Running title: Effect of 1-MCP on ascorbate metabolism in broccoli

Effect of 1-methylcyclopropene on the expression of genes for ascorbate metabolism in post-harvest broccoli

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Abstract

The effects of 1-methylcyclopropene (1-MCP) on ascorbate (AsA) metabolism were studied and the possible molecular mechanisms were discussed in two cultivars of broccoli (*Brassica oleracea* L. var. *italica*), 'Haitsu' and 'Ryokurei'. The results showed that 1-MCP treatment delayed the yellowing and suppressed the ethylene production in 'Haitsu' and 'Ryokurei'. Meanwhile, the AsA content declined to a lower level in the control during storage, and the reduction of AsA was significantly suppressed by the treatment with 1-MCP in the two broccoli cultivars. Gene expression analyses by real-time PCR showed that 1-MCP treatment down-regulated the gene expression of *BO-APX1* and *BO-APX2*, and up-regulated the gene expression of *BO-DHAR* and *BO-GLDH* compared with the control. The regulation of these genes expression might contribute to the suppression of AsA reduction by the 1-MCP treatment in 'Haitsu' and 'Ryokurei'. The results arising from this study might provide new insights into the possible mechanism, by which the treatment with 1-MCP delayed the senescence in broccoli.

Key words: 1-Methylcyclopropene (1-MCP), Ascorbate, Broccoli, Real-time PCR.

1. Introduction

Broccoli (*Brassica oleracea* L. var. *italica*) is a highly perishable horticultural crop. It has been reported that senescence of broccoli florets is accelerated by exogenous and endogenous ethylene (King and Morris, 1994; Tian et al., 1996; Fan and Mattheis, 2000). Thus, treatments that reduce ethylene synthesis or inhibit its perception might be effective in improving their shelf-life. 1-methylcyclopropene (1-MCP), which is an effective inhibitor of ethylene action and binds irreversibly to ethylene receptors, has been shown to be potentially useful for delaying ripening, maintaining quality, and extending the shelf-life of fruits, vegetables, and ornamental crops (Watkins, 2008). Application of 1-MCP can delay yellowing, decrease respiration and decay, alleviate certain ethylene-induced postharvest physiological disorders, and extend the shelf-life of broccoli (Ku and Wills, 1999; Fan and Mattheis, 2000; Able et al., 2002; Gong and Mattheis, 2003). In our previous study, we found that 1-MCP delayed the senescence process of broccoli by inhibiting the activities of enzymes involved in ethylene biosynthesis and the gene expression of these enzymes and of ethylene receptors at the transcription level (Ma et al., 2009).

Senescence in plant tissues is associated with excess production of reactive oxygen species (ROS), including superoxide radicals and hydrogen peroxide. To counteract the toxicity of ROS, plants contain complements of enzymatic and non-enzymatic antioxidants to scavenge ROS (Apel and Hirt, 2004; Sairam and

62 Tyagi, 2004; Moller et al., 2007). It has been suggested that the
63 ascorbate–glutathione cycle plays a crucial role in the antioxidant defense
64 system of plants (Mittler, 2002; Mori et al., 2009). The ascorbate-glutathione
65 cycle involves ascorbate peroxidase (APX), monodehydroascorbate reductase
66 (MDAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and
67 two reducing substances, reduced ascorbate (AsA) and glutathione (GSH). As
68 shown in Fig.1, the metabolic pathway of AsA has been proposed (Noctor and
69 Foyer, 1998). In this pathway, AsA is synthesized from L-galactono-1,4-lactone
70 (GL) by GL dehydrogenase (GLDH), and in the ascorbate-glutathione cycle, the
71 enzymatic action of APX produces monodehydroascorbate (MDA), which can
72 dismutate spontaneously to AsA and dehydroascorbate (DHA) or be reduced
73 enzymatically to AsA by NADPH-dependent MDAR. DHA is also reduced to
74 AsA enzymatically in a reaction mediated by DHAR, using GSH as an electron
75 donor. The resulting oxidized glutathione is then converted back to the reduced
76 form (GSH) by an NADPH-dependent GR (Nishikawa et al., 2003a). The
77 enzymes in the ascorbate-glutathione cycle exist as isoenzymes distributed in
78 distinct cellular organelles: chloroplasts, plastids, mitochondria, and
79 peroxisomes (Mittova et al., 2000).

80 Shigenaga et al. (2005) reported that enhancement of the action of the
81 ascorbate-glutathione cycle might relate to the suppression of senescence in
82 heat-treated broccoli florets. It has been confirmed that treatment with 1-MCP
83 could extend the shelf-life of broccoli at low concentration, even if in the

84 presence of exogenous ethylene (Ku and Wills, 1999; Fan and Mattheis, 2000;
85 Able et al., 2002; Gong and Mattheis, 2003; Ma et al., 2009). However, to our
86 knowledge, most of these studies mainly focused on the inhibitory effects of
87 1-MCP on the ethylene biosynthesis and perception, while limited information is
88 available regarding the responses of the ascorbate-glutathione cycle to the
89 1-MCP treatment in the senescence process of broccoli (Yuan et al., 2010). To
90 date, the effects of 1-MCP on AsA metabolism and gene expression of the
91 enzymes involved in the ascorbate-glutathione cycle still remain unclear. In the
92 present study, the effects of 1-MCP on the AsA content were investigated in the
93 two broccoli cultivars, ‘Haitsu’ and ‘Ryokurei’. Moreover, the expression of
94 genes (*BO-APX1*, *BO-APX2*, *BO-sAPX*, *BO-MDAR1*, *BO-MDAR2*, *BO-DHAR*
95 and *BO-GLDH*), which were directly related to the AsA metabolism, were
96 analyzed by real-time PCR. The results arising from this study will help to
97 further clarify the possible mechanism, by which the treatment with 1-MCP
98 delayed the senescence in broccoli.
99

2. Materials and Methods

2.1. Plant materials and treatments

Two cultivars of broccoli (*B. oleracea* L. var. *italica*), 'Haitsu' and 'Ryokurei', were grown at the Fujieda Farm of Shizuoka University, Shizuoka, Japan. They were grown under the same field conditions. The cultivar of 'Haitsu' is an early cultivar, which can be harvested approximately 65 days after planting. The cultivar of 'Ryokurei' is a medium cultivar, which can be harvested approximately 105 days after planting. Mature broccoli heads of uniform size, shape, and maturity, were selected and randomly divided into two groups. The heads were continuously treated as follows: in air as a control; with 5 μL L^{-1} of 1-MCP (Rohm and Hass, Japan). All treatments were conducted in airtight chambers at 20 °C under humidified conditions (RH > 95%). Florets were excised from the heads with a single-edged razor every day after harvest, three replicates of three heads of broccoli being taken at each sample time. The excised florets were immediately frozen in liquid nitrogen except for the sample for ethylene production analyses, and stored at -80 °C until used.

2.2. Assessment of broccoli yellowing

The colour of florets in broccoli was scored by a visual assessment of changes from green to yellow. A rating scale of senescence from 5 to 0 was adopted: 5, all green; 4, 20% yellowing; 3, 40% yellowing; 2, 60% yellowing; and 1, 80% yellowing. Intermediate numbers were assigned where appropriate according to the yellowing rate. The decrease in the colour scores almost paralleled the

122 decline in chlorophyll content in florets extracted with ethanol and determined
123 by spectrophotometry (Hyodo et al., 1994).

124 *2.3. Measurements of ethylene production*

125 A 1-g sample of florets was placed into a 15-mL vial, the vial was sealed
126 using a silicon rubber cap, and sample was incubated for 30 min at 20 °C. The
127 headspace gas in the vial was sampled using a 1-mL plastic hypodermic syringe
128 and injected into a gas chromatograph (Hitachi 163) equipped with an alumina
129 column at 70 °C and a flame ionization detector, in accordance with the
130 procedures described by Nishikawa et al. (2001). The rate of ethylene
131 production was expressed as nmol ethylene per h per g FW.

132 *2.4. Extraction and assays of ascorbate*

133 The ascorbate content of the reduced and oxidized forms was assayed by
134 HPLC in accordance with the method described by Nishikawa et al. (2001).
135 Each frozen sample (0.4 g) was homogenized using a mortar and pestle in 5 mL
136 of 2% metaphosphoric acid. The homogenate was centrifuged at 14,000 ×g for
137 20 min, and then the supernatant was filtered through Miracloth (Calbiochem).
138 The pH of the filtrate was adjusted by adding an equal volume of 0.2 M
139 K-phosphate buffer (pH 7.5). Total ascorbate was assayed by adding 1 mL of 2
140 mM dithiothreitol (DTT) to an aliquot of filtrate and incubating the mixture for
141 15 min (Masuda et al., 1988). After the sample was filtered through a 0.2-μm
142 cellulose acetate filter (Advantec), a 20 μL aliquot was injected onto a TSK-GEL
143 (Amide-80) column (TOSOH) attached to a LC-10AD pump (Shimadzu). The

144 column kept at 20 °C was eluted with 80% acetonitrile: 0.04% phosphoric acid
145 at a flow rate of 1.0 mL min^{-1}. Ascorbate was monitored at 245 nm (retention
146 time 5.3 min) using an SPD-10A spectrophotometric detector (Shimadzu)
147 attached to a chart recorder (C-R6A, Shimadzu). Peaks were converted to
148 concentrations by using the dilution of stock ascorbate to construct a standard
149 curve. AsA content was determined in a similar manner without the addition of
150 DTT. DHA content was calculated by subtracting the AsA value from the total
151 ascorbate.

152 *2.5. Isolation and sequence analysis of genes related to ascorbate metabolism*

153 Total RNA was extracted from florets of broccoli in accordance with the
154 method described by Kato et al. (2000). First-strand cDNA was synthesized
155 from 2 μg of total RNA using TaqMan Reverse Transcription Reagents (Applied
156 Biosystems).

157 For each cultivar, the cDNA fragments of genes related to ascorbate
158 metabolism were amplified by PCR using cDNA template and a set of primers
159 designed from the published sequences (Table 1). The amplified cDNAs were
160 sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied
161 Biosystems, Foster City, CA, USA) with an ABI PRISM 3100 Genetic Analyzer
162 (Applied Biosystems). TaqMan MGB probes and sets of primers for *BO-APX1*,
163 *BO-APX2*, *BO-sAPX*, *BO-MDAR1*, *BO-MDAR2*, *BO-DHAR* and *BO-GLDH*,
164 were designed on the basis of the common sequences between the two cultivars
165 of broccoli for each gene using Primer Express software (Applied Biosystems)

(Table 2).

2.6. Total RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted from the florets of broccoli after harvest in accordance with the method described by Kato et al. (2000). The total RNA was cleaned up using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with DNase digestion on a column in accordance with the manufacturer's instructions. Reverse transcription was performed with 2 μg of purified RNA and random hexamer in 60 min at 37 °C using TaqMan Reverse Transcription Reagents (Applied Biosystems).

TaqMan real-time PCR was carried out with TaqMan MGB Probe, a set of primers, and TaqMan Universal PCR Master Mix (Applied Biosystems) in accordance with the manufacturer's instructions. As an endogenous control, TaqMan Ribosomal RNA Control Regents VIC probe (Applied Biosystems) was used. The thermal cycling conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The levels of gene expression were analyzed with ABI PRISM 7000 Sequence Detection System Software (Applied Biosystems) and normalized with the results for 18S ribosomal RNA. Real-time quantitative RT-PCR was performed with three replicates for each sample.

2.7. Statistical analysis

All values are shown as the mean ± SE for three replicates. The data were analyzed, and Tukey's HSD test were used to compare the treatment means at $P < 0.05$.

3. Results

3.1 Effect of 1-MCP on yellowing and ethylene production

The transition in floret colour from green to yellow is a direct character of the senescence in broccoli. As shown in Fig. 2, the florets displayed obvious yellowing from the second and third day after harvest in ‘Haitsu’ and ‘Ryokurei’, respectively. Compared with the control, the yellowing process was delayed by the treatment with 1-MCP in the two cultivars.

In the control of ‘Haitsu’, ethylene production increased rapidly after harvest, reaching a peak on the third day (Fig. 3A). With the treatment of 1-MCP, ethylene production was significantly suppressed by the treatment with 1-MCP and no clear peaks were detected in ‘Haitsu’. In the control of ‘Ryokurei’, ethylene production increased rapidly after harvest with a peak on the first day, and then decreased. On the third day, there was a second peak of ethylene production, which coincided with the appearance of floret yellowing in ‘Ryokurei’. With the treatment of 1-MCP, the ethylene production was clearly suppressed on the second and third days, and the second peak of ethylene production was delayed by 1 day.

3.2 Effect of 1-MCP on AsA content

The AsA content decreased rapidly from the first day after harvest in ‘Haitsu’ and ‘Ryokurei’. Compared with the control, the AsA reduction was suppressed clearly by the treatment with 1-MCP from the first day after harvest in the two cultivars (Figs. 4 A and B).

The content of DHA was relatively low in the two cultivars investigated in the present paper, exhibited less than 10% of total ascorbate throughout the experimental period. The content of DHA was kept almost constant in the control or 1-MCP treated ‘Haitsu’ and ‘Ryokurei’ during the experimental period (Figs. 4 A and B).

3.3 Effect of 1-MCP on expression of genes related to AsA metabolism

In the control of ‘Haitsu’, the mRNA levels of the two cytosolic genes, *BO-APX1* and *BO-APX2*, increased significantly with a peak on the second day after harvest. In contrast to the *BO-APX1* and *BO-APX2*, the mRNA level of *BO-sAPX*, which is the stromal APX in chloroplasts, decreased rapidly on the first day, and then kept almost constant. Compared with the control, the mRNA levels of *BO-APX1* and *BO-APX2* in the treatment of 1-MCP were lower after harvest, while the gene expression of *BO-sAPX* was not affected by the 1-MCP treatment (Fig. 5). In ‘Ryokurei’, the mRNA level of *BO-APX1* decreased after harvest and then increased significantly from the first day, while the mRNA level of *BO-APX2* increased gradually throughout the experimental periods in the control. The gene expression of *BO-sAPX* decreased significantly after harvest and then increased on the third day. Similar to the ‘Haitsu’, the mRNA levels of *BO-APX1* and *BO-APX2* were lower in the treatment of 1-MCP than that of the control. Meanwhile, the application of 1-MCP had no obvious effects on the gene expression of *BO-sAPX* compared with the control during the experimental period (Fig. 6).

233 The mRNA levels of the genes (*BO-MDAR1*, *BO-MDAR2* and *BO-DHAR*),
234 which are related to the AsA regeneration, decreased rapidly rafter harvest in the
235 control of 'Haitsu'. With the treatment of 1-MCP, the mRNA level of
236 *BO-MDAR2* was lower than that of the control, while the mRNA level of
237 *BO-MDAR1* was not affected by the 1-MCP treatment in 'Haitsu'. Compared
238 with the control, the mRNA level of *BO-DHAR* in the treatment of 1-MCP was
239 higher than that of the control on the third and fourth days in 'Haitsu' (Fig. 5). In
240 the control of 'Ryokurei', the mRNA levels of *BO-MDAR1*, *BO-MDAR2* and
241 *BO-DHAR* decreased after harvest and then increased rapidly from the third day.
242 With the treatment of 1-MCP, the mRNA level of *BO-MDAR2* was much lower
243 than that of the control on the fourth day, while the gene expression of
244 *BO-MDAR1* was not affected by the 1-MCP treatment after harvest. Compared
245 with the control, the mRNA level of *BO-DHAR* was much higher in the
246 treatment of 1-MCP on the second and third days (Fig. 6).
247 In the control of 'Haitsu', the mRNA level of the mitochondrial gene
248 (*BO-GLDH*) decreased gradually during the experimental period. In the control
249 of 'Ryokurei', the mRNA level of *BO-GLDH* decreased rapidly after harvest and
250 then increased slightly (Figs. 5 and 6). With the treatment of 1-MCP, the mRNA
251 level of *BO-GLDH* was higher than that of the control after harvest in 'Haitsu'
252 and 'Ryokurei' (Figs. 5 and 6).
253

4. Discussion

The compound 1-MCP, which is an inhibitor of ethylene perception, is now used extensively to maintain the postharvest quality of fresh fruits and vegetables. At ambient temperature, broccoli senesces rapidly after harvest. The appearance of yellowing is thought to be a direct and important index during the senescence of broccoli (Ma et al., 2009; Yuan et al., 2010). In addition, endogenous ethylene is closely associated with the senescence of broccoli after harvest (Hyodo et al., 1994; King and Morris, 1994; Kasai et al., 1996). Suzuki et al. (2004) found that the suppression of ethylene production by ethanol vapor treatment led to the inhibition of some metabolic activity and the senescence of broccoli. In the present study, the yellowing process was suppressed clearly by the treatment with 1-MCP in ‘Haitsu’ and ‘Ryokurei’. In ‘Haitsu’, the ethylene production was suppressed significantly by the treatment with 1-MCP after harvest. In the control of ‘Ryokurei’, there were two peaks of ethylene production, and the second ethylene peak coincides with the appearance of yellowing. It can therefore be assumed that the second ethylene peak might be related to the onset of senescence. With the treatment of 1-MCP, the ethylene production was clearly suppressed on the second and third days, and the second peak of ethylene production was clearly delayed by 1 day. These results indicated that the application of 1-MCP was effective for delaying the senescence in the two broccoli cultivars (Figs. 2 and 3).

Recently, the mechanisms of 1-MCP involved in the senescence of vegetables

276 and fruits have been discussed in several studies that were mainly focused on
277 ethylene biosynthesis and perception, while little information was available
278 about the effects of 1-MCP on the AsA metabolism (Voesenek et al., 1997; Chen
279 et al., 1998; Lashbrook et al., 1998; Sato-Nara et al., 1999; Wang et al., 2002;
280 Ma et al., 2009). AsA, a critical component of antioxidative processes in fruits
281 and vegetables, has been proven to contribute to delaying senescence and
282 prolonging the shelf-life of fruits and vegetables by interacting enzymatically
283 and non-enzymatically with ROS (Mittler, 2002; Apel and Hirt, 2004; Sairam
284 and Tyagi, 2004; Moller et al., 2007; Mori et al., 2009). Decreased AsA content
285 have been proven to link with the development of physiological disorders in
286 pears and apples (Fawbush, et al., 2009). In the florets of broccoli, the AsA
287 content declined to a lower level during the process of senescence (Nishikawa et
288 al., 2003a). Moreover, the reduction of AsA could be suppressed by the
289 post-harvest handing, such as heat and ethanol vapor treatments (Shigenaga et
290 al., 2005; Mori et al., 2009). In the present study, we found that the treatment
291 with 1-MCP significantly slowed the reduction of AsA during the process of
292 senescence in ‘Haitsu’ and ‘Ryokurei’, suggesting that the regulation of AsA
293 content might contribute to the beneficial effects of 1-MCP on the senescence of
294 broccoli. Additionally, although the reduction of AsA was observed in the two
295 broccoli cultivars during the senescence process, the content of DHA kept
296 almost constant at a low level in the control or 1-MCP treated broccoli cultivars.
297 This similar phenomenon was also observed in the cultured cells of tobacco

298 (Kato and Esaka 1999; De Pinto et al., 2000). Saito et al. (1997) has reported
299 that the different metabolites produced by the AsA breakdown by means of
300 enzymatic cleavages of paculiar carbon-carbon bond might lead to the reduction
301 of AsA without an increase in DHA. Alternatively, the instability of the DHA
302 could also explain that the decrease in AsA was not accompanied with the
303 increase in DHA (De Pinto et al., 2000).

304 The enzymes in the ascorbate-glutathione cycle, such as APX, MDAR and
305 DHAR, exist as isoenzymes encoded by discrete genes and distribute in distinct
306 cellular compartments. In the recent years, the gene expression at the
307 transcription level, which might give a more precise estimate of antioxidant gene
308 activation than enzyme activity, was extensively studied (Nishikawa et al., 2003b; Tokunaga et al., 2005). To date, APX, which is the major enzyme
310 responsible for the AsA breakdown, is the most extensively studied enzymes in
311 the ascorbate-glutathione cycle. In the present paper, the results showed that the
312 expression of two cytosolic genes (*BO-APX1* and *BO-APX2*) increased
313 significantly during the experimental period, which tended to coincide with the
314 reduction of AsA after harvest in 'Haitsu' and 'Ryokurei'. In contrast to the
315 cytosolic APX, the gene expression of *BO-sAPX* decreased to a low level after
316 harvest in the two broccoli cultivars. In addition, the changing patterns of the
317 gene expression of cytosolic APX and chloroplastic APX were different in
318 response to the treatment of 1-MCP. Compared with the control, the expression
319 of *BO-APX1* and *BO-APX2* was significantly down-regulated by the 1-MCP

320 treatment, which led to the higher AsA content in the two broccoli cultivars
321 treated with 1-MCP. While the gene expression of *BO-sAPX* was not affected by
322 the 1-MCP treatment in ‘Haitsu’ and ‘Ryokurei’. Panchuk et al. (2002) have also
323 reported that various APX isoforms behave in a different way at the transcription
324 level during the stress treatment in Arabidopsis. These results imply that the
325 APX isogenes in different cell compartments might play different roles during
326 senescence in broccoli.

327 It has been confirmed that MDAR and DHAR are the enzymes responsible
328 for AsA regeneration in plants (Wheeler et al., 1998; Mittler, 2002; Apel and
329 Hirt, 2004; Sairam and Tyagi, 2004). Over-expression of MDAR and DHAR led
330 to the increase in the AsA content in tomato and maize (Chen et al., 2003;
331 Eltayeb et al., 2006). In the present study, we found that the gene expression of
332 *BO-MDAR1*, *BO-MDAR2* and *BO-DHAR* decreased rapidly in the early stage of
333 post-harvest along with the AsA content reduction during the senescence process
334 of ‘Haitsu’ and ‘Ryokurei’. With the treatment of 1-MCP the gene expression of
335 *BO-MDAR2* was down-regulated, while the gene expression of *BO-MDAR1* was
336 not affected compared with the control in the two cultivars. The changes in gene
337 expression of *BO-MDAR1* and *BO-MDAR2* were not in consistent with the
338 higher AsA content in the 1-MCP treatment, indicating that the regulation of
339 MDAR at the transcription level might be not necessarily involve in the
340 modulation of AsA content by the 1-MCP treatment in the two broccoli cultivars.
341 In contrast to the *BO-MDAR*, the gene expression of *BO-DHAR* was

342 up-regulated clearly by 1-MCP, which coincided with the higher AsA content in
343 the two broccoli cultivars treated with 1-MCP. Differential regulations of
344 MDAR and DHAR in response to postharvest treatments have been also
345 reported in the heat-treated broccoli (Shigenaga et al., 2005).

346 GLDH, an enzyme located on the inner mitochondrial membrane catalyzes
347 the oxidation of last precursor L-galactono-1,4-lactone to AsA. The enzyme
348 activity and transcription level of GLDH have been reported to positively
349 correlated with the AsA content in tobacco and *Arabidopsis thaliana* (Kato and
350 Esaka 1999; Tabata et al., 2001; Gatzek et al., 2002). Tokunaga et al. (2005)
351 reported that in the tobacco BY-2 cells over-expression of GLDH resulted in up
352 to 4-fold increase in the AsA content. However, in tomato and wheat leaves the
353 overexpression of GLDH did not alter the AsA content (Bartoli et al., 2005;
354 Alhagdow et al., 2007). Our results showed that in 'Haitsu' and 'Ryokurei' the
355 expression of *BO-GLDH* decreased significantly after harvest along with the
356 reduction of AsA. Meanwhile, compared with the control the decrease in the
357 gene expression of *BO-GLDH* was delayed by the 1-MCP treatment, which
358 presumably led to the suppression of AsA reduction in the two broccoli cultivars
359 treated with 1-MCP.

360 **5. Conclusion**

361 In the present study, the results demonstrated that the 1-MCP treatment
362 suppressed the reduction of AsA, and this suppression might contribute to the
363 beneficial effects of 1-MCP on the senescence in 'Haitsu' and 'Ryokurei'.

Additionally, the modulation of the AsA reduction by the 1-MCP treatment was highly regulated at the transcription level. The down-regulation of the gene expression of *BO-APX1* and *BO-APX2*, and up-regulation of the gene expression of *BO-DHAR* and *BO-GLDH* led to the suppression of AsA reduction in 'Haitsu' and 'Ryokurei' treated by 1-MCP. These results might provide new insights into the mechanisms by which 1-MCP delayed the senescence in plants.

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500 **Table 1.** Primers used for RT-PCR and lengths of the cDNA of genes related to

501 ascorbate metabolism.

cDNA	Sense primers (upper) and antisense primers (lower)	Length (bp)
BO-APX1	TACCCAGCTGTTAGCGAGGAGTACCAGAAG	724
	CGAGCTCAGAAAGCTTCAAGTGGGCCTCAG	
BO-APX2	AGCTACCCAACGGTGACCGAAGATTACCAG	730
	ACCCGAGCTCAGAAAGCTTCAAGTGTGCCT	
BO-sAPX	TGGCCGTATCAATCGCCGTTTAATCGCACTC	1321
	TGACACGAACGACCAATAGCTAAGGGCCTCA	
BO-MDAR1	TCCCTCTGTTGTTGCAGTTCGAAGAGCCA	1487
	ACTCTGTAGAGCGGCTTGAGCAATCTCGA	
BO-MDAR2	ACCAACTTCAGCGACAACCTCTGACGATCG	1478
	TGCGGTTCATGTTTCCTCTGCCATAGACTC	
BO-DHAR	AGTCGCGCCGGATTTATCAAGCGGTGCGGT	787
	GAGAGAAGCTGATCGGTCGGTCCCTTCGCT	
BO-GLDH	ATGCTCCGATCACTTCTCCTCCGCCGCTC	1749
	TGTTTGGGTCCAGCTCCCTTCGTGCTTTG	

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506 **Table 2.** TaqMan MGB probes and primer sequences used for the real-time

507 quantitative RT-PCRs of genes related to ascorbate metabolism.

cDNA	TaqMan MGB probe	Primers sequence	Orientation
BO-APX1	CCTACCATCTCCC	AGCCCATCAGGGAGCAGTT	Sense
		CCAGCAAGCTGATGGAAAGCA	Antisense
BO-APX2	AGAGAGCAGTTCCC	TGCTCTTAGGTTGTTGGAGCCTAT	Sense
		GGAAATCAGCAAAGGAGATGGT	Antisense
BO-sAPX	CCACCCAATTCTGG	CAAAGAGCTCCTCAACACCAAGT	Sense
		ATCATGCCATCCCAAACGA	Antisense
BO-MDAR1	TTTGTATTGTCACCAAAGAG	ACGGAATGGCCGATGGT	Sense
		GGTCTCTCATAAGGCGCGTAA	Antisense
BO-MDAR2	CGCTGCCAAGACT	GAGCACAGAAATAGTGAAAGCAGATC	Sense
		TCCCCAGCTGCACTGACAA	Antisense
BO-DHAR	CTCGGAGACTGCCC	TCCATCACCACACCCAACAA	Sense
		GCAACACCCTTTGGCAAAA	Antisense
BO-GLDH	TCACGTTGGAAAAGTGAA	TTGCCCTAGATCCTCTCAATGAC	Sense
		TTCCAAAACTCAGCCTCAGCTT	Antisense

508

509 **Figure legends**

510 **Fig. 1.** The figure showing ascorbate metabolism in plants adapted from Noctor
511 and Foyer (1998). Not all reactions are depicted stoichiometrically. AsA is
512 synthesized from L-galactotno-1,4-lactone (GL) by GL dehydrogenase (GLDH)
513 which is assumed to be in mitochondria. AsA is oxidized to
514 monodehydroascorbate (MDA) by ascorbate peroxidase (APX) located in the
515 cytosol and chloroplasts. MDA is converted to AsA by MDA reductase (MDAR)
516 found in the cytosol and chloroplasts. MDA disproportionates
517 non-enzymatically to AsA and dehydroascorbate (DHA), if not rapidly reduced
518 by MDAR. DHA is hydrolyzed to 2,3-diketogulonate unless reduced by DHAR
519 appearing in the cytosol and chloroplasts, using glutathione (GSH) as the
520 reductant. Oxidized glutathione (GSSG) is reduced by glutathione reductase
521 (GR) in the cytosol and chloroplasts.

522 **Fig. 2.** Effect of 1-MCP on the colour score of broccoli heads: (A) 'Haitsu' and
523 (B) 'Ryokurei'. The results shown are the mean ± SE for triplicate samples.
524 Means denoted by the same letter did not differ significantly at $P < 0.05$
525 according to Tukey's HSD test. Some error bars and symbols are hidden by
526 symbols.

527 **Fig. 3.** Effect of 1-MCP on the ethylene production in broccoli: (A) 'Haitsu' and
528 (B) 'Ryokurei'. The results shown are the mean ± SE for triplicate samples.
529 Means denoted by the same letter did not differ significantly at $P < 0.05$
530 according to Tukey's HSD test. Some error bars and symbols are hidden by

symbols.

Fig. 4. Effect of 1-MCP on the ascorbate content in broccoli: (A) 'Haitsu' and (B)'Ryokurei'. The results shown are the mean ± SE for triplicate samples. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Tukey's HSD test. Some error bars and symbols are hidden by symbols.

Fig. 5. Effect of 1-MCP on the expression of genes related to the AsA metabolism in 'Haitsu'. The isoenzymes encoded by discrete genes and distribute in distinct cell organelles. According to the putative localization of the encoding proteins, *BO-APX1*, *BO-APX2* and *BO-MDAR2* are cytosolic genes; *BO-GLDH* is mitochondrial gene; and *BO-sAPX*, *BO-MDAR1* and *BO-DHAR* are chloroplastic genes. The mRNA levels were analyzed by TaqMan real-time quantitative RT-PCR. Real-time RT-PCR amplification of 18S ribosomal RNA was used to normalize the expression of the genes under identical conditions. TaqMan MGB probes and sets of primers used for analysis are shown in Table 2. The results shown are the mean ± SE for triplicate samples. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Tukey's HSD test. Some error bars and symbols are hidden by symbols.

Fig. 6. Effect of 1-MCP on the expression of genes related to the AsA metabolism in 'Ryokurei'. The isoenzymes encoded by discrete genes and distribute in distinct cell organelles. According to the putative localization of the encoding proteins, *BO-APX1*, *BO-APX2* and *BO-MDAR2* are cytosolic genes;

BO-GLDH is mitochondrial gene; and *BO-sAPX*, *BO-MDAR1* and *BO-DHAR* are chloroplastic genes. The mRNA levels were analyzed by TaqMan real-time quantitative RT-PCR. Real-time RT-PCR amplification of 18S ribosomal RNA was used to normalize the expression of the genes under identical conditions. TaqMan MGB probes and sets of primers used for analysis are shown in Table 2. The results shown are the mean ± SE for triplicate samples. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Tukey's HSD test. Some error bars and symbols are hidden by symbols.

Fig. 1

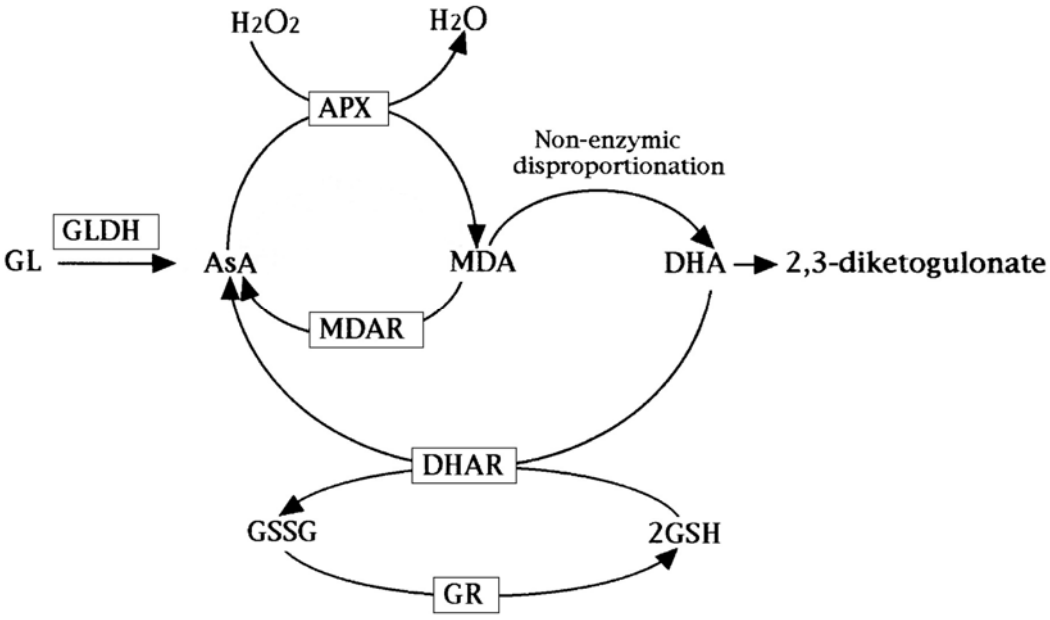

H2O2
H2O
APX
Non-enzymic
disproportionation
GLDH
GL
AsA
MDA
DHA
2,3-diketogulonate
MDAR
DHAR
GSSG
2GSH
GR

Fig. 2

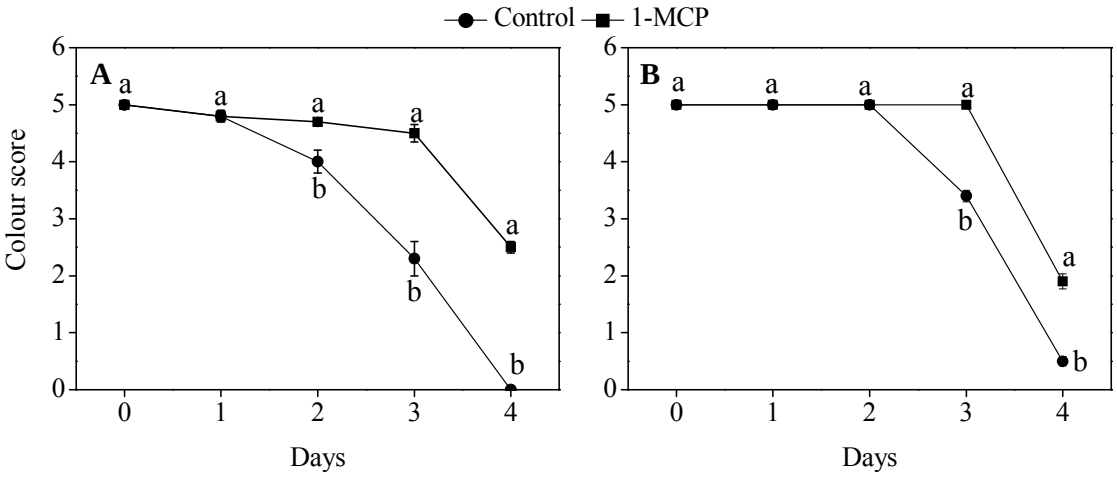


Fig. 3

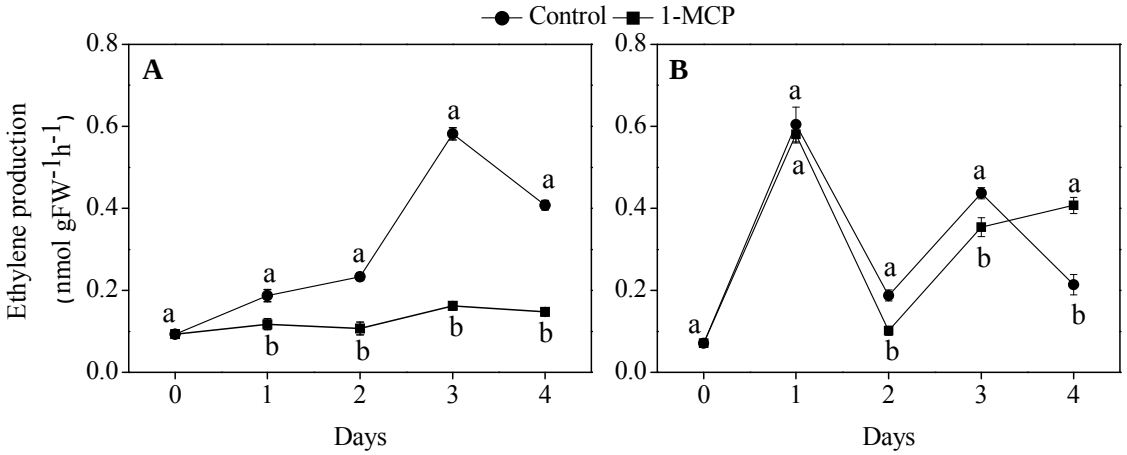

Control
1-MCP
A
B
Ethylene production
(nmol gFW-1h-1)
0.0
0.2
0.4
0.6
0.8
0
1
2
3
4
Days
a
b

Fig. 4

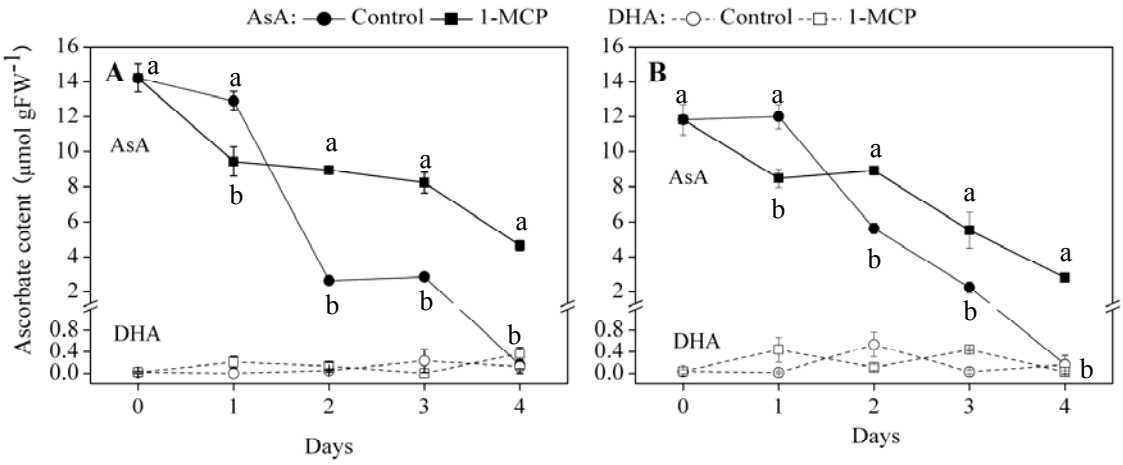

AsA: Control 1-MCP
DHA: Control 1-MCP
A
B
AsA
DHA
Ascorbate cotent (μmol gFW-1)
Days

Fig. 5

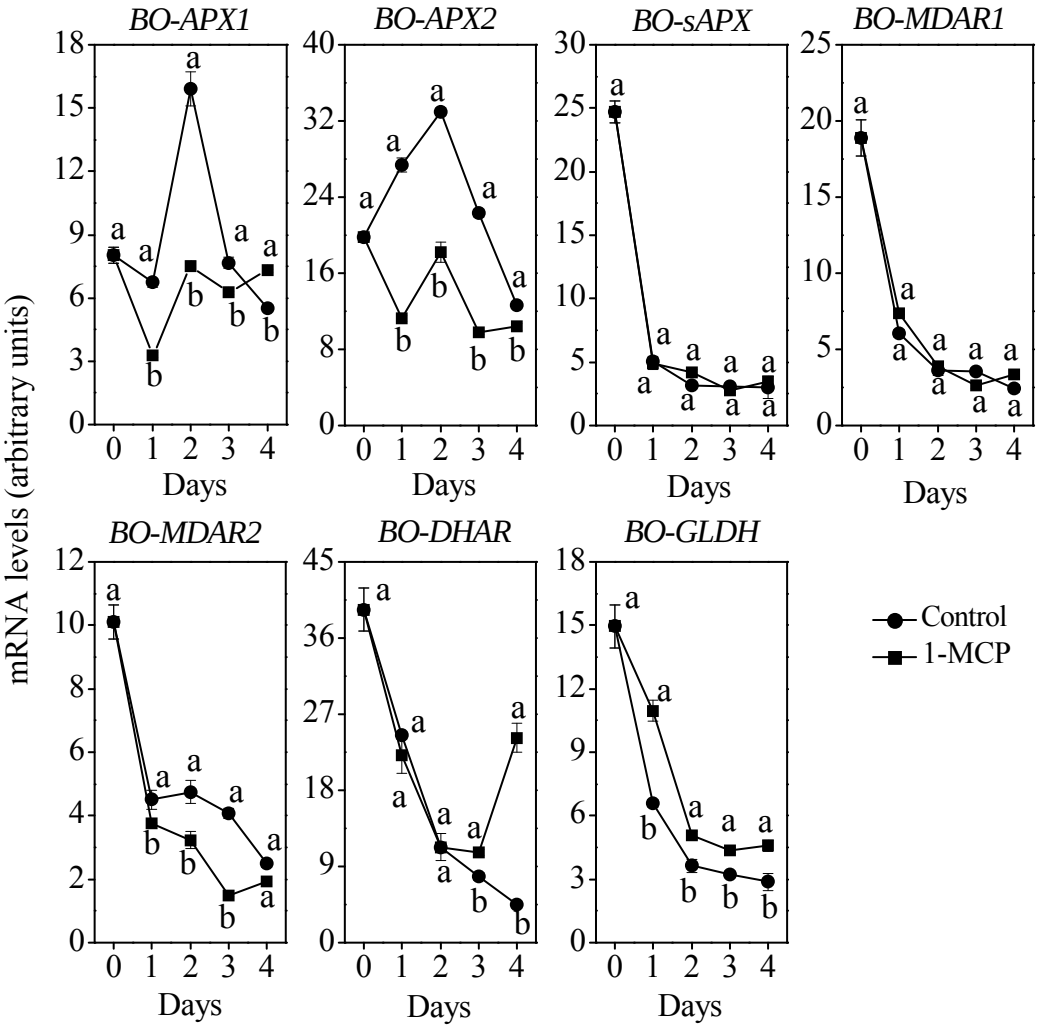
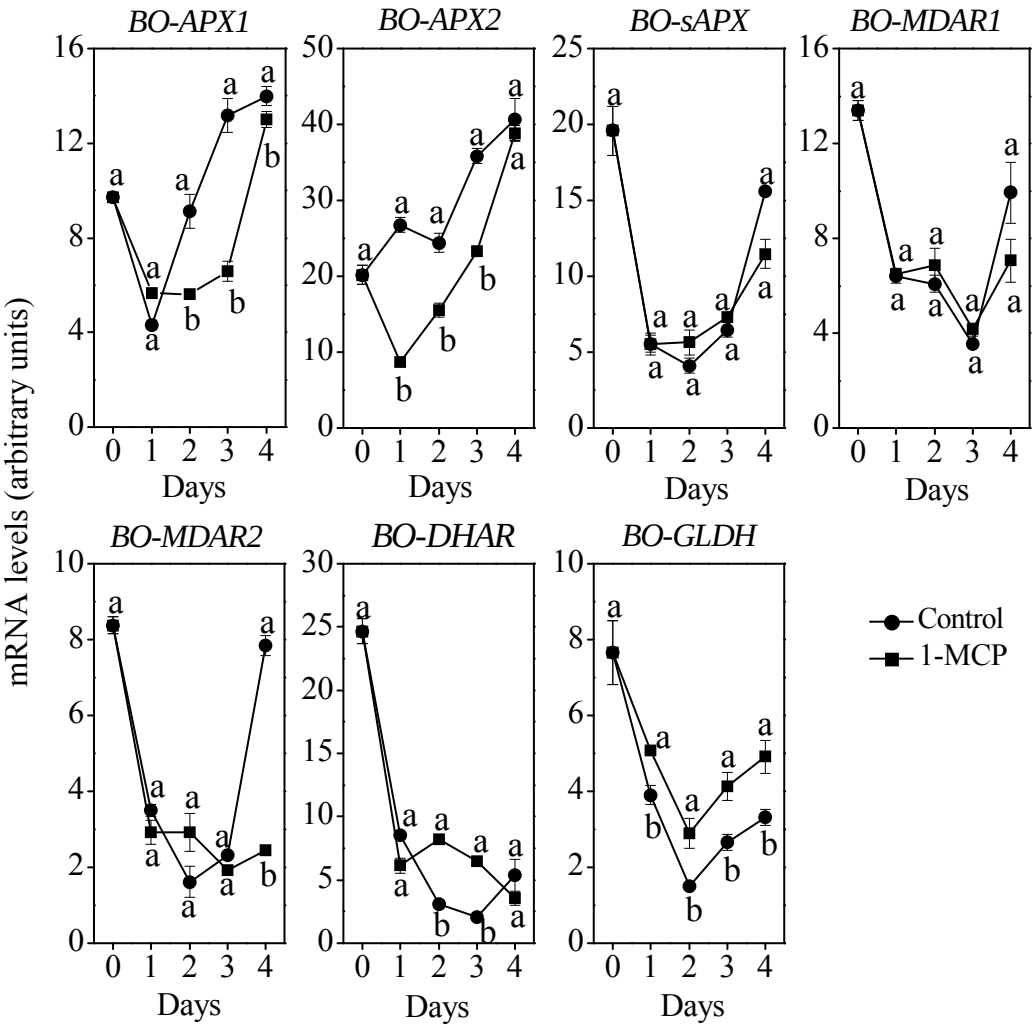

BO-APX1
BO-APX2
BO-sAPX
BO-MDAR1
BO-MDAR2
BO-DHAR
BO-GLDH
mRNA levels (arbitrary units)
Days
Control
1-MCP

Fig. 6


BO-APX1
BO-APX2
BO-sAPX
BO-MDAR1
BO-MDAR2
BO-DHAR
BO-GLDH
mRNA levels (arbitrary units)
Days
Control
1-MCP