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Full Length Article

Pre-Planting Technique Promoted Tobacco Root Growth due to Differentially Expressed Functional Proteins

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Abstract

Pre-planting technique is proven as the practical cultivation way of tobacco with the advantages of lower cost, higher operability and reliable survival rate. Some studies have focused on using tobacco genes to reflect the physiological changes caused by pre-planting treatment. In this study, a comprehensive metabolic profile of tobacco roots was discovered by comparing floating seedlings treatment and pre-planting treatments. A total of 5,080 proteins were identified in the roots of tobacco and 361 proteins were considered to be differentially expressed. Among these 361 proteins, 342 were up-regulated and 19 were down-regulated. Differentially expressed functional proteins including heat shock proteins (HSPs), glutathione-, glycerol kinase (GK), tyrosine-protein kinase (TK), serine/threonine protein kinase-like protein kinases (STK) and peroxiredoxin were significantly higher than floating seedlings technique. Most of these were involved in signal transmission, metabolism, stress and defense. Parallel reaction monitoring (PRM) analyses indicated the reliability of the proteomic results. The present study provided differential modulation of pathways by pre-planting treatment. In conclusion, pre-planting technique promoted tobacco root growth due to differentially expressed functional proteins involved in signal transmission, plant metabolism, stress tolerance and defense system.

Keywords: iTRAQ; Functional proteins; Glycerol kinase; Up-regulated; Metabolism

Introduction

Tobacco (*Nicotiana tabacum* L.) was widely grown in more than 100 countries as cultivated non-food crops (Peeran and Alsaid, 2016). Large-scale floating system was first found by Shan (1999) and widely used in high value-added crops seedling (Gao and Zhou, 2001). Large-scale floating seeding system of tobacco has gradually become the trend of tobacco production and used to improve tobacco yield and quality (Zhang, 2016). However, floating system technology gradually exposed some problems in the process of promotion and application. Fang (2015) reported that plant roots which cultured from floating system were slow to develop and weak regarding disease resistance. However, pre-planting technique could promote the growth of seedling roots and increase the survival rate (Fang, 2015). Temporary planting treatment was the key treatment of pre-planting technique and changed almost every aspect of plant physiology such as ion and osmotic homeostasis, photosynthesis activity, and the growth of plants (Gao and Zhou, 2001). Germinated seedlings were temporary grown in moist nutrient soil when the floating seedlings have four leaves of same size. This process may change quite a number of stress related gene expressions (Zhu, 2002) and influence the synthesis of diverse functional proteins (Christophe *et al.*, 2010).

With the rapid development of bioinformatic tools, the proteomic analysis became a cost-effective way to gain insight into plant response to changes of living environment. Mass spectrometry-based proteomic analyses were widely used in studying crops responses to environmental stress. Many reports have showed that kinds of protein expression changes in response to drought stress in plants (Hussain *et al*., 2018). Moreover, the drought stress-induced proteins involved in photosynthesis (Vincent, 2010), signaling pathways (Ali and Komatsu, 2006) and oxidative stress (Zang and Komatsu, 2007) have been identified. This study was designed to evaluate the changes in protein expression between floating system and pre-planting technique by comparing profiled changes of proteins accumulated using isobaric tags for relative and absolute quantification (iTRAQ).

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Materials and Methods

Plant Materials and Culture Conditions

A model plant of tobacco species, Yunyan 87, was used in this study (Li *et al.*, 2001). For control group (T_1), germinated seeds were grown in floating trays (55 cm × 35 cm, 51 holes / plate) with roots immersed in water for 35 days (Dong *et al.*, 2002). For test treatment (T_2), germinated seedlings were grown in floating trays with roots immersed in water for 10 days and then transplanted in moist nutrient soil for an additional 25 days. All plants were grown in greenhouses in a 16/8 h light/dark cycle, 25°C and 40% humidity. Roots were harvested and washed with distilled water for three times then flash-frozen in liquid nitrogen.

Phenotypic Characterization and Enzyme Activity Test

According to the YC/T 142-2010 standard, the plant height, leaf length and fresh weight (FW) were measured at maturation stage (Liu *et al.*, 2015). The whole plant was put in the forced hot air draft oven at 70±5°C for 5 days to measure dry weight (DW). Root morphology analysis was performed using the Epson Expression 1680 Scanner and Win RHIZO root system (Zhu and Guo, 2012). Root vitality and nitrate reductase were measured by triphenyl tetrazolium chloride (TCC) method (Bat *et al.*, 1994; Li and Gong, 2009). Free proline contents were measured by acid ninhydrin colorimetry (Shao *et al.*, 2008).

Proteome Extraction, Digestion and iTRAQ Labeling

Proteome extraction and iTRAQ analysis were performed by Wuhan Genecreate Bioengineering Co., Ltd. One g sample was ground into powder in liquid nitrogen and suspended in 200 μL of TEAB (triethylammonium bicarbonate). The samples were sonicated thrice on ice for 15 min using a high intensity ultrasonic processor. The remaining debris was removed by centrifugation at 12,000 × g at 4°C for 20 min, and the supernatant was transferred to a new tube to add 4 times volume of pre-cooled acetone (containing 10 m*M* DTT), which was added and precipitated for 2 h. The centrifugation step was repeated, and the pellet was collected and dissolved in 100 μL of TEAB. Protein concentration was determined by the Bradford assay using bovine serum albumin (BSA) as a standard. Three biological replicates were combined for iTRAQ analysis.

Proteins were digested with trypsin (Promega, U.S.A.) at 37°C at a ratio of 1:50 (enzyme/substrate) overnight and cleaned up using solid-phase extraction. The samples were acidified with 0.1% fluoroacetic acid (FA) and loaded on Strata-X C18 column (Phenomenex, Torrance, C.A., U.S.A.). After three washes with 0.1% FA, the peptides were eluted using 80% acetonitrile (ACN) with 0.1% FA. Peptides were dried by vacuum centrifugation, re-dissolved in 0.5 *M* of TEAB, and processed with an 8-plex iTRAQ Reagent-8 plex Multiplex Kit (AB Sciex U.K. Limited, UK).

iTRAQ Analysis

The labeled samples were divided into 12 components by using an HPLC system (Thermo DINOEX Ultimate 3000 BioRS, Thermo DINOEX , USA) connected to an Durashell C18 column (5 mm, 100 A, 4.6 × 250 mm). The iTRAQ-labeled samples were analyzed using an AB SCIEX nano LC-MS/MS mass spectrometer (Triple TOF 5600 plus, AB Sciex, USA) coupled to an AB Sciex column (75 μm×12 cm, AB Sciex, U.S.A.). Each sample (5 μL) was injected onto the LC−MS/MS system. The samples were first loaded onto PicoFrit emitter. The mobile phase B proportion was initially 5% with ramping gradually to 80% in 80 min and was kept for another 5 min at 80%. Finally, the proportion reached 5% in 5 min and was held for another 5 min (mobile phase A, 0.1% FA in 5%ACN; mobile phase B, 0.1% FA in 95%ACN). All the mass spectral data were processed and analyzed using Proteinpilot™ V4.5.

Parallel Reaction Monitoring Analysis (PRM)

PRM analyses were performed by Wuhan Genecreate Bioengineering Co., Ltd. One g samples were grinded to powder in liquid nitrogen and suspended in 200 μL of lysis solution (7 *M* Urea/2 *M* Thiourea/4% SDS/40 m*M* Tris-HCl, pH 8.5/1 m*M* PMSF/2 m*M* EDTA). The samples were sonicated thrice for 15 min on ice using a high intensity ultrasonic processor. The supernatant was collected by centrifugation at 12,000×g at 4℃ for 30 min and added DTT to final concentration was 10 m*M* which was reacted for 30 min. IAM was added to final concentration 55 m*M* which was dark reacted for 30 min. Protein concentration was determined by the Bradford assay using BSA as a standard. 100 μg proteins were digested with 2 μg trypsin at 37°C overnight and cleaned up using solid-phase extraction. The sample was further dissolved into loading buffer (0.1% FA, 2% ACN) for analysis by using HPLC system (Thermo DINOEX Ultimate 3000 BioRS, Thermo Fisher, Waltham, MA, USA). Each sample was injected onto the LC−MS/MS system. The samples were first loaded onto PicoFrit emitter (New Objective). Then, gradient elution was performed on a C18 column (3 μm, 75 μm × 150 mm) at a flow rate of 300 nL/min (mobile phase A, 0.1% FA, 2% ACN, 98% H_2O; mobile phase B, 0.1% FA, 98% ACN, 2% H_2O).

Data Analysis

All the mass spectral data were processed and analyzed by Proteinpilot™ V4.5 and the subsequent database searched from the UniProt *Solanaceae* databases (http://www.uniprot.org/) (Pundir *et al.*, 2015). Proteins were recorded which had at least one unique peptide and unused score≥1.3 (confidence over 95%). For quantitative characterization, the differential proteins which appeared in all three biological replicates with consistent expressions

were selected, 1.5-fold cutoff and *P* value ≤ 0.05 were set to determine differentially expressed. Functional analysis was conducted by Gene Ontology annotation (GO) (http://www.geneontology.org/) (Ashburner *et al.*, 2000). The differential accumulated proteins were imported and analyzed by the Clusters of Orthologous Groups (COG) of proteins database (http://www.ncbi.nlm.nih.gov/COG/) (Tatusov *et al.*, 1997) for phylogenetic classification and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/pathway.html) database (Minoru *et al.*, 2004) for metabolic pathway analysis.

Results

Phenotypic Characterization and Enzyme Activity Test

At maturity stage, pre-planting technique treatment observed more plant height, leaf lengths, leaf widths, fresh weight and dry weight compared with control (Table 1). The content of proline in plants could reflect the resistance of them. Compared to control, root vitality, nitrate reductase and proline content of pre-planting technique treatment were 1.99 mg•g^{-1}•h^{-1}, 20.72 μg•g^{-1}•h^{-1}, and 82.09 μg•g^{-1}•FW. Above all, pre-planting technique improved tobacco plant in both growth and yield.

Primary Data Analysis and Protein Identification by iTRAQ

Total proteins in samples were analyzed by iTRAQ with total number of 447,408 spectra were generated (Fig. 1). Among them, 151,742 spectra were matched to known peptides and 33,386 unique peptides were identified. The above data were separated into homology groups by aligning all proteins and grouping proteins recursively with highly significant alignment scores, and a total of 5,080 unique proteins were identified. 3,787 unique peptides were identified that they were included at least two peptides. The experimental expression data for each quantified protein was subjected to a *t*-test. Only those proteins that *P*-value ≤ 0.05 and exhibited a fold change greater than the threshold of significance (up regulate ≥ 1.5 and down regulate ≤ 0.67) were considered to be significantly changed with high confidence. Total of 361 differential proteins were identified which 342 were up-regulated and 19 species were down-regulated (Fig. 2).

The GO annotations were annotated 4905 proteins using *Blast2 go* (http://www.geneontology.org) according to the biological process, cellular component and molecular function (Fig. 3). The biological process annotation of the proteins was summarized in Fig. 3A, which showed that 16.83% of proteins were associated with cellular process, 16.29% with metabolic, and 9.9% were response to stimulus. The cellular component proteins were shown that 49.94% in the cell and cell part, 32.49% in the organelle and organelle part, 6.2% in macromolecular complex, and 4% in symplast

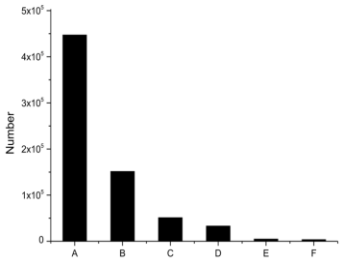

Fig. 1: Protein information of iTRAQ
Here **A**: Total spectra number; **B**: Spectra number; **C**: Unique spectra number; **D**: Peptide number; **E**: Unique peptide number; **F**: Protein number

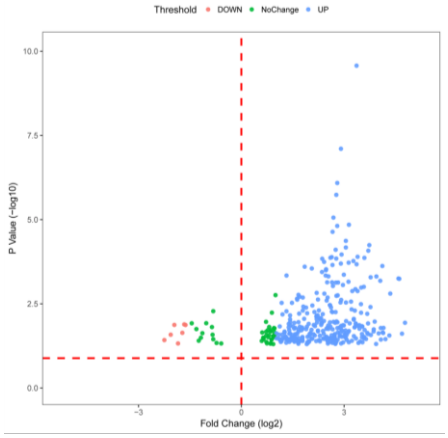

Fig. 2: Volcano Plot analyses of the differentially changed proteins
Each graph plots $\log_2$ (fold change in protein expression) versus $-\log_{10}$ (*P* test probability). *Red dots* correspond to proteins that are significantly up-regulated, *blue dots* to those that are significantly down-regulated, *green dots* to proteins that do not change significantly in expression (*i.e.*, that did not meet the *P*-value and fold-change criteria)

(Fig. 3B). As given in Fig. 3C, the highest number of proteins was associated with binding (42.83%), catalytic activity (40.47%), transporter activity (5.81%), and structural molecule activity (4.55%). Differentially proteins were classified into three groups including biological process, cellular component and molecular function based on GO analysis (Fig. 4). The up-regulated proteins with the highest number were response to cellular process, metabolic process, response to stimulus, binding, structural molecule activity and enzyme regulator activity. Significant differences in the classification of GO functions were shown between up-regulated and down-regulated differential proteins. The structural molecule activity and the enzyme regulator activity were existed in the differential proteins but not appeared in the GO annotation result of down-regulated (Fig. 4).

Analysis of differentially accumulated proteins by KEGG database was shown in Fig. 5. The up-regulated proteins were mainly including metabolic pathways (109, 45.61%), biosynthesis of secondary metabolites (68, 28.45%), microbial metabolism in diverse environments (37, 15.48%), and ribosome (23, 9.62%) in pathway analysis. The

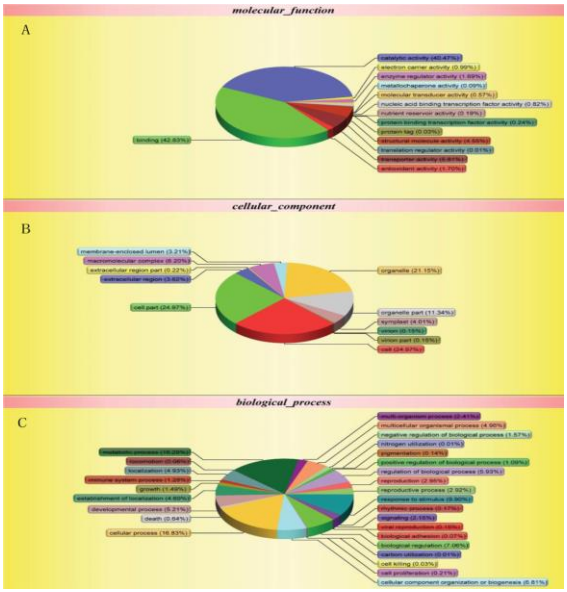

Fig. 3: Gene ontology enrichment analysis of differentially expressed proteins
The category number is displayed with biological process, cellular component, and molecular function

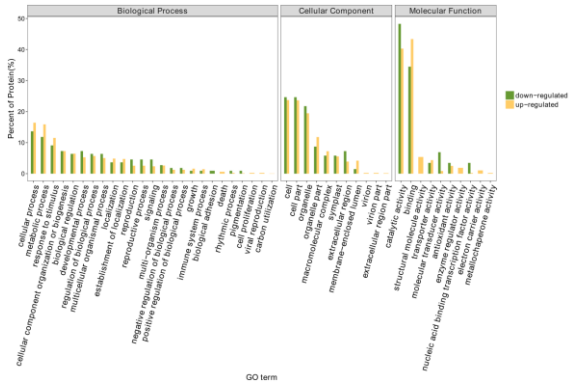

Fig. 4: Gene ontology enrichment analysis of functional classification of the differentially expressed proteins
Differentially expressed proteins were categorized according to their gene ontology terms, and in each category, the numbers of proteins up- and down-regulated are shown (Y-axis indicates the number of proteins with increased or decreased abundance)

down-regulated proteins were mainly including biosynthesis of secondary metabolites (5, 45.45%), metabolic pathways (4, 36.36%), and methane metabolism (2, 18.18%). The top ten pathways with the highest number between up-regulated and down-regulated in pathway analysis were metabolic pathways, biosynthesis of secondary metabolites, and methane metabolism (Fig. 5).

Validation of Protein Expression Changes by PRM

Most of the genes such as glutathione S-transferase (tr|A0A1S4BZT5, tr|A0A1S4A868, tr|A0A1S4AR76), cinnamoyl-CoA reductase (tr|A0A1S4BXI5), leucine

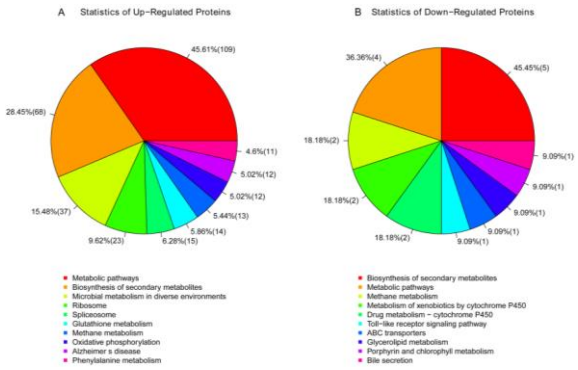

Fig. 5: KEGG pathways analysis of differentially expressed proteins
The top ten proteins with the highest number between up-regulated and down-regulated in pathway analysis are shown

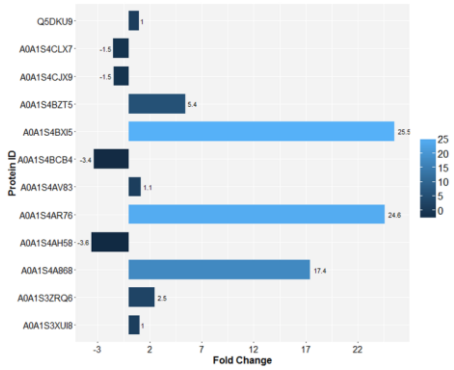

Fig. 6: PRM analysis of differentially expression protein and corresponding genes
The Y-axis (left) represents the expression level of the down-regulated protein, the Y-axis (right) represents the expression level of the up-regulated protein
The number on the column represents the difference multiple (non-logarithmic)

aminopeptidase (tr|A0A1S4AV83), and 6-phosphogluconate dehydrogenase (tr|A0A1S3ZRQ6) showed similar patterns of expression and regulation (Fig. 6). No significant difference in expression was observed for the adenosine kinase (tr|Q5DKU9) and isocitrate dehydrogenase (tr|A0A1S3XUI8). However, peroxidase (tr|A0A1S4BCB4, tr|A0A1S4AH58) and two kinds of glutathione S-transferase (tr|A0A1S4CJX9, tr|A0A1S4CLX7) were significantly down-regulated (Fig. 6).

Discussion

External ecological factors would affect plant gene expression in plant growth and development (Lobo *et al.*, 2014). Top ten enrichment of proteins occurred mainly including cytosol, plastid, chloroplast, proteasome, regulatory particle, proteasome accessory complex, chloroplast stroma, cytosolic part, cell-cell junction, plasmodesma, and cell junction (Table 2). Therefore, it was likely that pre-planting technique treatment strongly affected the related protein of cytosol, chloroplasts and proteasome responded to stress.

Table 1: Effects of two cultivation systems on tobacco plant fresh and dry weight, leaf length, plant height, root vitality, nitrate reductase and proline contents

Cultivation systems	Plant fresh weight (g)	Plant dry weight (g)	Leaf length (cm)	Leaf width (cm)	Plant height (cm)	Root vitality (mg g^{-1} h^{-1})	Nitrate reductase (μg g^{-1} h^{-1})	Proline content (μg g^{-1} FW)
Floating seedling	5.04±0.31b	0.41±0.03b	17.05±1.16b	6.80±0.42b	5.07±0.81b	1.74^{NS}	18.40^{NS}	69.14b
Pre-planting technique	10.34±1.27a	0.91±0.03a	18.61±1.06a	8.73±0.81a	9.47±2.01a	1.99	20.72	82.09a

Means following same letter within each column did not significantly differ at *P* 0.05
Values (± SD) represent the means of three replicates of each treatment; NS = Non-significant

Table 2: Gene ontology analysis of top ten accumulated proteins in the roots of pre-planting technique in cluster frequency, protein frequency of use and *p*-value

Gene Ontology term	Cluster frequency	Protein frequency of use	*P*-value[a]
cytosol	180 out of 331 genes, 54.4%	1924 out of 4685 genes, 41.1%	2.798843e-07
plastid	154 out of 331 genes, 46.5%	1656 out of 4685 genes, 35.3%	9.182625e-06
chloroplast	139 out of 331 genes, 42.0%	1479 out of 4685 genes, 31.6%	2.197422e-05
proteasome regulatory particle	9 out of 331 genes, 2.7%	29 out of 4685 genes, 0.6%	0.0001110509
proteasome accessory complex	9 out of 331 genes, 2.7%	29 out of 4685 genes, 0.6%	0.0001110509
chloroplast stroma	50 out of 331 genes, 15.1%	428 out of 4685 genes, 9.1%	0.0001811142
cytosolic part	36 out of 331 genes, 10.9%	276 out of 4685 genes, 5.9%	0.0001883814
cell-cell junction	77 out of 331 genes, 23.3%	746 out of 4685 genes, 15.9%	0.0001943199
plasmodesma	77 out of 331 genes, 23.3%	746 out of 4685 genes, 15.9%	0.0001943199
cell junction	77 out of 331 genes, 23.3%	746 out of 4685 genes, 15.9%	0.0001943199

[a]Hypergeometric's t-test *P*-value ($P < 0.05$)

Recent experiments have shown that many proteins or processes regulated by plant were involved in drought responses (Rampino *et al.*, 2012). Heat shock proteins (HSP) played important roles in many cellular processes in plant which was subjected to drought stress (Kregel, 2002; Swindell *et al.*, 2007). Glycerol kinase (GK) and tyrosine-protein kinase (TK) were all up-regulated at the protein level under drought stress to adapt to adverse environmental conditions (Chitteti and Peng, 2007). Signals were transmitted to the cells through the receptors when cells were stimulated by the external environment, and the signal was further amplified and transmitted by phosphorylation and dephosphorylation of TK and STK (Qu *et al.*, 2004). Noticeably, HSPs tr|A0A1S3ZAW0), GK (tr|A0A1S3ZDZ2), TK (tr|A0A1S4CHM8), and STK (tr|A0A1S4CBD4) detected in this study were up-regulated (Table 3). Our proteomic data further indicated that modulation of HSP, GK, TK and STK were essential part of physiological regulation in response to living environment changes.

In addition, many peroxisome proteins with antioxidant activity have been linked to cell structure, protein translation, protein biosynthesis, and plant development (Yao *et al.*, 2006). Overexpression of peroxidase protein could enhance antioxidant capacity in maize (Yu *et al.*, 2015). Glutathione metabolism was a major component of the enzymatic reaction oxygen scavenging system. Studies showed that low levels of H_2O_2 in the early stage of drought stress could improve the activity of glutathione metabolism and peroxisome (Hatzios, 2001). Pre-planting technique treatment roots up-regulated isocitrate dehydrogenase (tr|A0A1S3XUI8, tr|A0A1S3XUI8), peroxiredoxin (tr|A0A1S4BCB4, tr|A0A1S4DK72), 6-phosphogluconate dehydrogenase (tr|A0A139ZSG9, tr|A0A1S3ZRQ6), glutathione reductase (tr|A0A1S4CS55), leucine aminopeptidase (tr|A0A1S4AV83), and glutathione S-transferase all of which significantly to enhance the plant environmental tolerance and survival probability. Drought, salt, low temperature and other stress treatment significantly induced the expression of ZmGST23 gene in the corn with the expression level was high in shoots and mature leaves (Li *et al.*, 2016). Our study found that GST (tr|A0A1S4A868, tr|A0A1S4AR76) and cinnamoyl-CoA reductase (tr|A0A1S4BXI5) were up-regulated, indicating that they had roles in response of tobacco roots to environmental changes. In brief, we postulated that pre-planting technique might be associated with cooperation of the above proteins for inducting basic defense responses in tobacco.

Conclusion

Pre-planting technique promoted tobacco root growth due to differentially expressed functional proteins involved in signal transmission, plant metabolism, stress tolerance and defense system. Integration of several signaling pathways/mechanisms was indicated by GO analysis, including glutathione metabolism, phenylptropanoid pathway, defense-related proteins accumulation, lignin biosynthesis and scavenging of reactive oxygen. Taken together, these results provide new insight into tobacco breeding and further proteomic research.

Acknowledgements

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Table 3: Identification of differentially accumulated proteins in the roots of pre-planting technique

Accession[a]	Protein name[b]	T2:T1[c]	*P*-value[d]
tr|A0A1S3XQB7	anionic peroxidase	2.8385	0.0265
tr|A0A1S4C1T9	anionic peroxidase	7.9602	0.0004
tr|A0A1S3X9W2	anionic peroxidase	7.9610	0.0139
tr|A0A1S3ZLW1	aspartate aminotransferase	6.4594	0.0108
tr|A0A1S4CNP0	aspartate aminotransferase	5.3373	0.0345
tr|A0A1S3ZAW0	heat shock protein 90-5	3.1910	0.0183
tr|A0A1S3ZDZ2	glycerol kinase-like	2.2844	0.0260
tr|A0A1S4CHM8	PTI1-like tyrosine-protein kinase 3	5.2194	0.0203
tr|A0A1S4CBD4	serine/threonine-protein kinase	24.0339	0.0006
tr|A0A1S4C9Q4	LRR protein 4-like	6.7806	0.0007
tr|B2YKT9	RNA-binding protein	1.0960	0.2304
tr|A0A1S3XUI8	isocitrate dehydrogenase	10.2980	0.0000
tr|A0A1S3XUI8	isocitrate dehydrogenase	10.2980	0.0000
tr|A0A139ZSG9	6-phosphogluconate dehydrogenase	5.7058	0.0161
tr|A0A1S3ZRQ6	6-phosphogluconate dehydrogenase	7.4379	0.0029
tr|A0A1S4A868	glutathione S-transferase	6.9691	0.0000
tr|A0A1S3YKT8	glutathione reductase	5.7068	0.0394
tr|A0A1S4AR76	glutathione S-transferase	10.5153	0.0001
tr|A0A1S4CJX9	glutathione S-transferase T1-like	7.6352	0.0193
tr|A0A1S4CQJ4	glutathione S-transferase U9-like	4.5893	0.0090
tr|A0A1S4CLX7	probable glutathione S-transferase	12.0663	0.0001
tr|A0A1S4BZT5	glutathione S-transferase GST 23-like	7.3683	0.0231
tr|A0A1S3YM66	probable glutathione S-transferase	6.6309	0.0250
tr|A0A1S4BQE0	probable glutathione S-transferase	2.0344	0.0335
tr|A0A1S4C8H1	probable glutathione S-transferase	0.5666	0.0357
tr|A0A1S4CS55	glutathione transferase	1.5253	0.0225
tr|A0A1S4BCB4	peroxiredoxin-2E-2,	6.9912	0.0112
tr|A0A1S4DK72	peroxiredoxin-2B-like	7.9458	0.0221
tr|A0A1S4B2V1	peroxidase N1-like	6.2422	0.0054
tr|A0A1S3WZE1	peroxidase 51-like	3.9829	0.0249
tr|A0A1S4ABQ5	peroxidase 64-like	2.1773	0.0149
tr|A0A1S4DNQ6	peroxidase 27-like	2.1161	0.0338
tr|A0A1S4AH58	peroxidase 44-like	0.3150	0.0129
tr|A0A1S4AV83	leucine aminopeptidase 2	10.9132	0.0137
tr|A0A1S4BXI5	cinnamoyl-CoA reductase	13.0596	0.0001
tr|O82151	Beta-D-glucan exohydrolase	3.2391	0.0402
tr|A0A1S4CX32	histidinol-phosphate aminotransferase	2.4923	0.0053

[a]Accession number in Uniprot database; [b]Name of the protein identified by MS/MS; [c]The ratio between protein levels in pre-planting technique and floating system plants; [d]hypergeometric's t-test *p*-value ($P < 0.05$)

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