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Analytical Methods

Differential protein profiles of postharvest *Gynura bicolor* D.C leaf treated by 1-methylcyclopropene and ethephon

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ABSTRACT

Proteins were extracted from *G. bicolor* that had been treated with 1-methylcyclopropene and ethephon and then stored at room temperature for 1, 3 and 7 days. More than 300 protein spots were detected by 2-DE and 38 differentially abundant spots ($P < 0.05$) were excised and analysed by using MALDI-TOF/TOF. Thirty-three proteins were finally confidently identified. According to the Clusters of Orthologous Groups of proteins, the proteins identified were classified into those responsible for metabolism (75.8%), information storage and processing (9.1%) and cellular processes and signaling (12.1%). Compared with ethephon and control treatments, 1-methylcyclopropene specifically increased the abundances of superoxide dismutase, peroxidase, carbonic anhydrase, nucleoside diphosphate kinases, glyceraldehyde 3-phosphate dehydrogenase, RuBisCO and ribulose bisphosphate carboxylase/oxygenase activase. 1-Methylcyclopropene protected leaf chloroplast and cells by enhancing stress response and defense, and delayed senescence by inhibiting substance and energy metabolisms. Therefore, 1-methylcyclopropene allowed better self-defense and delayed senescence of *G. bicolor* leaf.

1. Introduction

G. bicolor (*Gynura bicolor* D.C), a cultivated leafy vegetable, belongs to Composite *Gynura Cass* and is mostly produced in southern China (Jiang, Hou, Yuan, Jiang, & Yu, 2010). More than 90% of the leaf content is water, and chlorophyll and anthocyanin give a unique green surface and a purple back. Since the living organism with high metabolic activity after harvest is prone to losing nutrients and appearance during senescence, proper post-harvest treatments are in need for *G. bicolor* production. However, most *G. bicolor*-related studies have focused on its nutritional value, antioxidant activity, and distilled anthocyanin and flavonoids, while effective preservation technologies were only reported by our group during the last 6 years (Jiang, 2010; Jiang et al., 2010).

Up to date, 1-methylcyclopropene (1-MCP), as a highly potent inhibitor of ethylene, has been widely applied to keep vegetables fresh (Blankenship & Dole, 2003). 1-MCP can dramatically slow senescence (Watkins, 2006), lower ethylene production and respiratory rates (Ella, Zion, Nehemia, & Amnon, 2003), reduce membrane lipid peroxidation (Blankenship & Dole, 2003) and maintain good quality of *G. bicolor* leaf (Jiang, 2010). However, most studies hitherto merely focused on the physiological and biochemical changes (Blankenship & Dole, 2003) and gene expression (Ziliotto, Begheldo, Rasori, Bonghi, & Tonutti, 2008).

Proteins, which execute physiological functions in an organism and directly reflect life phenomena, have specific activity patterns and provide indirectly genetic information (Komatsu et al., 2011). Evaluating protein structures and functions may clarify the mechanisms under physiological or pathological conditions. Liu, Li, Lai, Lo, and Chen (2013) found that N^6-benzylaminopurine decreased the quantity of broccoli proteins involved in energy, carbohydrate and amino acid metabolisms. Zhang et al. (2012) reported that differentially abundant peach proteins, in response to 1-MCP and ethephon, were involved in energy and metabolism, cell structure, protein fate, stress response and defense, and ripening and senescence. A recent study on papaya fruit suggested that differentially accumulated proteins were related to cell wall degrading, oxidative damage protection and protein folding, and cell growth and survival were induced by 1-MCP during fruit ripening (Huerta-Ocampo et al., 2012). At present, the proteomics of postharvest leafy vegetables is critical, particularly for evaluating the regulatory effects of 1-MCP on the protein files during leafy vegetable senescence.

We herein determined biological parameters and protein profiles of postharvest *G. bicolor* to analyze the functions of

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differentially abundant proteins to explore the relationship between protein profiles and biological parameters, and to elucidate the *G. bicolor* response to 1-MCP. Since this is the first proteomics study involving postharvest *G. bicolor*, the results are feasibly beneficial to analogous studies.

2. Materials and methods

2.1. Plant materials and treatments

G. bicolor leaf were obtained on 10th Dec 2010 from the markets in Nanjing, Jiangsu, China. Harvested leaves (CK0) were immediately delivered to the laboratory and sorted to remove those with visual defects (pest-damaged, bruised and defective). The leaves were then divided into a control group (900 g), a 1-MCP treated group (900 g) and an ethephon treated group (900 g), and were immediately subjected to the following treatments: (a) control group (CK): without any treatment; (b) 1-MCP treatment (MT): leaves were closed up in desiccators in the presence of 500 μL L^{-1} 1-MCP (Agrofresh, USA) for 6 h. KOH (Sinopharm Chemical Reagent Beijing Co., Ltd., China) solution (1% (w/v)) was placed inside the desiccators to absorb CO_2 during treatment. After being ventilated for 30 min, the leaves were packaged; (c) ethephon treatment (ET): leaves were dipped in a 200 μL L^{-1} ethephon (Sinopharm Chemical Reagent Beijing Co., Ltd., China) solution for 10 min before being air-dried and packed. Leaves from each treatment group were divided into nine samples, each containing 100 g (100 g × 9) and packed in sealed plastic bags. Thereafter they were placed in a room held at 20–25 °C with 80–90% relative humidity for 1, 3 and 7 days.

Stalk-free leaves from each treatment group were immediately frozen in liquid nitrogen and stored at −20 °C prior to further analysis. CK0, CK1, CK3, CK7, MT1, MT3, MT7, ET1, ET3 and ET7 are abbreviations of control, 1-MCP and ethephon treatments on Day 0, 1, 3 and 7 respectively.

2.2. Determination of respiratory rate, and anthocyanin and chlorophyll contents

The respiratory rate of *G. bicolor*, which was determined with the small skep method based on CO_2 absorption (Wu et al., 2009), was expressed as milligrams of CO_2 per kilogram per hour fresh weight (FW). The chlorophyll and anthocyanin contents in the fresh leaf samples was determined by the method of Jiang (2010). The absorbance of the extracts was determined using a spectrophotometer (WFJ UV-2802 PC). The chlorophyll content was expressed as mg g^{-1} FW, and that of anthocyanin was expressed as μg g^{-1} FW.

2.3. Protein sample preparation

To minimize errors, the proteomic analysis at each treatment time point was conducted in triplicate. Total proteins of *G. bicolor* leaf were extracted according to a slightly modified phenol extraction method (Zhang et al., 2011). The frozen leaf tissue (0.5 g + 10% PVPP) was finely powdered in liquid nitrogen and extracted with 5 ml of extraction solution (1.05 mol L^{-1} sucrose, 60 mmol L^{-1} Tris, 10 mmol L^{-1} EGTA, 1 mmol L^{-1} PMSF, 1 mmol L^{-1} DTT and 1% (v/v) TritonX-100, pH 8.35), and left for 2 h. The homogenate was centrifuged at 5000*g* for 30 min at 4 °C, the supernatant was collected for subsequent extraction using different solvents. After adding Tris-saturated phenol (15 ml, pre-ice-cold, pH 7.8), samples were left for 2 h before the mixture was centrifuged at 5000*g* at 4 °C for 30 min. The collected upper phenolic phase was precipitated overnight with five folds of chilled acetone at −20 °C. After being washed twice with ice-cold methanol and twice with chilled acetone, the pellets were then air-dried at room temperature and stored at −20 °C. The dried protein powders were finally solubilised overnight at 4 °C in lysis buffer (1 mg pellets in 100 μL of lysis buffer) containing 7 mol/l urea, 2 mol/l thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT and 0.5% (v/v) pH 4–7 IPG buffer. The samples were then centrifuged at 5000*g* for 10 min at 4 °C. The supernatant protein contents were determined according to the method of Zhang et al. (2012) by using bovine serum albumin as the standard, and the samples were stored at −20 °C until 2-DE.

2.4. 2-DE and staining

Sample aliquots (350 μL) containing 300 μg proteins were applied to pH 4–7 IPG (17 cm) strips, and were isoelectrically focused on a PROTEAN IEF system (Bio-Rad, Hercules, CA, USA) for a total run of 60 kV h at 20 °C. Then the focusing was performed on an IPGphor apparatus under the following conditions: 100 V, 200 V, 500 V and 1000 V for 1 h, respectively; 4000 V and 8000 V for 2 h and 8000 V until final volt-hours (60 kV h). Before SDS–PAGE, the strips were equilibrated for two periods of 15 min with 2% (w/v) DTT and 2.5% (w/v) iodoacetamide in equilibration buffer (1.5 mol/l Tris–HCl, pH 8.8, 6 mol/l urea, 20% glycerol, 4% SDS) respectively, after which the strips were run on 12% SDS–PAGE self-cast gels. Electrophoresis was carried out at 20 °C and 1.0 W/gel for 2 h, and at 15 W/gel until the dye front reached about 1 cm from the bottom of the gel using an Ettan Six vertical set (GE Healthcare). The gels were stained with silver nitrate as described by Blum, Beier, and Gross (1987). At least three replicates were performed for each treatment.

2.5. Image acquisition and data analysis

All the stained gels were imaged by a Versdoc 3000 scanner (Bio-Rad) at 300 dpi resolution, and analysed by PDQuest Version 8.0 (Bio-Rad) (Zhang et al., 2012). The images were cropped and optimised, and protein spots were matched automatically and then carefully manually edited and confirmed. For each spot, the mean percentage volume (PV) was computed at every stage, and the spots showing a mean PV that increased/decreased at least twofold (statistically significant as calculated by one-way ANOVA ($P < 0.05$)) in different stages/treatments were considered as differentially abundant proteins.

2.6. Identification of protein in-gel digestion by MALDI-TOF/TOF

The differentially abundant protein spots were digested with trypsin as described by Shevchenko, Wilm, Vorm, and Mann (1996). MALDI-TOF/TOF tandem MS analyses were performed by a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, USA) in the m/z range of 700–3500 with an accelerating voltage of 20 kV in reflectron mode and a delayed extraction set to 120 ns. Thereafter, a combined search (MS plus MS/MS) was performed using GPS Explorer™ v3.5 (Applied Biosystems) over NCBI databases using the MASCOT search. Only the best matches with high confidence levels (confidence interval = 100%) were chosen when the software gave more than one eligible result. The differentially abundant proteins were functionally classified according to Functional Catalogue (http://mips.helmholtz-muenchen.de/proj/funcatDB) and Clusters of Orthologous Groups of proteins (COG, http://www.ncbi.nlm.nih.gov/COG/). The subcellular localisation predictions of 33 differentially abundant proteins were based on PSORT (http://wolfpsort.org).