited significant upregulation at both the transcriptomic and proteomic levels, which demonstrated that arginases might be a critical parameter for ammonification. A previous study reported that ammonia from microbes was very beneficial for plants when colonizing plant roots. Notably, ammonium ions were considered to be signaling substances involved in the interactions between plant-growth-promoting fungi and plants (Narula et al. 2009). Even though the conjugation effect of ammonium ions as signaling substances or nutrients for plants has not been elucidated, a deeper investigation of the molecular mechanism governing ammonium production is merited.

Secretomes of NJAU 4742 and cucumber roots during the interaction process.

Some extracellular functional proteins (antioxidant proteins, GH family, β -glucosidase, expansion-like proteins, chitinase, and secondary metabolism proteins) (Supplementary Dataset S2) secreted by NJAU 4742 were significantly regulated in TCE compared with TE; among these proteins, GHs and β -glucosidase exhibited the highest levels. These results provide important evidence that NJAU 4742 might colonize the outer root surface by secreting β -glucosidases to degrade the host cell walls and subsequently occupy this ecological niche. These results were consistent with the findings of previous studies, which observed that GHs could directly influence the colonization of *T. virens* on plant roots and then improve plant defense (Morán-Díez et al. 2015; Lamdan et al. 2015). Also, Rubio et al. (2012) found that the genes of *Trichoderma* spp. involved in carbohydrate metabolism, nutrient exchange with the plant, building block generation, and cell wall synthesis were upregulated after interacting with tomato. Taken together, these findings indicated that *Trichoderma* spp. could secrete various enzymes and were necessary for colonizing host tissues or loose cell walls to promote root growth and regulate root architecture.

Some *Trichoderma* strains exhibited a better ability to tolerate oxidative stress than many other soilborne organisms and obtained an advantage over other rhizosphere residents in surviving in the rhizosphere ecosystem by upregulating peroxidase (Morán-Díez et al. 2010). Dietz (2016) also found that upregulated peroxidase could participate in the scavenging of oxygen species (ROS) and immune response of the plant, which could enhance the stress resistance and photosynthetic capacity without causing damage to beneficial microbes. Additionally, some peroxidases in cucumber roots were also upregulated in the TCE treatment compared with the CE treatment. Typically, peroxidases are the principal components in the plant defense response against pathogens. These enzymes may strengthen the plant cell walls or induce plant oxidative bursts in the apoplast and create suitable conditions for microbe interactions (Brotman et al. 2013). Several pathogenesis-related (PR)-like proteins (Table 1; Supplementary Dataset S3) were also identified in CE treatment, which suggested that these functional proteins could be specifically induced by the colonization of *T. virens*. Parallel findings were observed during the interaction between *T. atroviride* and host plants, where PR proteins were specifically activated for recognition (Marra et al. 2006). These findings indicated that *Trichoderma* spp. can secrete enzymes to interact with plant root, which may represent a strategy for maintaining ROS stability to influence further plant immune responses.

MATERIALS AND METHODS

Cucumber germination.

Cucumber seed (*Cucumis sativus* L. 'Jinchun-No. 4' obtained from Tianjin Cucumber Research Center, China) were surface sterilized with 75% (vol/vol) ethanol for 1 min followed by 10% (vol/vol) sodium hypochlorite for 20 min, and the seed were subsequently washed three times with sterile water. The cucumber seed were placed in sterile Petri dishes containing 0.2× MS and incubated in the dark for 36 h at 30°C for germination. The germinated seed were cultured with 0.2× MS solution in a humidity-controlled plant growth chamber for 7 days, and the culture conditions were set as 25°C, 80% humidity, and 16 h of light and 8 h of darkness per cycle.

Inoculum preparation.

T. guizhouense NJAU 4742 was originally isolated from a mature compost sample, and its genome sequence has already been published in the NCBI database (accession number LVVK000000000.1). Spore suspensions were obtained after cultivation on potato dextrose agar for 7 days and later diluted to 1×10^6 spores·ml⁻¹. Next, NJAU 4742 spores were inoculated into MS liquid medium (1:100, vol/vol) with 1% (wt/vol) glucose in a 500-ml triangular flask and cultured at 28°C and 180 rpm for 24 h. The newly germinated spores were filtered with a filter membrane (0.45 μ m), then washed twice with 100 ml of sterile distilled water. The newly germinated spores were resuspended in 5 ml of 0.2× MS solution for the subsequent experiment.

Inoculation of NJAU 4742 to cucumber roots.

In total, 50 germinated seedlings with similar-sized roots and shoots were placed on the stands in each 250-ml triangular flask containing 100 ml of 0.2 × MS liquid medium. After 4 days of cultivation, the 0.2 × MS was replaced by fresh medium, and the preprepared newly germinated spores were inoculated into the cucumber roots. The plants were maintained in an artificial climate chamber with the following conditions: a photoperiod of 16 and 8 h at 26 and 22°C, respectively, assuring photosynthetic photon flux density of 300 μ mol m⁻² s⁻¹, constant relative humidity of 80%, and moderate shaking on an

orbital shaker (100 rpm) to furnish sufficient oxygen for NJAU 4742 growth. The growth medium was supplemented to 100 ml with fresh $0.2 \times$ MS solution over time. Three different treatments, including TC, T, and C, were performed.

Protein extraction from different treatments.

Samples from different treatments in hydroponic medium on the 25th day were used for protein extraction for proteome analysis. In total, 50 triangular flask replicates were performed for each treatment to collect enough samples. The liquid media from the coculture system TCE, TE, and CE were collected after 25 days, during which 16 randomly selected replicates from the same treatment were combined as a new replicate (three replicates for each treatment) to ensure enough proteins for LS-MS/MS analysis. The combined solutions from different treatments were centrifuged at 10,000 rpm for 10 min at 4°C, and the supernatants were filtered through a 0.22- μ m filter (Millex-GP; Syringe-Driven Filter Unit, 041237-LRL-001). After that step, the filtered supernatants were concentrated by use of the ammonium sulfate precipitation method described by Wingfield (2016). The precipitates were redissolved in $0.5\times$ phosphate-buffered saline (PBS; pH 7.0) and then transferred into a dialysis bag (MD44-3500D; Solarbio) in PBS (pH 7.0) overnight at 4°C. The intracellular proteins of TCI and TI were also collected for LS-MS/MS analysis. Fifty individual triangular flasks were randomly divided into two parts; then, the separated triangular flasks were combined as a new replicate (two replicates for each treatment) for intracellular protein extraction. The intracellular proteins of NJAU 4742 mycelium from TCI and TI treatments were extracted according to Cilia et al. (2009), with some modifications. The mycelium was collected and washed three times with ice-cold $0.5\times$ PBS (pH 7.0); then lysed in 50 μ l of modified RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% Na-deoxycholate, and 1% nonionic surfactant glycol octyl phenyl-polyethylene) containing protease and phosphatase inhibitors (A32959; Thermo Fisher). The mixtures were collected by centrifugation at 5,000 rpm for 10 min at 4°C, and the lysates were transferred to clean 2-ml microcentrifuge tubes, then stored at -80°C for the subsequent experiment. The frozen cells were ground into a fine powder in liquid nitrogen using a mortar and pestle, then resuspended in ice-cold extraction buffer (500 mM NaCl, 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.5], with KOH, 5 mM EDTA, 1 mM dithiothreitol, 5 mM NaF, polyvinyl pyrrolidone at 1 $\text{mg}\cdot\text{ml}^{-1}$, 0.4 mM phenylmethylsulfonyl fluoride, and one tablet of protease inhibitor cocktail [Roche Complete Mini, EDTA-free] per 10 ml of buffer) at 1 ml per gram of fresh weight, after which the system was mixed thoroughly by vortexing and then incubated on a rotating platform for 1 h at 4°C. TCA-acetone protein precipitation was applied to concentrate the proteins, and the extracts were resuspended in the remaining 2 ml of PEM buffer (0.1 M piperazine-*N,N'*-bis(2-ethanesulfonic acid), 2 mM EGTA, and 1 mM MgSO_4 , pH 8.5). Finally, the extracellular and intracellular protein contents were determined by the Bradford method (Kruger 2009) by using bovine serum albumin as a standard.

Protein digestion and LC-MS/MS analysis.

The SWATH analysis was applied to identify and quantify the proteins in different treatments as follows: 100 μ g of protein of each sample was digested by trypsin (GIBCO; Thermo Fisher) at 37°C overnight (1:50, wt/wt), after which an equal volume of 0.1% formic acid (FA) was added to acidify the reaction system; peptides were loaded onto Strata-X C18 cartridges three times, washed with 0.1% FA + 5% acrylonitrile (ACN) twice, and eluted with 1 ml of 0.1% FA + 80% ACN; finally, the eluted peptides were dried using a vacuum concentration meter. LC-electrospray ionization-MS/MS analysis was performed on a

Triple TOF 5600 plus LC-MS/MS system (SCIEX, Ontario, Canada). Data-dependent acquisition was followed by SWATH acquisition with the same amount and gradient conditions; then, the peptides were loaded onto a C18 trap column (5 μ m, 100 μ m by 20 mm) with an elution rate of $300 \text{ nl}\cdot\text{min}^{-1}$ onto a self-packed C18 analytical column (3 μ m, 75 μ m by 150 mm) over a 120-min gradient. Buffer A (2% acetonitrile, 0.1% FA, and 98% H_2O) and buffer B (98% acetonitrile, 0.1% FA, and 2% H_2O) were used as the mobile phases; MS1 and MS2 spectra were collected in the ranges of 350 to 1,500 and 100 to 1,500 $m\cdot z^{-1}$, respectively; and precursor ions were excluded from re-selection for 15 s. SWATH data were acquired with a 250-ms MS1 scan followed by 90-ms MS2 scans with 32×25 unified atomic mass unit (amu) isolation windows covering the mass range of 400 to 1,200 amu.

SWATH data analysis.

Spectral library generation and SWATH data processing were performed using skyline software (V3.5). Prior to targeted data extraction, a spectral library document was automatically generated complying with the rules implemented by Haudry et al. (2013) and used to create a decoy spectral library for targeting peak extraction. Based on the previous instructions, each ion was automatically extracted with a retention time width of 5 min, and the area under the curve for individual ions was calculated. Fragment ion areas belonging to one peptide were summed to obtain the peptide's abundance, and the summed peptide abundance of a specific protein was considered its abundance. All raw data among different treatments were normalized by using a median normalization method to eliminate random errors and sample bias (Oberg and Mahoney 2012). The mProphet algorithm was employed for each extracted peak by skyline software (V3.5) to assess the data confidence and control the FDR, and ProteoWizard (V3.0.9134) was used to transform Wiff files to the mzXML file type. Subsequently, SWATH targeted data analysis was completed by using OpenSWATH, which could automatically estimate the FDR by integrating a decoy scoring system and various peak group extractions by mProphet (Weisser et al. 2013). The peak groups obtained from SWATH maps at an FDR (1%) and fold-change ≥ 2 were aligned between different treatments based on the clustering behaviors of the retention time. Specifically, features were always considered alignment on the basis of a nonlinear alignment algorithm under the maximum FDR quality of 1% or the further constraint of a retention time difference of less than 60 s in the LC gradient after iRT normalization (Escher et al. 2012).

Bioinformatic analysis.

Signal peptides were identified using SignalP4 (Gomes et al. 2017). The CAZyme annotation was performed by HMMER software (Finn et al. 2015) with the dbCAN database. KEGG pathway annotation was performed using the KEGG Orthology-Based Annotation System 2.0 (Xie et al. 2011). GO annotation was also performed, and all differentially expressed proteins were assigned according to their biological process, cellular component, and molecular function. Cytoscape software (V3.5.1) was used to analyze PPIs based on the STRING database. All identified proteins in different treatments were separated by using a Venn diagram. All data were visualized according to the NJAU 4742 protein Atlas database and the cucumber genome database.

Quantification of NJAU 4742

by quantitative reverse-transcription PCR.

Based on the proteomic analysis results, the total RNA from the NJAU 4742 mycelium (TCI and TI) was extracted and then reverse transcribed into cDNA as a template. Quantitative

reverse-transcription PCR was carried out on a PCR system (7500 fast real-time PCR system; Applied Biosystems) by using SYBR premix ExTaq II (TaKaRa, Dalian, China). The primers for verification of shikimate metabolism are listed in Supplementary Table S1, and the primers for verification of nitrogen metabolism are listed in Supplementary Table S2. Cycle threshold values were calculated with the β -tubulin gene as an endogenous control, and the data were expressed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The auxin in the cucumber roots was extracted as follows: after liquid nitrogen grinding and high-speed centrifuging, the supernatants (50 ml) of cucumber roots from the TC and T treatments were adjusted to pH 2.5 with 1.0 M HCl and extracted three times with ethyl acetate (1:3, vol/vol), with rotational evaporation at 30°C, and the residue of organic solvents was dissolved in 1 ml of methanol and filtered through a 0.22- μ m membrane filter. The quantification of the extracted auxin was performed with high-performance liquid chromatography by using a reversed-phase C18 analytical column (9.4 by 150 mm; Agilent Technologies, Santa Clara, CA, U.S.A.) according to Chen et al. (2003). The characteristics of nitrogen transformation in the hydroponic system were determined by using $^{15}\text{N-KNO}_3$ as the sole nitrogen source, and the abundance of ^{15}N -total nitrogen in the plants was determined by using an automated isotope ratio mass spectrometer (IRMS; SERCON, 20-20, 209 UK), as described by Rashti et al. (2017). The relative abundance of ^{15}N -total in plants was calculated by the ratio of $^{15}\text{N}/^{15}\text{N} + ^{14}\text{N}$ (Herridge et al. 1990), while the relative abundance of secreted $^{15}\text{N-NH}_4^+$ in the liquid medium of the T, C, and TC treatments was estimated from the ^{15}N -total nitrogen content by multiplying it based on the ratio of the calculated NH_4^+ /total N, as determined by a continuous flow analyzer (Auto Analyzer 3; Germany).

AUTHOR-RECOMMENDED INTERNET RESOURCES

Atlas database: <http://bioinfo.njau.edu.cn/tgn4742/>

Cucumber genome database:

<https://www.ncbi.nlm.nih.gov/genome/?term=Cucumis+sativus>

STRING database: <https://string-db.org/cgi/about>

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