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EFFECTS OF INTERCROPPING OF FLUE-CURED TOBACCO WITH AMERICAN PEPPERMINT ON PROTEOMICS OF FLUE-CURED TOBACCO

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Abstract

Following intercropping the flue-cured tobacco plant with American peppermint the changes in tobacco leaf proteins were investigated. Further, the effect of intercropping tobacco with peppermint on the tobacco leaf proteome was preliminarily explored. The results showed that the expression of anti-biotic stress-related proteins such as chitinase and β-1,3-glucanase decreased in the intercropped tobacco leaves. While the expressions of anti-abiotic stress-related and glycolytic enzymes such as ribulose-1,5-bisphosphate carboxylase/oxygenaseactivase, fructose-bisphosphatealdolase, and phosphoglycerate kinase increased. The results demonstrated that the intercropping of flue-cured tobacco with American peppermint was beneficial to the resistance of tobacco against abiotic stress, but not biotic stress.

Introduction

Tobacco (*Nicotiana tabacum* L.,) is one of the major cash crops in Yunnan Province, China. Cultivation of the plant as monoculture is the primary method of tobacco production in the area. However, the effect of long-term monoculture of *N. tabacum* results in degradation of soil quality, increases the attack of serious diseases and insect pests to the crop and as a result the quality of tobacco leaf gets reduced. In addition, the excessive use of chemical fertilizers and pesticides in the cultivated fields of tobacco causes serious environmental contaminations. Intercropping can make use of environmental resources in a more rational way. Under such condition light, heat, water and fertilizer can be used more effectively. This would decrease the amount of fertilizers, could reduce the rate of infestation by pests and diseases thereby ensuring a high yield of quality tobacco crops. Aromatic plants not only synthesize raw materials for aroma but when intercropped with tobacco crops, can manage the infestation of tobacco diseases. Study has shown that intercropping of flue-cured tobacco with garlic controls black shank disease of tobacco significantly in a better way (Xue*et al*. 2015). Furthermore, it has been seen that intercropping with peanuts reduces the incidence of tobacco bacterial wilt (Shi*et al*. 2011). On the otherhand, intercropping of tobacco with various aromatic plants such as lemon grass, sweet basil, peppermint and geranium could improve the agronomic traits and quality of flue-cured tobacco (Peng*et al*. 2014). Li *et al*. (2014) reported that intercropping tobacco with herbal plants such as lavender, rosemary, geranium, catmint and rose could enrich the tobacco leaves with special aromatic flavors, improving their quality. Intercropping tobacco with rose was found to enhance the expression of resistance-related proteins, while decreasing the expression of photosynthesis-related proteins ina tobacco plant (Yu *et al*. 2015). In the present study, two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) techniques were used to compare the leaf proteomes between monocropped and peppermint (*Mentha* × *piperita* L., Lamiaceae) intercropped tobacco plants to investigate the effects of aromatic plants on the resistance and growth of tobacco plants.

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Materials and Methods

The tested aromatic plant was American peppermint, and the tobacco variety used was Honghua Dajinyuan (HHDJY). The work was carried out from May to August, 2017. The field work was done in the Yunyanyinxiang Base, Shilin County, Yunnan Province. The area has a warm temperature and subhumidcongtinenyal monsoon climate zone. The tobacco was intercropped with American peppermint, with 12 rows of tobacco plants and four rows of American peppermint plants with three replicates for each treatment. The row spacing and plant spacing were 1.2 m and 50 cm, respectively. The monocropped flue-cured tobacco plants were treated as controls. Both the tobacco plants and the American peppermint plants were transplanted in May, 2017. On June 25, 2017, during the rosette stage of the tobacco plants, the tips of the top 4th tobacco leaf of each treatment and the control were collected using the five-point sampling method. The five tips of each treatment were mixed together and treated as a composite single sample for the study. The sample was stored at –80°C for further laboratory analyses.

The TCA/acetone precipitation method was used to extract the leaf total protein (Yu *et al*. 2015). In brief, an amount of 1.0 g of monocropped and intercropped tobacco leaves were weighed, ground in liquid nitrogen and suspended in 20 ml of 10% TCA/acetone [containing 0.1% (w/v) DTT and 1% (w/v) PMSF], respectively. After overnight precipitationat −20°C, the samples were centrifuged at 12,000 rpm, 4°C for 40 min. Each pellet was suspended in 20 ml of 80% cold acetone [containing 0.1% (w/v) DTT and 1% (w/v) PMSF] and allowed to stand for 2 hrs at −20°C. Further, the samples were centrifuged at 12,000 rpm, 4°C for 40 min. The above process was repeated three times, and then the samples were air-dried at room temperature. Each precipitate was dissolved in 1 ml of hydration solution (7 urea, 2 mol/lthiourea, 4% CHAPS, 1% DTT, and 0.5% biolyte) and allowed tostand at room temperature for 1 hr. Next the samples were centrifuged at 12,000 rpm, 20°C, for 15 min. Four volumes of pre-cooled acetone were added to each sample and kept at −20°C for 2 hrs. Then the samples were centrifuged at 12,000 rpm, 4°C for 40 min. The precipitated samples were dissolved in the hydration solution and subjected to protein concentration determination using the method ofBradford (1976).

The precast IPG strips (24 cm, pH 4-7) from GE Healthcare were used for 2-DE. The protein samples were kept at room temperature for 14 hrs for full denaturation and solubilization before loaded with an amount of 600 μg and 500 μl. The isoelectric focusing (IEF) parameters were set up following the protocol of Yu *et al*. (2015). After the IEF completed, the strips were incubated first in the equilibration buffer with 1% DTT and then in the equilibration buffer with 2.5% iodoacetamide for 15 min, respectively. Next, they were transferred to 12.5% separation gels for vertical plate electrophoresis. After electrophoresis was complete, the gels were removed and stained with Coomassie Brilliant Blue G-250 (Yu *et al*. 2015).

The stained gels were scanned using a GE Image Scanner with a resolution of 300 dpi. The Image Master 2D Platinum 7.0 software was used for analysis with parameters setting as follows: Smooth, 2; Min area, 5; Saliency, 100.

The selected differentially expressed protein (DEP) spots were excised, decolored, and hydrolyzedenzymically. The products of hydrolysis were extracted and desalted and sent to the Applied Protein Technology Co., Ltd. for the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-TOF/MS) analysis. The obtained mass spectral peaks were searched against NCBI (National Center for Biotechnology Information) non-redundant protein database using Mascot (http://www.matrix.com).

The subcellular localization prediction was performed for those protein sequences that meet the MS identification requirements with Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/). The functional classification was performed with Gene Ontology (GO,

http://amigo.genontology.org/cgi-bin/amigo/blast.cgi). The metabolic pathway analysis was performed with KEGG (http://www.kegg.jp/). The protein-protein interaction networks analysis is performed in the STRING database (http://www.string-db.org/).

Results and Discussion

The 2-DE of the tobacco leaf total protein was performed using 24 cm IPG strips with a pH range 4-7. The Image Master 5.0 was used for profile analysis, exhibiting about 400 reproducible clear protein spots on each profile. In details, there were 412 proteins detected in the profile of monocultured tobacco (control), and 461 proteins detected in that of the intercropped tobacco. The comparison between them revealed 25 distinct protein spots with significant differences in abundance (Fig. 1). Among them, 21 were successfully identified, of which 8 were down regulated and 13 were up regulated in the intercropped tobacco leaves (Table 1).

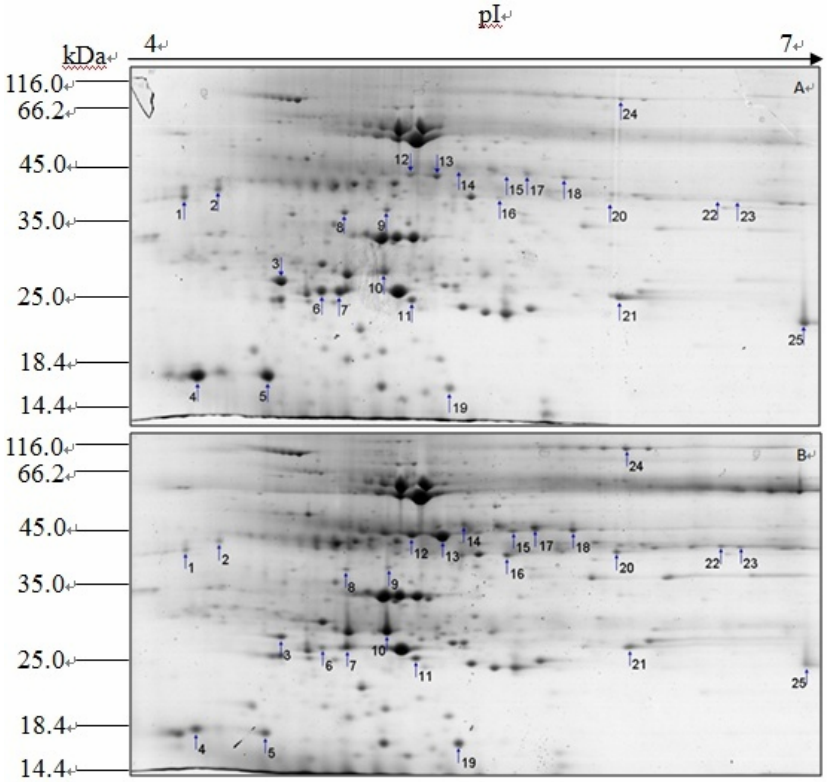


Fig. 1.Showing the 2-DE profiles of total proteins of tobacco leaves. A. Monocultured tobacco, B. Tobacco intercropped with peppermint.

As shown in Fig. 2, 21 proteins were distributed in three parts of the cell. A number of 17 proteins (~81%) was from the chloroplast. For the remaining four proteins, Protein 3, 8 and 9 were located in the extracellular region, and protein 25 was localized to the cell wall.

To clarify the functions of the DEPs, the 21 DEPs were classified depending on their biological process and molecular function by using the GO functional classification system (Fig. 3). Proteins of the biological process are primarily involved in the glycolytic process and response,

each accounting for 19%, followed by metabolic process and photosynthesis, with a percentage of 14.3% each. However, for the molecular function, ATP binding was the dominant one, with a percentage of 23.8%, and the rest were distributed more or less evenly and acted mostly for binding and enzyme activity.

Table 1. DEP spots between monocultured tobacco and tobaccointercropped with peppermint identified by MALDI-TOF/TOF MS.

Spot	Locus No.	Protein	Mr/pI	Pep.count	Scores	Change
3	XP_016446027.1	Endochitinase P	27.51/4.89	10	139	-
4	prf||0905192A	Carboxylase	10.17/5.30	8	91	-
5	AIF75367.1	Cchloroplastribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small unit	17.39/7.85	11	91	-
7	XP_009792415.1	Chlorophyll a-b binding protein 37	28.66/5.70	8	166	-
8	AAA63542.1	β-1,3-glucanase	37.70/5.12	9	137	-
9	XP_016437252.1	Glucan endo-1,3-β-glucosidase	37.44/5.30	11	121	-
10	XP_009790936.1	Chlorophyll a-b binding protein 40	28.27/5.48	9	207	+
11	CAA41712.1	Photosystem II 23 kDa polypeptide	27.87/7.03	12	350	-
12	NP_001312578.1	Rubiscoactivase 2	48.31/8.14	26	398	+
13	XP_016477286.1	Rubiscoactivase 2	48.28/7.57	25	422	+
14	XP_009773609.1	Glutamine synthetase	47.48/6.73	13	125	+
15	XP_016480950.1	Rubiscoactivase 1	48.63/8.43	20	357	+
16	XP_009631311.1	Fructose-bisphosphate aldolase1	42.81/6.38	15	205	+
17	XP_016476662.1	Phosphoglycerate kinase	49.99/8.48	19	242	+
18	XP_016494189.1	Phosphoglycerate kinase	50.54/8.66	25	418	+
19	YP_398869.1	ATP synthase CF1ε subunit	14.57/4.99	6	244	+
20	XP_009613926.1	Fructose-bisphosphate aldolase1	42.57/7.59	20	230	+
22	XP_009619213.1	41 kDa chloroplast stem-loop binding protein b	41.68/7.18	20	248	+
23	XP_016483564.1	41 kDa chloroplast stem-loop binding protein a	43.93/6.77	19	187	+
24	ACF60500.1	Plastid transketolase	80.05/6.16	26	129	+
25	OIT32766.1	Auxin binding protein abp19a	21.97/6.26	3	130	-

To understand their involvement in the metabolic pathways, the 21 DEPs were analyzed using the KEGG database to determine their primary relationships within the biochemical metabolic pathways. The KEGG analysis mapped 21 proteins into 15 metabolic pathways, but they are primarily associated with biosynthesis of amino acids (Protein 14, 16, 18 and 24), carbon metabolism (Protein 4, 16, 18 and 24), carbon fixation in photosynthetic organisms (protein 4, 16, 18, and 24), as shown in Fig. 4.

So far, severalresearcheshave been carried out in the prevention and control of pests and diseases through intercropping with aromatic plants. This process is valuable and ecologically significant for agricultural production.β-glucosidase is a key enzyme which hydrolyzes and releases glycosidic aroma substances. Studies have shown that β-glucosidase converted the

glycosidic aroma precursors in tea leaves to generate aroma substances increasing the aroma of tea leaves (Tu*et al*. 1999). In addition, this enzyme has important biological functions in pest defense and regulating plant growth and development. Yao *et al*. (2007) showed that the heterologous expressionof β-glucosidase gene in tobacco may reduce the infection of tobacco mosaic virus (TMV) by increasing the salicylic acid level. In the present study, it was found that the expression of β-glucosidase was reduced in intercropped tobacco leaves compared to the monocropped ones.

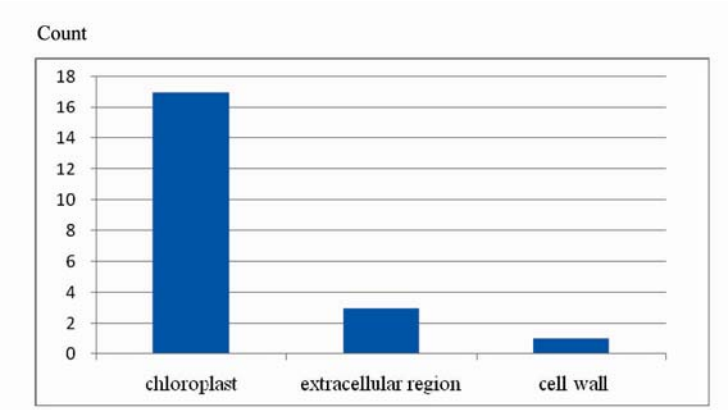


Fig. 2.Subcelluar locations of DEPs (chloroplast, extracellular region, cell wall).

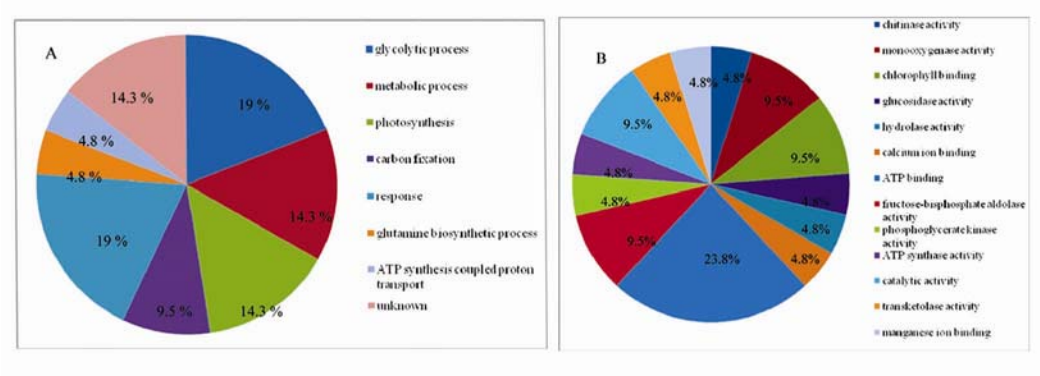
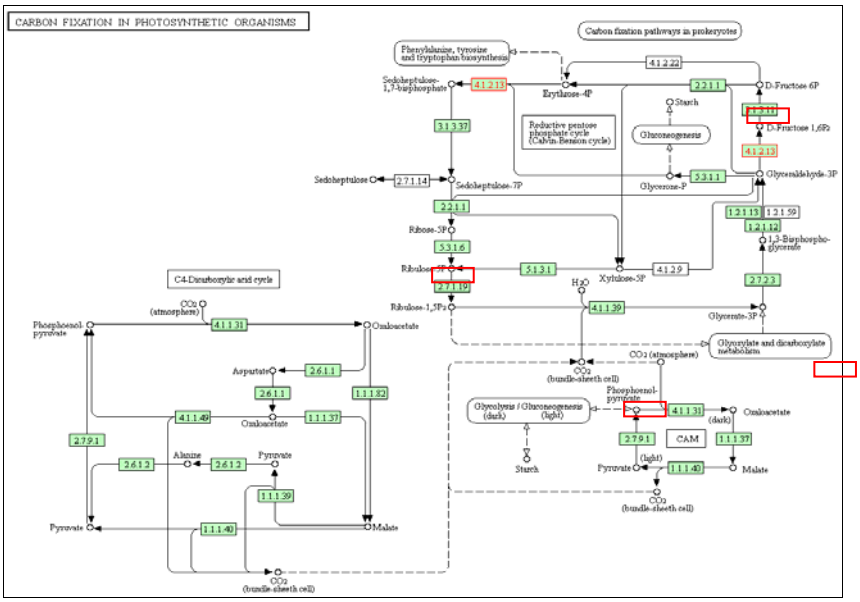
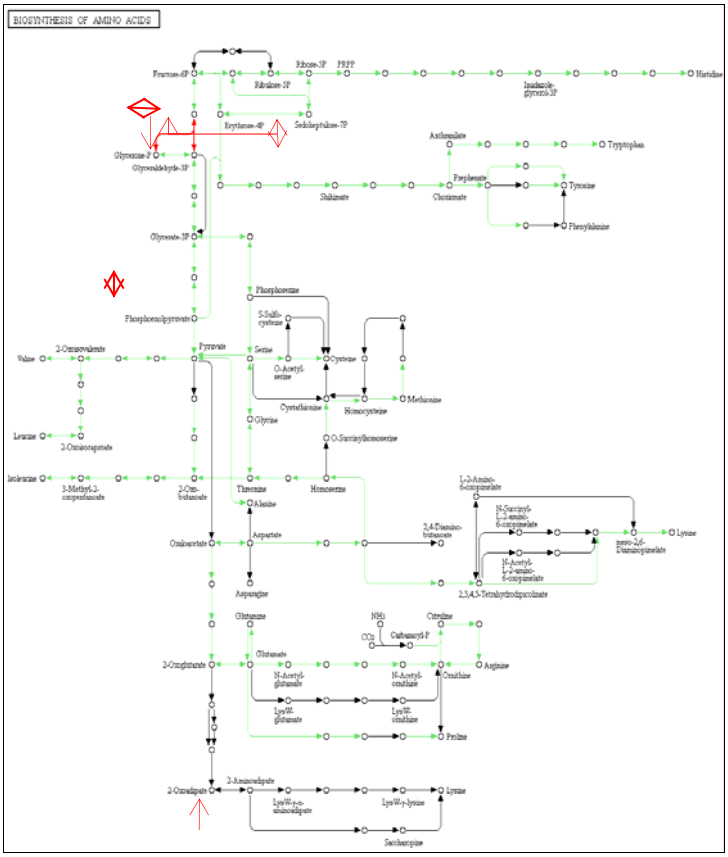


Fig. 3. GO annotations of DEPs.A. Biological process, B.Molecular function.

Chitinase and β-1,3-glucanase are two essential disease-related proteins. Through hydrolysis, they can dissolve fungal cell walls, inhibit fungal growth and increase plant resistance to pathogens. Interestingly, the expressions of the enzymes mentioned above in the intercropped tobacco leaves of the present study were lower than that of the monocultured ones. Nevertheless, the expressions of ribulose-1,5-bisphosphate carboxylase/oxygenaseactivase (RCA), fructose-bisphosphatealdolase, plastid transketolase, glutamine synthetase, and phosphoglycerate kinase were all increased in tobacco whenintercropped with peppermint. RCA is a key enzyme regulating the activity of rubisco and plays a pivotal role in the resistance of plants against various abiotic stress, such as temperature (Jurczyk*et al*. 2016), salt (Zhang *et al*. 2012), drought (Rollins *et al*. 2013) and heavy metals (Son *et al*. 2014). Fructose-diphosphatealdolaseand phosphoglycerate


CARBON FIXATION IN PHOTOSYNTHETIC ORGANISMS
Carbon fixation pathways in prokaryotes
Phenylalanine, tyrosine and tryptophan biosynthesis
Reductive pentose phosphate cycle (Calvin-Benson cycle)
Gluconeogenesis
Starch
Erythrose-4P
D-Fructose 6P
D-Fructose 1,6P2
Glyceraldehyde-3P
Glycerone-P
Sedoheptulose-7P
Ribose-5P
Ribulose-5P
Xylulose-5P
Ribulose-1,5P2
1,3-Bisphospho-glycerate
Glycerate-3P
C4-Dicarboxylic acid cycle
Phosphoenol-pyruvate
Oxaloacetate
Aspartate
Alanine
Pyruvate
Malate
Glycolysis / Gluconeogenesis
CAM
Glyoxylate and dicarboxylate metabolism


BIOSYNTHESIS OF AMINO ACIDS
Fructose-6P
Ribose-5P
PRPP
Erythrose-4P
Sedoheptulose-7P
Glyceraldehyde-3P
Shikimate
Chorismate
Tryptophan
Tyrosine
Phenylalanine
Glycerate-3P
Phosphoenolpyruvate
Pyruvate
Serine
Cysteine
Methionine
Homocysteine
Glycine
Threonine
Homoserine
Aspartate
Asparagine
Lysine
Glutamate
Glutamine
Arginine
Ornithine
Proline
Histidine

(Contd.)

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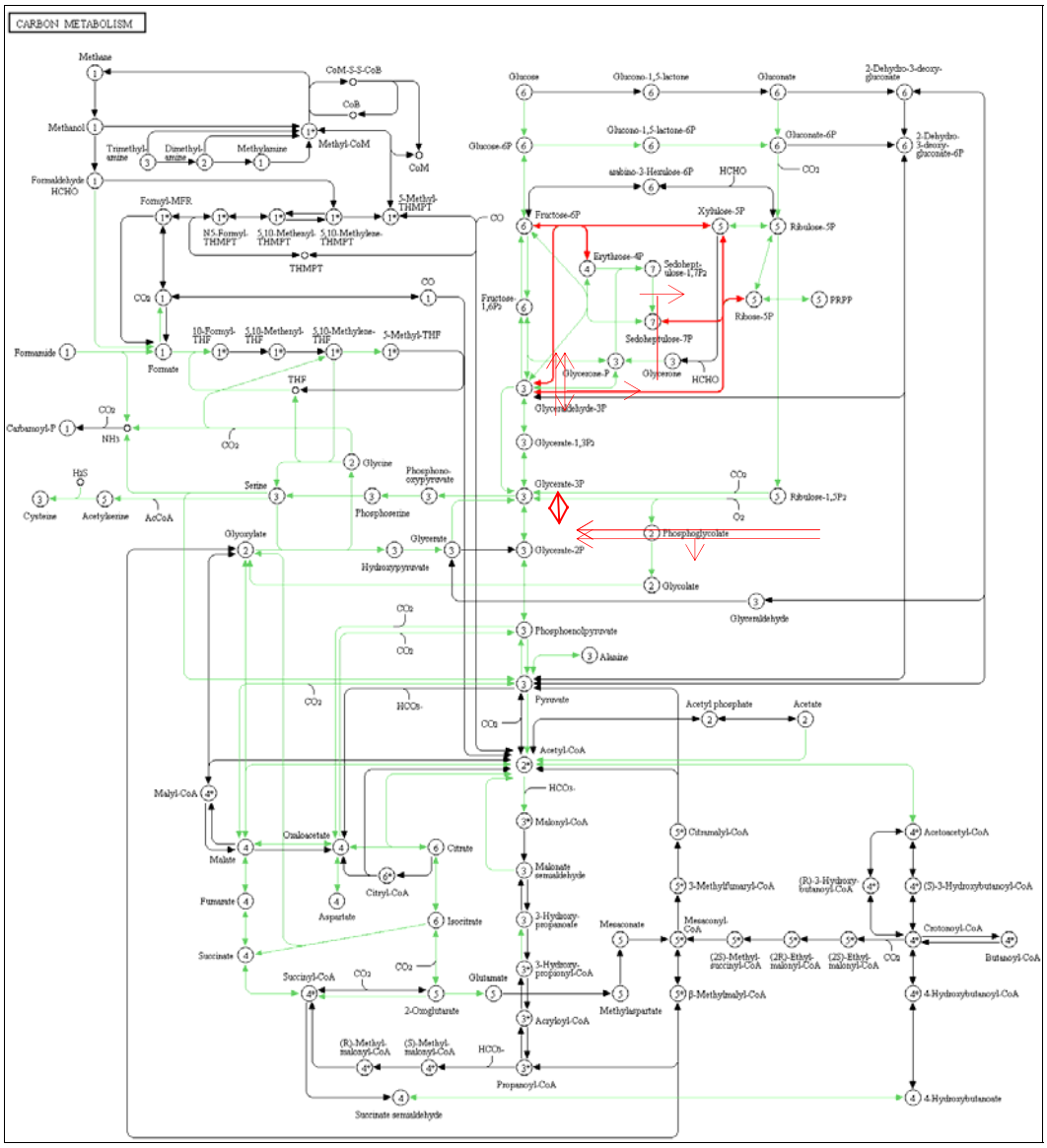

Fig. 4.Primary metabolic pathways of DEPs.

kinase are key enzymes for glycolysis. The increased or decreased expressions of various enzymes indicated minor effects of the aromatic plants on the resistance of tobacco against biotic stress and might even contribute to the disease-susceptibility of tobacco plants. Nevertheless, the upregulated expression of glycolytic enzymes and RCA is beneficial to the resistance of tobacco against abiotic stress.

Acknowledgments

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