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EFFECTS OF INTERCROPPING WITH *PERILLA FRUTESCENS* (L.) BRITT. ON FLUE-CURED TOBACCO PROTEOME

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Abstract

Tobacco and Perilla were used to study the effects of aromatic plants intercropping on the protein expression of tobacco leaves. The differences in leaves total protein between monocropped and intercropped tobacco with two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) techniques were explored. The results demonstrated that the expressions of antibiotic stress-related proteins such as chitinase, β-1,3-glucanase, oxygen-evolving enhancer protein and iron superoxide dismutase decreased, whereas the expressions of abiotic stress related protein such as ribose-1,5-bisphosphate carboxylase/ oxygenase activase, and the proteins involved in glycolysis such as fructose 1,6-bisphosphate aldolase all enhanced. These results indicated that the intercropping method favored the tobacco resistance to abiotic stress but did not favor the resistance to antibiotic stress.

Introduction

Tobacco is one of the main economic crops in Yunnan Province, and its primary cultivation method is monocropping. However, long-term using of this practice results in soil degradation, reduced quality of tobacco leaves, as well as serious pests and diseases. In addition, the excessive chemical fertilizers and pesticide applications cause environment pollution. Intercropping can improve the effective use of environmental resources such as light, heat, water and fertilizer, reduce the application of fertilizers, enhance crop yield and quality, and reduce the incidence of pests and diseases. The intercropping with aromatic plants cannot only integrate natural aroma to tobacco, but also effectively control the incidence of diseases. Studies have shown that the intercropping with garlic worked significantly against tobacco black shank in tobacco (Xue *et al.* 2015). The intercropping with peanut significantly reduced the incidence of tobacco bacterial wilt in tobacco (Shi *et al.* 2011). Tobacco intercropped with aromatic plants such as lemongrass, sweet basil, mint and sweet-scented geranium may enhance the agronomic traits and the quality of flue-cured tobacco (Peng *et al.* 2014). It was found that the aromatic ingredients of the intercropped plants such as lavender, rosemary, sweet-scented geranium, catnip and rose, in the tobacco leaves improved their quality. After intercropping with rose, the expressions of resistance-related proteins increased but the expressions of photosynthesis-related proteins decreased in tobacco (Yue *et al.* 2015). In this study, two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS) was used to compare the proteins of leaves of the monocropped tobacco and the tobacco intercropped with perilla. The purpose of this study was to explore whether the perilla had an effect on tobacco disease resistance and growth.

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Materials and Methods

The aromatic plant to be tested was *Perilla frutescens*, and the tobacco variety was Honghuadajinyuan (HD). The test time was from May to August, 2017. The test site was at the base of the YunYan Impression Manor, Shilin County, Yunnan Province, a subtropical low-latitude, mountain and high plateau, monsoon climate zone. The tobacco was intercropped with perilla, with 12 rows of tobacco and four rows of perilla. There were three replicates for each treatment. The row spacing was 1.2 m and the plant spacing was 50 cm. The monocropped flue-cured tobacco was used as a control. The distance was 100.0 m between the intercropped and monocropped plots. The tobacco and perilla plants were all transplanted in May, 2017. On June 25, 2017, the top 4th leaf tips of the tobacco plants at the rosette stage from each treatment were collected by the 5-point sampling method. The five tips collected for each treatment were mixed together as one representative sample and stored at a –80°C freezer.−

The leaf total protein was extracted by the TCA/acetone precipitation method (Yu *et al.* 2015). In brief, a weight of 1.0 g leaf sample from each treatment (monocropping and intercropping, respectively) was ground in liquid nitrogen and then suspended in 20 ml 10% TCA/acetone [containing 0.1% (w/v) DTT and 1% (w/v) PMSF]. After precipitated overnight at –20°C, it was centrifuged at 12,000 rpm, in 4°C, for 40 min. The pellet was resuspended in 20 ml 80% cold acetone [containing 0.1% (w/v) DTT and 1% (w/v) PMSF] and allowed to stand still at –20°C for 2 hrs, centrifuged at 12,000 rpm, in 4°C for 40 min. The last two steps were repeated three times, and then the pellet was air-dried at room temperature and dissolved in 1 ml rehydration solution (7 mol/l urea, 2 mol/l thiourea, 4% CHAPS, 1% DTT and 0.5% biolyte), standing still at room temperature for 1 hr, and centrifuged at 12,000 rpm, in 20°C for 15 min. Then the pellet was added with four volumes of –20°C pre-cooled acetone, standing still at –20°C for 2 hrs and centrifuged at 12,000 r/min, 4°C for 40 min. The precipitated samples were dissolved in the hydration solution and subjected to protein concentration determination using the method of Bradford (1976).

The 24 cm, pH 4-7 prefabricated IPG strips (GE Healthcare) were used for isoelectric focusing. An amount of 600 µg, 500 µl protein was loaded and hydrated at room temperature for 14 hrs. The isoelectric focusing (IEF) parameter setting was as the method of Yu *et al.* (2015). After finishing the IEF, each strip was placed in an equilibrium solution containing 1% DTT and 2.5% iodoacetamide for 15 min and then transferred to 12.5% for vertical gel electrophoresis (SDS-PAGE). After the completion of electrophoresis, the gel was removed and stained with Coomassie brilliant blue G-250 (Yu *et al.* 2015).

The stained gel was scanned with a GE Image scanner with a resolution of 300 dpi. Further it was analyzed with the Image Master 2D Platinum 7.0 software with parameters as Smooth, 2; Min Area, 5; and Saliency, 100.

The selected differential protein spots were cut out, followed by decolorization, enzymatic hydrolysis, extraction and desalting. Then they were sent to the Shanghai Zhongke Xinshengming Biotechnology Co., Ltd. Xuhui for MALDI-TOF/TOF-MS analysis. The spectrometry data were sent to the National Center for Biotechnology Information (NCBI) non-redundant database for protein retrieval using the Mascot software (http://www.matrix.com) from the species of Nicotiana.

The protein sequences that met the identification criteria for MS were submitted to protein subcellular localization prediction using the Plant-mPLoc server (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). The gene ontology tool was used for functional classification (http://amigo.geneontology.org/cgi-bin/amigo/blast.cgi). The KEGG was used for metabolic pathway analysis (http://www.kegg.jp). The STRING database was used for protein-protein interaction network analysis (http://www.string-db.org).

Results and Discussion

The pH 4 - 7, 24 cm IPG strips were used for 2-DE for the total protein of tobacco leaves. The MapMaster 5.0 software was used to analyze the protein profiles. By profile comparison, it was found that about 400 reproducible and clear protein spots could be detected on each gel. In detail, a total of 412 proteins were detected for the control sample of monocropped tobacco, and a total of 462 protein were found for the sample of tobacco intercropped with perilla. The comparison of tobacco leaf proteins between monocropping and intercropping revealed 20 distinct protein spots with significantly different expression abundance (Fig. 1). A number of 19 of the 21 differentially expressed proteins (DEPs) in the intercropped sample were successfully identified, with 11 down-regulated and 8 up-regulated (Table 1).

Table 1. DEPs between monocropped tobacco and tobacco intercropped with perilla identified by MALDI-TOF/TOF MS.

Spot	Locus No.	Protein	Mr/pI	Pep. count	Scores	Change
1	prf||0905192A	Carboxylase	10. 17/5. 30	8	91	-
2	AIF75367. 1	Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	17. 39/7. 85	11	91	-
3	XP_016446027. 1	Endochitinase P	27. 51/4. 89	10	139	-
4	AAA63542. 1	β-1,3-glucanase	37. 70/5. 12	9	137	-
5	XP_009588754. 1	ATP synthase δ chain	26. 81/8. 96	14	343	-
6	XP_009790936. 1	Chlorophyll a-b binding protein 40	28. 27/5. 48	9	207	+
7	YP_398869. 1	ATP synthase CF1εsubunit	14. 57/4. 99	5	114	-
8	NP_001311962. 1	oxygen-evolving enhancer protein 2-1	28. 56/7. 66	10	221	-
9	XP_016437252. 1	Glucan endo-1,3-β-glucosidase	37. 44/5. 30	11	121	-
10	NP_001312578. 1	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase 2	48. 31/8. 14	26	398	+
11	XP_016477286. 1	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase 2	48. 28/7. 57	25	422	+
12	XP_009767240. 1	Photosystem II stability/assembly factor HCF136	43. 08/6. 86	16	341	-
13	XP_009631311. 1	Fructose 1,6-bisphosphate aldolase 1	42. 81/6. 38	15	205	+
14	XP_016480950. 1	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase 1	48. 63/8. 43	20	357	+
15	OIT33056. 1	Chlorophyll a-b binding protein 6a	26. 24/6. 43	7	187	-
16	CAA00826. 1	Iron superoxide dismutase	25. 43/5. 39	10	222	-
17	XP_009613926. 1	Fructose 1,6-bisphosphate aldolase 1	42. 57/7. 59	20	230	+
18	ACF60500. 1	Plastid transketolase	80. 05/6. 16	26	129	+
19	XP_009619213. 1	41 kDa chloroplast stem-loop binding protein b	41. 68/7. 18	20	248	+

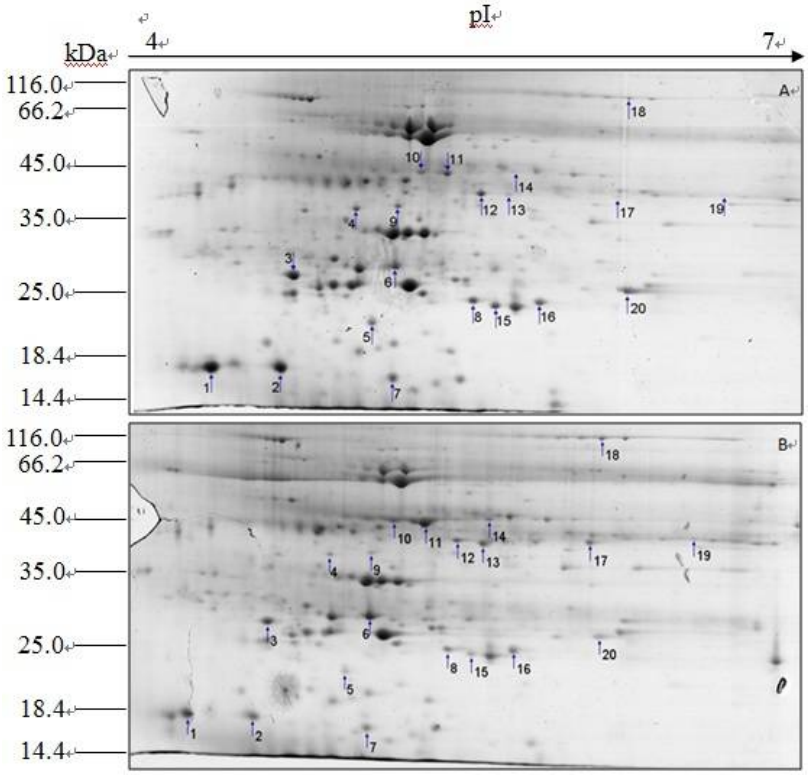

Fig. 1. The 2-DE profiles of total protein of tobacco leaves. A. 2-DE profile of monocropped tobacco; B. 2-DE profile of tobacco intercropped with perilla.

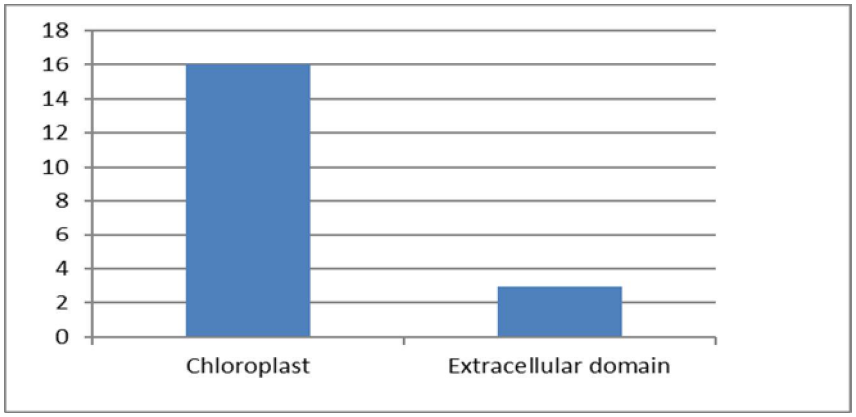

Fig. 2. Subcellular localization of DEPs. (Chloroplast, extracellular domain).

As shown in Fig. 2, 19 proteins have two localization sites, wherein 84% of the proteins are localized in chloroplasts, and the remaining three proteins, protein Nos. 3, 4 and 9 are localized in the extracellular domain.

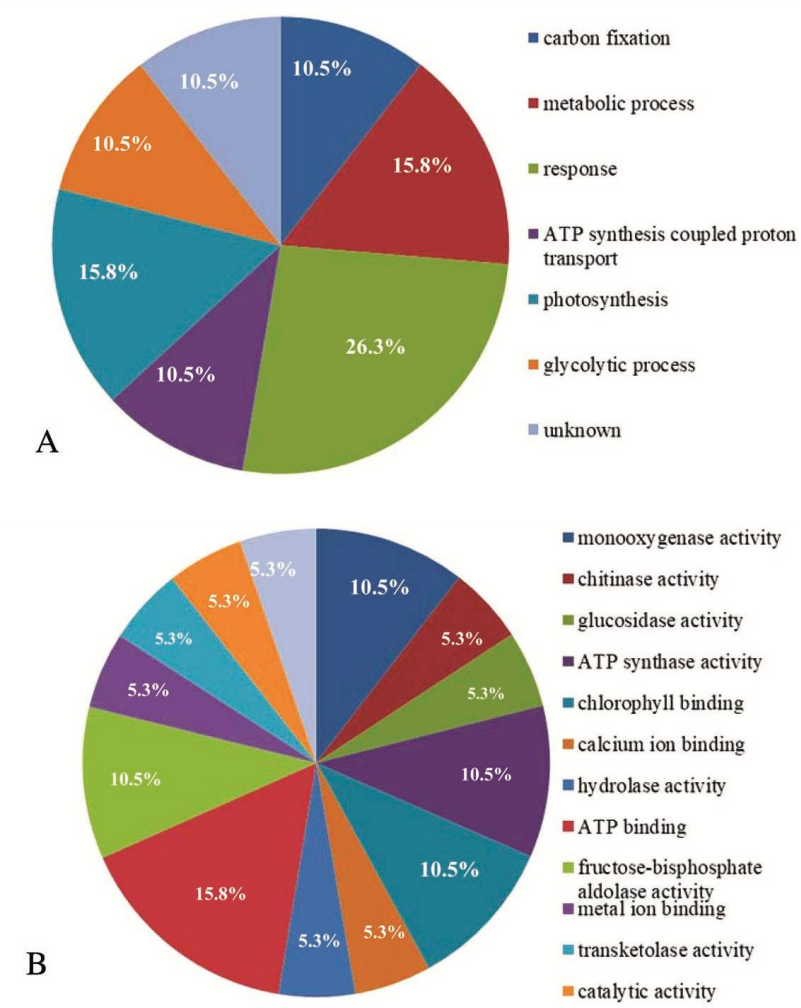

Fig. 3. The GO annotations of DEPs. A. Biological process, B. Molecular function.

To clarify the functions of DEPs, the GO annotations were applied to the 19 identified DEPs to classify their related biological processes and molecular functions (Fig. 3). These 19 proteins are mainly involved in the stress response in biological process, accounting for 26.3% of total protein, followed by metabolic process and photosynthesis, accounting for 15.8% each. Regarding the molecular function, ATP binding accounts for 15.8%, and the rest is distributed relatively evenly, mainly about binding and enzymatic activity.

To understand the metabolic pathways that the DEPs were involved in, we used the KEGG database to analyze the 19 DEPs to determine their primarily related biochemical metabolic pathways. The KEGG analysis localizes these 19 DEPs to 11 metabolic pathways, primarily the carbon metabolism (protein Nos. 1, 13 and 18) and carbon fixation in photosynthetic organisms (protein Nos. 1, 13 and 18) (Fig. 4).

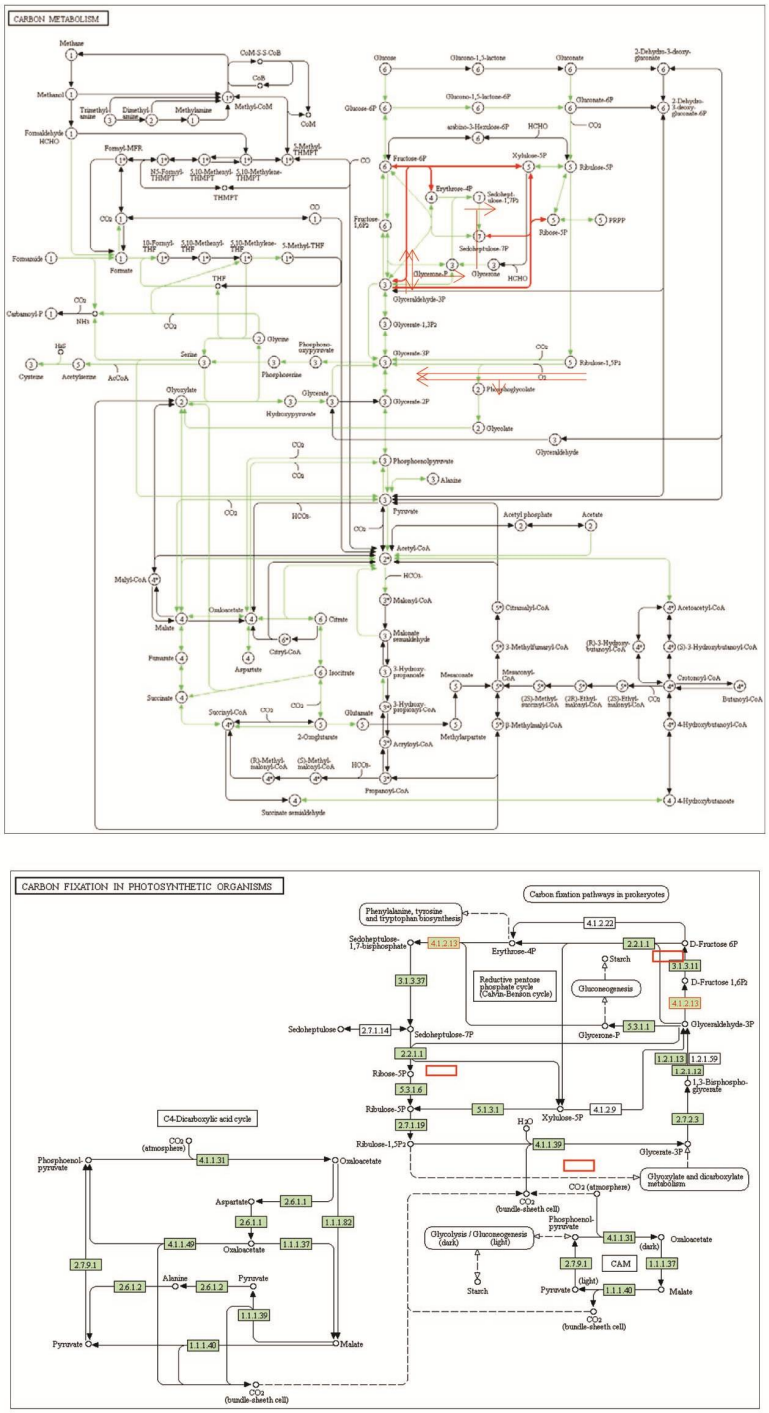

Fig. 4. Metabolic pathways in which DEPs were mainly involved in.

A lot of studies have been conducted on the effects of aromatic plants and intercropping on the prevention and control of pests and diseases, and they are valuable for agricultural production and important for ecology（Liang *et al.* 2015.

β-glucosidase is a key enzyme for the hydrolysis of glycosidic to release aroma substances. Studies have shown that the enzyme enhanced the aroma of tea by transforming the glycoside precursors of aroma in tea leaves to aroma substances. In addition, the enzyme has important biological functions in protecting against pests and regulating plant growth and development. It has been reported that the heterologously expressed β-glucosidase genes in tobacco could reduce the tobacco mosaic virus (TMV) infection by increasing the level of salicylic acid (Yao *et al.* 2007). In this study, the expression of β-glucosidase was actually reduced in the leaves of tobacco intercropped with perilla compared to the monocropped tobacco.

Chitinase and β-1,3-glucanase are two important pathogenesis-related proteins that dissolve fungal cell walls by hydrolysis, inhibit fungal growth, and boost plant resistance to pathogens. Interestingly, the expression levels of these two enzymes in this study were lower than those of the monocropped tobacco. In addition, the expressions of resistance-related proteins such as oxygen-evolving enhancer protein and iron superoxide dismutase trended to decrease in the intercropped tobacco. In contrast, the expressions of ribulose-1,5-bisphosphate carboxylase/ oxygenase activase (RCA), fructose 1,6-bisphosphate aldolase and plastid transketolase were all enhanced in the tobacco intercropped with perilla. RCA is a key enzyme regulating the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase, and plays an essential role in the resistance of plants against various abiotic stress such as temperature (Jurczyk *et al.* 2016), salinity (Zhang *et al.* 2012), drought (Rollins *et al.* 2013) and heavy metals (Son *et al.* 2014). Fructose 1,6-bisphosphate aldolase is a key enzyme of glycolysis. The elevated and reduced expressions of these enzymes indicated that the aromatic plants had little effects on the antibiotic stress resistance of tobacco and may even benefit the susceptibility of tobacco. Nevertheless, the improved expressions of glycolytic enzymes and RCA favored the abiotic stress resistance of tobacco.

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