1 Effect of 1-methylcyclopropene on senescence and sugar
2 metabolism in harvested broccoli florets

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Abstract

The effect of 1-methylcyclopropene (1-MCP) treatment on superficial color, sugar content and the activities of sugar metabolism enzymes, the expression of sucrose transporters and carbohydrate metabolizing enzymes in broccoli florets were investigated. Broccoli florets treated with $2.5 \mu\text{L L}^{-1}$ of 1-methylcyclopropene (1-MCP) inhibited the increase of lightness (L^*) value and retarded the decrease in hue angle (H) value and chlorophyll content. This treatment maintained higher levels of sugars comparing with control florets. In general, the activities of sucrose synthase-synthesis direction (SS-S) were enhanced in florets treated with 1-MCP, whereas the activities of sucrose synthase-cleavage direction (SS-C), glucokinase (GK) and UDP-glucose pyrophosphorylase (UGPase) were reduced. In addition, the expression of genes encoding sucrose transporters (*BoSUC1* and *BoSUC2*) and carbohydrate metabolizing enzymes (*BoINV1*, *BoHK1* and *BoHK2*) was induced upon 1-MCP treatment. These results indicated that 1-MCP can delay senescence of broccoli florets in the present study may be attributed to maintaining higher sugar content through regulation of sugar metabolism.

Keywords: Broccoli; 1-Methylcyclopropene (1-MCP); Sugar metabolism; Yellowing

1. Introduction

Broccoli (*Brassica oleracea* L. var. *italica*) is harvested at the immature stage and its florets senescence quickly after harvest at ambient temperatures. During broccoli senescence, a loss of the green color is observed due to the degradation of chlorophyll in florets, which reduces the commercial acceptance of this product. Various postharvest techniques such as controlled atmosphere storage (Izumi et al., 1996) and application of ethanol (Han et al., 2006) or 6-BA treatment (Xu et al., 2012) have been demonstrated to delay postharvest yellowing, improve visual quality and extend shelf life of broccoli.

In addition to act as energy substances, sugars have pivotal hormone-like functions in sensing and signaling mechanisms as signaling molecules and have been implicated in plant development and senescence (Rolland et al., 2002). It has been found that a fast accumulation of soluble sugars in banana peel led to repression of chlorophyll catabolism and the development of stay-green ripe fruit (Yang et al., 2009). A change in the sugar concentration was demonstrated to be closely associated with epicarp degreening and regreening of certain citrus fruit (Huff, 1984). In leaves of roquette, the extension in postharvest shelf life was related to higher sucrose level (Clarkson et al., 2005). In broccoli florets, a rapid loss of sucrose was observed during postharvest storage (Downs and Somerfield, 1997). It has been shown that exogenous sucrose supply (Irving and Joyce, 1995), visible light treatment (Büchert et al., 2011) or harvested at sunset time (Hasperué et al., 2011) can delay postharvest senescence and increase the shelf life of broccoli, possibly by maintaining higher endogenous

soluble sugar levels. In broccoli tissues treated with cytokinin, the concentrations of sucrose, glucose and fructose remain higher than in controls (Downs et al., 1997). Meanwhile, it is generally accepted that the senescence of broccoli florets is closely associated with endogenous ethylene. The ethylene action inhibitor 1-MCP, has been found effective in delaying senescence and increasing storage life of broccoli (Ku and Wills, 1999; Yuan et al., 2010). However, it is still unclear whether sugars are involved in mechanisms by which 1-MCP delaying senescence in broccoli florets. The objectives of this study were to investigate the effect of 1-MCP on superficial color, chlorophyll and sugar contents, and activities of sugar metabolism enzymes, the expression profiles of genes associated with carbohydrate transport and metabolism after harvest in broccoli florets and to evaluate the possible role of sugars in delaying senescence of broccoli florets.

2. Materials and methods

2.1. Plant material and 1-MCP treatment

Broccoli (*Brassica oleracea* L. var. *italica*, cv. Chaoda No 1) heads were obtained from local producers (Nanjing, province of Jiangsu, China), top iced and immediately transported to the laboratory within 2 hour of harvest. Broccoli heads were randomly divided into two groups, untreated (control) and 2.5 $\mu\text{L L}^{-1}$ 1-MCP for 6 h at 20 °C. This specific 1-MCP treatment condition was chosen as optimal for extending the shelf life of broccoli according to our previous study (Xu et al., 2013). 1-MCP was released from a commercial Smart FreshTM powder (a.i. 0.14%, Agrofresh

Ltd, Philadelphia, PA, USA) by adding distilled water according to the manufacturer's instructions. After treatment, the broccoli heads were stored in the dark at 15 °C and 95% relative humidity for 4 days. There are three replicates of 30 heads each per treatment, and the whole experiment was conducted twice. Florets of three heads from each replicate were excised every day during storage, frozen with liquid nitrogen and stored at -80 °C for enzyme assay and RNA extraction.

2.2. Superficial color and chlorophyll content determination

The superficial color parameters were evaluated with a colorimeter (Minolta, Model CR-400, Osaka, Japan) by measuring the lightness (L^*) and hue angle (H) values. Chlorophyll concentrations were determined as described by Lichtenthaler (1987) with slight modifications. Frozen broccoli florets (0.16 g) were crushed by a mill in 10 mL of 95% ethanol, stirred and centrifuged at 12 000 g for 10 min, and then the residue was removed. Chlorophyll quantification was performed spectrophotometrically at 665 and 649 nm and the chlorophyll content was expressed as chlorophyll mass on a fresh weight basis (mg/g).

2.3. Sugar analysis

Broccoli samples (2 g) was mixed with 10 mL distilled water and centrifuged at 12 000 g for 10 min, and then the residue was removed. Sucrose, glucose and fructose were determined by high performance liquid chromatography (HPLC) using a Sugar-D column (250 × 4.6 mm) at a flow rate of 1.0 mL min⁻¹. Sucrose, glucose and fructose were identified by their retention times and were quantified according to standards.

2.4. Enzyme assay

For glucokinase (GK), UDP-glucose pyrophosphorylase (UGPase), soluble acid invertase (SAI) and sucrose-phosphate synthase (SPS) and sucrose synthase (SS), broccoli sample was extracted using a buffer containing 400 mM Tris-HCl (pH 8.5), 5 mM EDTA, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, 1 mM phenylmethylsulphonyl fluoride, 10 mM ascorbic acid, 1% (v/v) Triton X-100, 10 mg L⁻¹ of leupeptin, and 10 mg L⁻¹ of chymostatin. In the case of the crude extract prepared for invertase measurements, 400 mM HEPES–NaOH (pH 8.8) was used instead of Tris–HCl (pH 8.5). In all cases, the samples were completely ground in a cold mortar in the presence of insoluble polyvinyl polypyrrolidone and centrifuged at 10 000 g for 15 min at 4 °C. These crude extracts were used for enzyme activity measurements.

GK was measured according to the method of Mustroph and Albrecht (2003), the reaction mixtures was 0.1 M Tris-HCl, pH 8.5, with 5 mM MgCl₂, 0.5 mM NAD, 10 mM glucose, 2 mM ATP and 2 U of G6PDH. The reaction was started with ATP. UGPase activity was determined in a reaction mixture containing 80 mM Hepes, pH 7.8, 5 mM MgCl₂, 0.6 mM NAD, 1 mM UDP-glucose, 0.5 mM Ppi, 1 U of G6PDH, and 1 U of phosphogluconmutase (Sowokinos et al., 1997). SAI and SPS were assayed according to the methods of Hubbard et al (1989) and Vargas et al (2008) with some modifications. SAI activity at 37 °C was assayed by adding 100 mM sodium acetate (pH 4.8) and 100 mM sucrose solution. Reaction was stopped at 30 min by adding 1.5 mL DNS solution. The assay solution of SPS contained 50 mM

Tris-HCl (pH 7.5), 15 mM MgCl₂, 1 mM EDTA, 1 mM fructose-6-P, 2 mM UDP-glucose and 500 µL of sample. Sucrose produced by these reactions was assayed using the anthrone assay (Van Handel, 1968). The SS activities were determined in the synthesis (SS-S) and cleavage (SS-C) direction. The procedure for the sucrose synthesis direction was identical to that of SPS except the reaction mixtures contained 10 mM fructose and did not contain fructose 6-P or glucose 6-P. The SS cleavage activity determination was the same as for SAI except the reaction mixtures contained 1 mM UDP.

2.5. Analysis of sucrose transport and carbohydrate metabolic genes

Reverse transcription PCR (RT-PCR) was used to analyse the expression pattern of the sucrose transporter and carbohydrate metabolic genes Broccoli sucrose transporter 1 (*BoSUC1*), Broccoli sucrose transporter 2 (*BoSUC2*), Broccoli acid invertase 1 (*BoINVI*), Broccoli hexokinase 1 (*BoHK1*) and Broccoli hexokinase 2 (*BoHK2*) in broccoli florets. Total RNA was extracted by the method of Chang et al (1993). Reverse transcription was conducted according to the manufacturer's instructions of PrimeScript™ 16 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). The RT-PCR was performed with a total volume of 25 µL which contained aliquots of cDNA samples, 1 µL forward primer and reverse primer (20 µM), 10 µL of 10 × PCR buffer (Mg²⁺ free), 1.5 µL MgCl₂ (25 mM), 15.7 µL ddH₂O, 2 µL deoxy-ribonucleoside triphosphate (dNTP) (2.5 mM), 0.3 µL Taq polymerase (5 U µL⁻¹). 18S was used as an internal control. Primers specific to the following genes were used: *BoSUC1* (accession no. AY065840), forward: 5'

-CCCGTTCGCAATGACCAA-3', reverse: 5'-CTCCCACTTTCTTACCAATCCA-3';
BoSUC2 (accession no. AY065839), forward: 5'-CTCGACGTGGCGAACAAC-3',
reverse: 5'-ACGGCCCATCCAATCAGT-3'; *BoHK1* (accession no. AF454961),
forward: 5'-CAGCGGGTAGACAAAGGG-3', reverse:
5'-GGAGTCGCCAAAGAAAGC-3', *BoHK2* (accession no. AF454962), forward:
5'-TTGTCGCCGCTGTTATTC-3', reverse: 5'-CCTCCACGCTGTCTGATG-3'
BoINV1 (accession no. AF274298), forward: 5'-AGGACCTCAACGACCCAC-3',
reverse: 5'-CCTCAGCCTCAACCACCA-3'; *18S* (accession no. AF513990), forward:
5'-CTTAGTTGGTGGAGCGATTT-3', reverse: 5'-AAGGGCAGGGACGTAGTC-3'.

Each measurement was performed in triplicate.

2.6. Statistical analyses

All statistical analyses were processed using SPSS package program version 16.0 (SPSS Inc., Chicago, IL, USA). All data were expressed as means $\pm$ the standard error (SE). The data were analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by Duncan's multiple-range tests, and the differences at $p < 0.05$ were considered to be significant.

3. Results and discussion

Florets attached to broccoli heads began to degreen immediately after harvest, and changes in superficial color during broccoli senescence was evaluated through the color parameters hue angle (H) and lightness (L^*). 1-MCP treatment significantly ($p < 0.05$) inhibited the decrease of H value and the increase of L^* value (Fig. 1a and b). The rate of chlorophyll diminution in 1-MCP treated florets was significantly slower

than that of control florets (Fig. 1c). This is consistent with the findings of Ku and Will (1999) and Yuan et al (2010).

Sugars as sources of carbon skeleton are necessary to maintain energy supply and extend the postharvest life of perishable horticultural commodities. Supplying exogenous sucrose was reported to increase the shelf life of broccoli (Irving and Joyce, 1995). Broccoli heads harvested at sunset maintained a higher soluble sugar content in comparison with those harvested in early morning, which resulted in a slower rate of senescence and higher levels of chlorophylls, antioxidants and phenolics (Hasperué et al., 2011). Continuous low-intensity light treatment delayed senescence and maintained photosynthetic activity for a longer time, which in turn contributed to higher levels of sugar in harvested broccoli heads (Büchert et al., 2011). In the present study, treatment of broccoli with 1-MCP effectively delayed broccoli senescence manifested as lower L^* value and higher H value and chlorophyll content (Fig. 1), the contents of sucrose, glucose and fructose maintained higher than in control florets (Fig. 2), perhaps providing access to a supply of sugars for respiration for a longer period. These results suggest that the effect of 1-MCP in delaying senescence of broccoli might be correlated to higher sugar levels.

Sucrose is the main form of sugar transported to the sink tissues in sugar metabolism (Nishikawa et al., 2005). The accumulation of sucrose depends on the balance between its synthesis and degradation. The activities of SPS and SAI were found to be the primary determinants of sucrose accumulation in developing muskmelon fruits (Hubbard et al., 1989). In citrus fruits, sucrose accumulation

coincided with an increase in SPS activity (Komatsu et al., 1999). SPS is an important enzyme in banana sink tissues that accumulate sucrose (Hubbard et al., 1990). The decrease of sucrose content was associated with increased SAI activity and decreased SPS activity in broccoli florets (Pramanik et al., 2004). In this work, 1-MCP resulted in induced higher activity of SS-S (Fig. 3d), the enzymes involved in sucrose biosynthesis, and maintained lower activity of SS-C (Fig. 3c), sucrose catabolizing enzyme, which may account for higher sucrose level in 1-MCP-treated florets (Fig. 2a). SAI activity increased gradually in both control and 1-MCP-treated broccoli florets during storage. No significant differences in SPS and SAI activities were observed between control and 1-MCP-treated broccoli florets (Fig. 3a and b). GK and UGPase are two enzymes involved in hexose sugar (glucose and fructose) catabolism. Meanwhile, UGPase constitutes a reversible enzymatic step for interconversions between starch and sucrose metabolites, and is responsible for synthesis and metabolism of UDP-glucose (Kleczkowski, 1994). In this investigation, 1-MCP treatment reduced GK and UGPase activities in comparison with the control florets (Fig. 4), which may partly account for higher glucose and fructose levels in 1-MCP-treated florets (Fig. 2b and c).

Recently, the role of soluble sugars as signaling molecules functioning in plant physiology has been intensively studied (Rolland et al., 2006). Sugars are involved in signal transduction and gene expression throughout all stages of the plant's life cycle (Smeekens, 2000). Gapper et al (2005) reported that exogenous cytokinin treatment caused a reduction in the expression of genes involved with carbohydrate transport

(*BoSUC1* and *BoSUC2*) and metabolism (*BoINV1* and *BoHK1*). In our work, the effect of 1-MCP treatment on the expression of sucrose transporter (*BoSUC*) genes in broccoli florets was shown in Fig 5. *BoSUC1* transcript levels were higher in florets treated with 1-MCP than in control, particularly in the first day postharvest. Similarly, 1-MCP treatment caused an increase in *BoSUC2* transcript levels in florets as compared with the control. *BoINV1* transcript levels in florets were detectable at harvest and had increased in 1-MCP-treated samples. The expressions of hexokinase genes, *BoHK1*, *BoHK2* were similar in postharvest broccoli florets. 1-MCP induced stronger expression of *BoHK1* and *BoHK2* transcript levels in florets when compared to the control samples. Furthermore, although 1-MCP-treated broccoli florets retained higher levels of sucrose, glucose and fructose and lower GK activity, 1-MCP treatment enhanced expression of genes involved in carbohydrate transport (*BoSUC1* and *BoSUC2*) and metabolism (*BoINV1*, *BoHK1* and *BoHK2*) (Fig. 5). The discrepancy may occur because transport of 1-MCP is insufficiently rapid through the florets to influence the very early changes in expression of genes involved in carbohydrate regulation during senescence. Take together, it is clear from the present work that 1-MCP treatment may directly or indirectly alter the expression of genes involved in sucrose transport and carbohydrate metabolism.

Sugars that accumulate in the peel tissue of banana fruit seem to be a major factor regulating chlorophyll degradation (Yang et al., 2009). In broccoli sprouts, sucrose may act as a key signaling molecule in inducing the accumulation of glucosinolates and anthocyanins (Guo et al., 2011). Sugars (mainly sucrose and fructose)

accumulations were under ethylene regulation during apples ripening, and the block of ethylene action (1-MCP treatment) caused similar effects on total sugars and sucrose accumulation (Defilippi et al., 2004). There was a complex interaction between ethylene and sugar metabolism in fruits and may be similar to the complexity observed in vegetative tissues, and ethylene seems to affect glucose signaling through abscisic acid (ABA) (León and Sheen, 2003). It has been proposed that acid invertase expression is regulated independently of ethylene in tomato (Rothan et al., 1997). Ethylene accelerates senescence of broccoli and involves in broccoli yellowing. It was found that increasing the endogenous sugar levels by exogenous sucrose supply resulted in decreased ethylene responsiveness and delayed senescence in harvested broccoli florets, implying that the decline of sugars can enhance the ethylene sensitivity and accelerate senescence (Nishikawa et al., 2005). 1-MCP was thought to occupy ethylene receptors to cease ethylene signaling and action (Blankenship and Dole, 2003). In this work, 1-MCP treatment prevented chlorophyll degradation and maintained higher levels of soluble sugars in broccoli florets (Fig.1c and Fig. 2), which suggested that 1-MCP blocked efficiently the binding sites for ethylene during the period in which metabolism sugars could have been affected. However, the intricate and complex webs that interrelates sugars and ethylene signaling pathways need further investigation.

4. Conclusion

In the present work, postharvest application of $2.5 \mu\text{L L}^{-1}$ of 1-MCP maintained visual quality in broccoli florets during storage at 15°C . The results indicate that

sugar may play an important role in regulating broccoli senescence, and 1-MCP may delay senescence of broccoli florets by maintaining higher sugar content through regulating sugar metabolism.

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Figure Captions:

Fig. 1. Changes in H (a), L^* (b) values and chlorophyll content (c) in control and 1-MCP-treated broccoli florets during storage at 15 °C. Vertical bars represent the standard errors of the means of triplicate samples. * $p < 0.05$, ** $p < 0.01$ based on Duncan' s multiple range test between 1-MCP-treated and control samples.

Fig. 2. Contents of sucrose (a), fructose (b) and glucose (c) in control and 1-MCP-treated broccoli florets during storage at 15 °C. Vertical bars represent the standard errors of the means of triplicate samples. * $p < 0.05$, ** $p < 0.01$ based on Duncan' s multiple range test between 1-MCP-treated and control samples.

Fig. 3. Activities of SPS (a), SAI (b), SS-C (c) and SS-S (d) in control and 1-MCP-treated broccoli florets during storage at 15 °C. Vertical bars represent the standard errors of the means of triplicate samples. * $p < 0.05$, ** $p < 0.01$ based on Duncan' s multiple range test between 1-MCP-treated and control samples.

Fig. 4. Activities of GK (a) and UGPase (b) in control and 1-MCP-treated broccoli florets during storage at 15 °C. Vertical bars represent the standard errors of the means of triplicate samples. * $p < 0.05$, ** $p < 0.01$ based on Duncan' s multiple range test between 1-MCP-treated and control samples.

413 Fig. 5. Expression of genes involved in sucrose transport (*BoSUC1* and *BoSUC2*) and
414 carbohydrate metabolism (*BoINV1*, *BoHK1* and *BoHK2*) in control and
415 1-MCP-treated broccoli florets during storage at 15 °C. Reverse
416 transcription-polymerase chain reaction (RT-PCR) was carried out using *18S-rRNA* as
417 a standard.

1 **Figure 1.**

12 **Figure 2.**

Figure 3.

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