Auxin induced carotenoid accumulation in GA and PDJ-treated citrus fruit after harvest

1 **Auxin induced carotenoid accumulation in GA and PDJ-treated citrus fruit after**

2 **harvest**

3 Gang Ma[a,b], Lancui Zhang[a], Rin Kudaka[b], Hayato Inaba[b], Kan Murakami[b], Masashi

4 Yamamoto[b], Nami Kojima[b], Masaki Yahata[a,b], Hikaru Matsumoto[c], Masaya Kato[a,b,*]

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6 [a] Department of Bioresource Sciences, Faculty of Agriculture, Shizuoka University, 836

7 Ohya, Suruga, Shizuoka 422-8529, Japan

8 [b] Graduate School of Integrated Science and Technology, Shizuoka University, 836 Ohya,

9 Suruga, Shizuoka 422-8529, Japan

10 [c] Institute of Fruit Tree and Tea Science, NARO, Shizuoka 424-0292, Japan

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14 *Corresponding author: Masaya Kato

15 Telephone: +81-54-238-4830   Fax: +81-54-238-4830

16 Email: kato.masaya@shizuoka.ac.jp

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Abstract

19

20   Combined spraying of gibberellin (GA) and prohydrojasmon (PDJ) was an effective

21   method to prevent the physiological disorder of peel puffing in citrus fruit. However, the

22   GA and PDJ combined treatment inhibited carotenoid biosynthesis during the fruit

23   ripening process, which led to the mature fruit with poor color. In the present study, to

24   improve the coloration of the GA and PDJ-treated fruit, the effects of postharvest

25   treatments of two auxins, indole-3-acetic acid (IAA) and   1-naphthaleneacetic acid

26   (NAA),   on   carotenoid accumulation were investigated in Satsuma mandarin

27   'Aoshima unshiu' (*Citrus unshiu* Marc.). The results showed that IAA and NAA

28   treatments induced carotenoid biosynthesis in the GA and PDJ-treated fruit after harvest.

29   With the treatments of IAA and NAA, the contents of β-carotene, β-cryptoxanthin, all-

30   *trans*-violaxanthin, and 9-*cis*-violaxanthin were enhanced in both flavedos and juice sacs.

31   The increase in the carotenoid accumulation was accompany with the up-regulation of

32   carotenoid biosynthetic genes in the IAA and NAA treatments. In addition, ethylene

33   production was induced after the IAA and NAA treatments, and the increase of the

34   endogenous ethylene might stimulate carotenoid biosynthesis in citrus fruit. The results

35   presented in this study suggested that the postharvest treatment of auxin was an effective

36   method for improving the coloration of the GA and PDJ-treated fruit.

37   **Key words:** Flavedo; Juice sacs; Gene expression; Carotenoid; IAA; NAA

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1. Introduction

Citrus fruit are one of the richest sources of carotenoids. To date, approximately 115 kinds of carotenoids have been identified in citrus. The carotenoid content and composition are one of the most important factors in determining the appearance and quality of citrus fruit. In carotenoid-abundant varieties, such as mandarins and sweet oranges, the massive accumulation of xanthophylls endows the fruit with appealing orange color (Kato et al., 2004). The accumulation of carotenoid in citrus fruit is not only responsible for the attractive color of peel and pulp, but also has an immense value for human health. Recent epidemiologic and clinical studies suggested that the citrus carotenoids, especially β-cryptoxanthin, which is specifically and massively accumulated in citrus fruit, have a broad of antioxidant and anticancer activities, and dietary intake of them is a promising strategy to prevent cancers, diabetes, and neurological diseases (Park et al., 2017; Dhuique-Mayer et al., 2020; Gansukh et al., 2020; Zacarías-García et al., 2020; Kim et al., 2021).

In past decades, carotenoid accumulation and the transcriptional regulation of carotenoid biosynthetic genes have been extensively investigated in citrus fruit (Kato et al., 2004; Rodrigo et al., 2004; Alós et al., 2006; Ma et al., 2013; Fujii et al., 2021). It was well characterized that carotenoid composition and the expression of carotenoid biosynthetic genes changed greatly during fruit ripening process (Fig. 1). With peel color turning from green to orange, β,ε-carotenoids contents decreased, while β,β-carotenoids contents gradually increased. In most citrus varieties, β-cryptoxanthin and 9-*cis*-violaxanthin were accumulated as the major β,β-carotenoids, and responsible for the appealing orange color of the peel and pulp (Oberholster et al., 2001; Ríos et al., 2010; Rodrigo et al., 2013). Kato et al. (2004) reported that the change from β,ε-carotenoid

67 accumulation to β,β-carotenoid accumulation in citrus fruit was accompanied with a

68 sharp decrease in the expression of *CitLCYe*, and a simultaneous increase in the

69 expression of *CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitHYb*, and *CitZEP*.

70   Compared to other tropical fruit, citrus fruit have a relatively long shelf-life after

71 harvest. Nowadays, to delay the deterioration and maintain the fruit quality and flavor

72 after harvest, several explorations on the optimum storage conditions and postharvest

73 technologies have been conducted (Kader et al., 2002; Matsumoto et al., 2009; Ma et al.,

74 2012; Moraes Bazioli et al., 2019). It was generally acknowledged that low temperature

75 was optimal for carotenoid accumulation, and hence improved fruit coloration in both

76 peel and pulp of citrus fruit after harvest (Cohen et al., 1978; Matsumoto et al., 2009;

77 Carmona et al., 2012). In addition, ethylene is a crucial factor for stimulating carotenoid

78 biosynthesis in citrus fruit (Young and Jahn, 1972; Wheaton and Stewart, 1973; Rodrigo

79 and Zacarías, 2007; Zhu et al., 2021). The postharvest application of ethylene has been

80 world widely used to promote peel degreening of citrus fruit. Moreover, Ma et al. (2015)

81 reported that the combination of ethylene treatment with red LED light irradiation

82 induced the accumulation of β-cryptoxanthin and lutein in the flavedos and led to a better

83 color of fruit after harvest. Auxin is an important plant hormone, which interacts with

84 ethylene in the regulation of plant growth and development. To date, however, the

85 information on the roles of auxin in citrus is still limited, and its effects on the carotenoid

86 accumulation has not been reported in citrus fruit after harvest (Ma et al., 2021).

87   Citrus fruit of the loose-skin varieties are prone to develop a physiological disorder

88 of peel puffing during the ripening process. The peel puffing is characterized by albedo

89 breakdown and separation between peel and pulp, which leads to the formation of air

90 spaces between the peel and pulp in the mature fruit. The occurrence of peel puffing

significantly affects fruit taste and quality, and makes fruit unmarketable. Moreover, the puffy fruit cannot be stored for a long period, because they are susceptible to postharvest decay and deteriorate rapidly after harvest. To prevent the peel puffing, a combination of gibberellin (GA) and prohydrojasmon (PDJ) was sprayed on tree at the color break stage in Japan (Kawase et al., 1981; Garcia-Luis et al., 1985; Sato et al., 2015). However, the GA and PDJ treatment reduced the loss of chlorophylls and delayed the accumulation of carotenoid, which led to the mature fruit with poor color (Ma et al., 2021). In the present study, to improve the coloration of the GA and PDJ-treated fruit, the effects of two auxins, indole-3-acetic acid (IAA) and 1-naphthaleneacetic acid (NAA), on the carotenoid accumulation were investigated in citrus fruit after harvest. Moreover, the changes in the expression of carotenoid biosynthetic genes in response to NAA and IAA treatments were discussed in this study. The results presented in this study will not only contribute to elucidating the roles of auxin in citrus fruit, but also provide an alternative strategy for enhancing the nutritional and commercial qualities of GA and PDJ-treated citrus fruit after harvest.

2. Materials and methods

2.1. Plant materials and treatments

The Satsuma mandarin 'Aoshima unshiu' (*Citrus unshiu* Marc.) cultivated at the Fujieda Farm of Shizuoka University (Shizuoka, Japan) were used as materials. To prevent puffing, fruit were sprayed with 5 mg L^{-1} of GA and 50 mg L^{-1} of PDJ on tree on September 16th, 2016. The mature fruit were harvested from the farm on December 6th (210 days after full bloom) and dipped in the solution of 500 μM IAA or NAA for 1 h. Fruit dipped in deionized water were used as the control. After air-dried, the fruit were

115   placed in plastic containers and stored at 20 °C for 6 d under 85–90 % RH. After storage,

116   the flavedos and juice sacs of the whole fruit in each treatment were separated,

117   immediately frozen in liquid nitrogen, and stored at −80 °C until use. In the present study,

118   the fruit of control used in Table 1 and Fig. 2 were untreated fruit without any treatment

119   during the ripening process.

120   *2.2. Color measurement*

121   The fruit color was measured with a colorimeter (NR-11, Nippon Denshoku, Japan),

122   and the CIE 1976 (L*, a*, b*) color scale was used. The citrus color index (CCI) = 1000

123   × a*/ (L* × b*) and hue angle $H°$ = arctangent (b*/a*) were calculated according to

124   previous methods (Ma et al., 2015).

125   *2.3. Ethylene measurements.*

126   Ethylene production of citrus fruit was measured after 3 d and 6 d of storage. Citrus

127   fruit were placed in 2 L sealed plastic containers, each equipped with a septum, and the

128   containers were kept sealed for 2 h at 20 °C before sampling. For each treatment, we used

129   three containers, and five fruit were placed in one container. Headspace samples were

130   taken with a 1 mL plastic hypodermic syringe and were injected into a gas chromatograph

131   (Hitachi 163) for ethylene analysis. The rate of ethylene production was expressed as

132   nmol ethylene per hour per kilogram fresh weight.

133   *2.4. Extraction and determination of chlorophylls*

134   Chlorophyll was extracted from the flavedos using 5 mL of *N,N*-dimethylformamide.

135   The samples were centrifuged at 3000 rpm for 10 min after the overnight incubation at

136   room temperature. The absorbances of each sample at 664 and 647 nm were determined

137   by spectrophotometer. The contents of chlorophyll a, chlorophyll b, and total chlorophyll

138   were calculated according to Moran's method and expressed as milligrams per kilogram

139   fresh weight (Moran,1982).

140   *2.5. Extraction and determination of carotenoids*

141   The identification and quantification of carotenoids in the flavedos and juice sacs

142   were conducted according to Kato's methods (Kato et al., 2004). Carotenoids were

143   extracted from the flavedos and juice sacs using the extraction solution (hexane: acetone:

144   ethanol, 2: 1: 1, *v/v*) containing 10 % (*w/v*) magnesium carbonate basic and 0.1 % (*w/v*)

145   2,6-di-tert-butyl-4-methylphenol. The organic solvents were evaporated by rotary

146   evaporator at maximum 35 °C under vacuum condition. After the organic solvents were

147   completely evaporated, the samples were saponified overnight with 20 % (*w/v*)

148   methanolic KOH. Then, the NaCl-saturated water was added to remove the water-soluble

149   extracts. The carotenoids repartitioned into the diethylether phase were collected and

150   evaporated to dryness. Subsequently, the residue was redissolved in 5 mL of solution

151   (TBME: methanol, 1:1, *v/v*), and an aliquot (20 μL) was injected to the reverse-phase

152   HPLC system (Jasco, Tokyo, Japan) fitted with a YMC Carotenoid S-5 column (Waters,

153   Milford, MA). The contents of the five major carotenoids (β-carotene, β-cryptoxanthin,

154   all-*trans*-violaxanthin, 9-*cis*-violaxanthin, and lutein) in citrus fruit were calculated by

155   the standard curves and expressed as milligrams per kilogram fresh weight (Kato et al.,

156   2004). The total carotenoid was calculated by summing the five major carotenoids.

157   Carotenoid quantification was performed in three replicates.

158   *2.6. Total RNA extraction and real-time quantitative RT-PCR*

159   Total RNA was extracted from the fruit according to Kato's method (Kato et al.,

160   2004). The total RNA was purified and on-column DNase digestion was performed by

161   using the RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was

162   synthesized from 1 μg of purified RNA using TaqMan reverse transcription Reagents

163   (Applied Biosystems, Foster City, CA).

164   In this study, TaqMan MGB probes and sets of primers for carotenoid metabolic

165   genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*, *CitLCYb2*, *CitLCYe*, *CitHYb*, *CitZEP*,

166   *CitNCED2*, and *CitNCED3*) and ethylene biosynthetic genes (*CitACS1*, *CitACS2*, and

167   *CitACO*) were designed using the Primer Express software. TaqMan Ribosomal RNA

168   Control Reagents VIC Probe (Applied Biosystems) was used as endogenous control to

169   normalize the raw data. TaqMan real-time PCR was performed by using TaqMan

170   Universal PCR Master Mix (Applied Biosystems) and StepOnePlus^{TM} Real-Time PCR

171   System (Applied Biosystems). The reaction mixture was made according to the previous

172   method (Ma et al., 2021), and template cDNA, a TaqMan MGB Probe (250 nM), and

173   primers (900 nM) were contained. The thermal cycling conditions consisted of 95 °C for

174   10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Real-time quantitative

175   RT-PCR was performed in three replicates for each sample.

176   *2.7. Statistical analysis*

177   All data were shown as the mean ± SE. Tukey's HSD test (at $p < 0.05$) and student's

178   t-test (at $p < 0.01$ and $p < 0.05$) were used to determine significant differences among

179   different treatments.

180

181   **3. Results**

182   *3.1. Changes of color, chlorophylls and carotenoids contents in the GA and PDJ-treated*

183   *fruit*

184   'Aoshima unshiu' is a loose-skin citrus variety, and prone to develop the

185   physiological disorder of peel puffing. In the mature fruit of 'Aoshima unshiu', albedo

186   broke down and peel severely separated from pulp (Fig. 2). To prevent the peel puffing,

187   a mixture of GA and PDJ was sprayed on tree at the color break stage in 'Aoshima unshiu'.

188   As shown in Fig. 2, the peel puffing was prevented in the GA and PDJ-treated fruit.

189   However, fruit coloration was delayed after the GA and PDJ combined treatment. In the

190   un-treated fruit, the peel turned orange completely in December. Whereas the peel of the

191   GA and PDJ-treated fruit was in light yellow color with green spots (Fig. 2). The hue

192   angle of the GA and PDJ-treated fruit was higher than that of untreated fruit (Table 1).

193   The color index CCI of the un-treated fruit reached 8.45 in December, while it was only

194   3.43 in the GA and PDJ-treated mature fruit. The GA and PDJ treatment delayed the loss

195   of chlorophyll, and the contents of chlorophylls in the GA and PDJ-treated fruit were

196   higher than the un-treated fruit (Table 1). In addition, carotenoid biosynthesis was

197   inhibited by the GA and PDJ treatment. The contents of the major carotenoids (β-

198   cryptoxanthin, lutein, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin) in the GA and PDJ-

199   treated fruit were much lower than those in the un-treated fruit (Table 1).

200   *3.2. Changes of carotenoid metabolism in the flavedos of GA and PDJ-treated fruit after*

201   *the postharvest treatments of IAA and NAA*

202   To solve the problem of the poor coloration of the GA and PDJ-treated fruit, the

203   effects of postharvest treatments of IAA and NAA on carotenoid metabolism were

204   investigated in this study. In the mature citrus fruit, 9-*cis*-violaxanthin was the major

205   carotenoid accumulated in the flavedos, followed by β-cryptoxanthin and lutein. The

206   contents of β-carotene and α-carotene in the flavedos were extremely low. As shown in

207   Fig. 3, the contents of carotenoids investigated in this study were simultaneously

208   enhanced by the IAA and NAA treatments. The total carotenoid content in the IAA and

209   NAA treatments was 1.6 and 1.9 times of the control, respectively (Fig. 3). In addition,

210   the expression of carotenoid metabolic genes (*CitPSY*, *CitPDS*, *CitZDS*, *CitLCYb1*,

211   *CitLCYb2*, *CitLCYe*, *CitHYb*, *CitZEP*, *CitNCED2*, and *CitNCED3*) was analyzed in this

212   study. As shown in Fig. 4, the expression of carotenoid biosynthetic genes *CitPSY*,

213   *CitPDS*, and *CitHYb* was up-regulated by the IAA and NAA treatments on the 6th day

214   after harvest. Whereas, the expression of carotenoid catabolic gene *CitNCED3* was

215   significantly down-regulated by the IAA and NAA treatments on the 6th day after harvest.

216   The up-regulation of the carotenoid biosynthetic genes and down-regulation of carotenoid

217   catabolic gene were well consistent with the enhanced carotenoid accumulation in the

218   IAA and NAA treatments.

219   *3.3. Changes of carotenoid metabolism in the juice sacs of GA and PDJ-treated fruit after*

220   *the postharvest treatments of IAA and NAA*

221   In this study, the effects of IAA and NAA on the carotenoid accumulation were also

222   investigated in the juice sacs after harvest. The contents of carotenoids in the juice sacs

223   were lower than those in the flavedos. In the juice sacs, β-cryptoxanthin was the major

224   carotenoid followed by lutein and 9-*cis*-violaxanthin. With the IAA and NAA treatments,

225   the contents of β-carotene, β-cryptoxanthin, all-*trans*-violaxanthin, 9-*cis*-violaxanthin, as

226   well as total carotenoid content were increased in the juice sacs (Fig. 5). Gene expression

227   results showed that expression of *CitPDS*, *CitZDS*, and *CitLCYb2* was simultaneously up-

228   regulated by the IAA and NAA treatments, which led to an increased accumulation of

229   carotenoid in the juice sacs (Fig. 6). Similar to the flavedo, the expression of *CitNCED3*

230   in the juice sacs was also significantly down-regulated by the IAA and NAA treatments

231   on the 6th day after harvest.

232   *3.4. Changes of ethylene production and ethylene biosynthetic genes in the GA and PDJ-*

233   *treated fruit after the postharvest treatments of IAA and NAA*

234   To elucidate the mechanism that auxin treatment improved carotenoid accumulation

235 in the GA and PDJ-treated fruit, ethylene production was investigated in this study. Citrus

236 fruit is non-climacteric, and produces small amounts of ethylene in the mature fruit.

237 During the storage period, ethylene production kept at a low level in the control (Fig. 7).

238 With the NAA and IAA treatments, ethylene production was induced during the storage

239 period. On the 6th day after harvest, ethylene production in the IAA and NAA treatments

240 was much higher than that of the control (Fig. 7). Gene expression results showed that

241 postharvest treatments of IAA and NAA induced the expression of ethylene biosynthetic

242 genes, *CitACS1*, *CitACS2*, and *CitACO*, in the GA and PDJ-treated fruit on the 6th day

243 after harvest (Fig. 8).

244 **4. Discussion**

245   Peel puffing is a serious physiological disorder occurred in loose-skin citrus varieties.

246 The peel puffing impairs fruit quality and taste, and thus reduces the marketability of

247 citrus fruit. In past decades, extensive efforts have been made to reduce the peel puffing

248 of citrus fruit (Kawase et al., 1981; Garcia-Luis et al., 1985; Shiraishi et al., 1999; Ibáñez

249 et al., 2014; Martinelli et al., 2015; Sato et al., 2015). Among them, the combined spraying

250 of GA and PDJ at the color break stage was found to be an effective method to prevent

251 the occurrence of peel puffing. However, the combined spraying of GA and PDJ treatment

252 delayed the color development of citrus fruit during the ripening process, which limited

253 its application in peel puffing (Fig. 2). In a previous study, to improve the coloration of

254 the GA and PDJ-treated fruit, we treated the fruit with NAA and ABA three times on tree

255 after the spraying of GA and PDJ. The results showed that NAA and ABA treatments

256 accelerated the color changes from green to orange in the GA and PDJ-treated fruit. In

257 the NAA and ABA treatments, chlorophylls contents were reduced rapidly, and β,β-

258 xanthophylls contents were increased (Ma et al., 2021). Although pre-harvest sprays of

259   NAA and ABA on tree were proven to promote the coloration of citrus fruit, they required

260   expensive labor and chemical costs, and had potential negative impacts on trees. In the

261   present study, to develop an alternative and practicable method, we investigated the

262   effects of postharvest treatments of IAA and NAA on the coloration of GA and PDJ-

263   treated fruit. The results showed that both IAA and NAA treatments improved the

264   coloration of the GA and PDJ-treated fruit after harvest. Compared with the control, the

265   fruit in the IAA and NAA treatments displayed attractive orange color (Fig. 3A).

266   Moreover, the postharvest treatments of IAA and NAA did not diminish the protective

267   effect of GA and PDJ on peel puffing, and the fruit in the IAA and NAA treatments did

268   not develop peel puffing during the storage period (Fig. 4A).

269   In the present study, the mature fruit harvested at 210 d after full bloom were used as

270   materials. During the storage period, chlorophylls contents decreased to a low level in the

271   control as well as the IAA and NAA treatments in the flavedos. Compared with the control,

272   postharvest treatments of IAA and NAA did not significantly affect the contents of

273   chlorophylls on the 6th day after harvest (Fig. S1). In citrus, carotenoids are the

274   predominant pigments responsible for the external and internal coloration of the mature

275   fruit, and their contents and compositions are important indexes for the maturity and

276   quality of citrus fruit. In the present study, to elucidate the roles of auxins in citrus fruit

277   coloration, the changes in carotenoid accumulation were investigated in the GA and PDJ-

278   treated fruit after harvest. In recent years, an increasing number of researches on the

279   postharvest storage of citrus fruit were conducted (Young and Jahn, 1972; Wheaton and

280   Stewart, 1973; Ma et al., 2012, 2015; Moraes Bazioli et al., 2019; Zhu et al., 2021).

281   Crucial factors, such as low temperature, ethylene, and red light, that enhanced carotenoid

282   accumulation during storage were identified, and novel postharvest treatments were

283   developed, which greatly improved the coloration and commercial quality of citrus fruit.

284   Auxin is one of the important plant hormones that are involved in plant growth and

285   development. In plants, the application of auxins exhibited contradictory roles in

286   regulating fruit ripening and carotenoid accumulation (Davies et al., 1997; Paul et al.,

287   2012; Ma et al., 2021). In tomato, auxin was found to be a negative regulator of fruit

288   ripening and carotenoid accumulation. In IAA-treated tomato fruit, ripening was retarded

289   and carotenoid biosynthesis was inhibited (Su et al., 2015). In citrus, in contrast, the spays

290   of NAA on tree accelerated the ripening of citrus fruit, and enhanced carotenoid

291   accumulation (Ma et al., 2021). In addition, the application of NAA in citrus has been

292   reported to stimulate plant growth, increase fruit setting and production, and improve fruit

293   quality (Greenberg et al., 1996, 2006, 2010). In the present study, the results showed that

294   both IAA and NAA effectively increased carotenoids contents in the flavedos and juice

295   sacs of citrus fruit after harvest (Figs. 3-6). The enhanced contents of carotenoids by the

296   IAA and NAA treatments contributed to improving the coloration of GA and PDJ-treated

297   fruit. Moreover, gene expression results suggested that that the changes of the carotenoid

298   accumulation in the IAA and NAA treatments was highly regulated at the transcriptional

299   level. In the flavedos, the expression of *CitPSY*, *CitPDS*, and *CitHYb* was up-regulated,

300   while the expression of *CitNCED3* was down-regulated by the treatments of IAA and

301   NAA. The up-regulation of the carotenoid biosynthetic genes and down-regulation of

302   carotenoid catabolic gene were well consistent with the increases of carotenoids contents

303   in the flavedos treated with IAA and NAA. In the juice sacs, the higher carotenoids

304   contents in the IAA and NAA treatments were attributed to the up-regulation of *CitPDS*,

305   *CitZDS*, and *CitLCYb2*, and down-regulation of *CitNCED3* after harvest. In addition, we

306   found that the expression of *CitNCED3* was simultaneously down-regulated in flavedos

307   and juice sacs by the IAA and NAA treatments. These results indicated that the inhibition

308   of carotenoid catabolism might be an important mechanism that IAA and NAA treatments

309   enhanced carotenoid accumulation in citrus fruit after harvest.

310   Although citrus is non-climacteric fruit producing small amounts of ethylene during

311   the ripening process, ethylene is a crucial factor that regulates carotenoid accumulation

312   in citrus fruit (Young and Jahn, 1972; Wheaton and Stewart, 1973; Rodrigo and Zacarías,

313   2007; Zhu et al., 2021). Nowadays, postharvest application of ethylene has been world

314   widely used for the degreening of citrus fruit. Exogenous treatment of ethylene effectively

315   induced carotenoid accumulation and improved the coloration of citrus fruit (Rodrigo and

316   Zacarías, 2007; Matsumoto et al., 2009; Ma et al., 2015). In addition, endogenous

317   ethylene plays an important role in carotenoid biosynthesis in citrus fruit, even though it

318   is at a low level during the ripening process. Carmona et al. (2012) reported that

319   endogenous ethylene was involved in the regulation of carotenoid biosynthetic gene

320   expression in the peel of citrus fruit during both natural ripening and postharvest. In plants,

321   there is a complex interaction between ethylene and auxin, and they act either

322   antagonistically or synergistically to regulate plant growth and development in a specie-

323   specific manner (Rahman et al., 2001; Paul et al., 2012; Böttcher et al., 2013). In the

324   present study, the results showed that the postharvest treatments of IAA and NAA induced

325   the expression of ethylene biosynthetic genes, *CitACS1*, *CitACS2*, and *CitACO*, and the

326   ethylene production in the GA and PDJ-treated fruit was significantly increased by the

327   IAA and NAA treatments on the 6th day after harvest (Fig. 7 and Fig. 8). The increase of

328   endogenous ethylene was well consistent with the up-regulation of carotenoid

329   biosynthetic genes and the enhanced carotenoids contents in the IAA and NAA treatments.

330   The similar phenomenon was also described by other plant species, such as apple, peach,

331 and grape (Kondo et al., 2004; Trainotti et al., 2007; Li and Yuan, 2008). Thus, these

332 results indicated that the regulation of carotenogenesis by auxins might be exerted

333 through the induction of ethylene production. In future study, further researches on the

334 effects of IAA and NAA on the ethylene perception and the expression of ethylene

335 response factors will be conducted to elucidate the regulatory mechanisms of auxins on

336 ethylene production in citrus fruit.

337

338 **5. Conclusion**

339 　　In this study, the effects of two auxins, IAA and NAA, on carotenoid accumulation

340 were investigated in the GA and PDJ-treated citrus fruit after harvest. The results showed

341 that the postharvest treatments of IAA and NAA were effective to induce carotenoid

342 biosynthesis in the flavedos and juice sacs, and improve the coloration of GA and PDJ-

343 treated fruit. Moreover, the enhanced carotenoid accumulation by the IAA and NAA

344 treatments might be mediated by increasing ethylene production in citrus fruit after

345 harvest. The results presented in this study suggested that postharvest auxin treatment has

346 a great potential for improving the carotenoid accumulation and coloration of GA and

347 PDJ-treated fruit. In future study, further research on the optimization immersion time of

348 IAA and NAA is still needed, which will contribute to commercial application of auxin

349 in the GA and PDJ-treated puffy fruit.

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351 **Declaration of competing interest**

352 The authors report no declarations of interest.

353

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359　**CRediT authors' contribution statement**

360　　M.K., H.M., and G.M. conceived and designed the experiments. G.M. and LC.Z

361　wrote the paper. R.K., K.M., Y.M., and N.K. carried out the experiments and analyzed the

362　data. M.Y. and H.I. contributed to sample collection. All authors approved the final

363　revision to be published.

364

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Table 1 Fruit color, chlorophyll and carotenoid contents in the GA and PDJ-treated and un-treated citrus fruit harvested in December. Asterisks indicate significant differences compared to the control by Student's *t*-test: *$p < 0.05$, **$p < 0.01$.

Color and pigment		Untreated	GA + PDJ
Fruit color	Hue angle	61.42±2.57	77.39±4.54**
	CCI	8.45±1.08**	3.43±1.18
Chlorophyll (mg kg^{-1})	Chlorophyll a	4.49±0.40	31.88±0.65**
	Chlorophyll b	4.10±0.43	8.81±0.11**
	Total chlorophyll	8.22±0.67	40.69±0.76**
Carotenoid (mg kg^{-1})	β-Carotene	0.32±0.11*	0.13±0.05
	α-Carotene	0.68±0.04*	0.25±0.01
	β-Cryptoxanthin	26.36±3.21**	7.48±0.55
	Lutein	25.44±2.46**	10.02±0.96
	All-*trans*-violaxanthin	6.20±0.59**	2.18±0.13
	9-*Cis*-violaxanthin	47.08±3.85**	19.08±1.12
	Total carotenoid	106.08±14.48**	39.15±3.90

Figure legends

Fig. 1. Metabolic pathway involved in biosynthesis of carotenoid via the MEP pathway in citrus. MEP pathway, methylerthritol-4-phosphate pathway; GGPP, geranylgeranyl diphosphate. The enzymes investigated in this study are: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYb, lycopene β-cyclase; LCYe, lycopene ε-cyclase; HYb, β-ring hydroxylase; ZEP, zeaxanthin epoxidase; NCED, 9-*cis*-epoxycarotenoid dioxygenase.

Fig. 2. The appearance of the GA and PDJ-treated and un-treated citrus fruit harvested in December. In un-treated fruit, the peel puffing occurred. In GA and PDJ-treated fruit, the peel puffing did not occur, while the coloring of fruit was delayed.

Fig. 3. Changes of the appearance (A) and carotenoid content (B) in the flavedos of GA and PDJ-treated citrus fruit on the 6th day after postharvest treatments of IAA and NAA. β-Car, β-carotene; α-Car, α-carotene; β-Cry, β-cryptoxanthin; Lut, lutein; T-vio, all-*trans*-violaxanthin; C-vio, 9-*cis*-violaxanthin; Total, total carotenoid. The total carotenoid was calculated by summing β-carotene, α-carotene, β-cryptoxanthin, lutein, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin. Columns and bars represent the means and SE (*n*=3), respectively. Different letters (a, b, and c) indicate significant differences at the 5 % level by Tukey's test.

Fig. 4. Changes of the carotenoid biosynthetic genes expression in the flavedos of GA and PDJ-treated citrus fruit on the 6th day after postharvest treatments of IAA and NAA. The mRNA levels were analyzed by TaqMan real-time RT-PCR, and the expression of 18S ribosomal RNA (rRNA) was used as a control to normalize the raw data. Columns and bars represent the means and SE (*n*=3), respectively. Different letters (a, b, and c) indicate significant differences at the 5 % level by Tukey's test.

Fig. 5. Changes of the appearance (A) and carotenoid content (B) in the juice sacs of GA and PDJ-treated citrus fruit on the 6th day after postharvest treatments of IAA and

NAA. β-Car, β-carotene; α-Car, α-carotene; β-Cry, β-cryptoxanthin; Lut, lutein; T-vio, all-*trans*-violaxanthin; C-vio, 9-*cis*-violaxanthin; Total, total carotenoid. The total carotenoids were calculated by summing β-carotene, α-carotene, β-cryptoxanthin, lutein, all-*trans*-violaxanthin, and 9-*cis*-violaxanthin. Columns and bars represent the means and SE (*n*=3), respectively. Different letters (a, b, and c) indicate significant differences at the 5 % level by Tukey's test.

Fig. 6. Changes of the carotenoid biosynthetic genes expression in the juice sacs of GA and PDJ-treated citrus fruit on the 6th day after postharvest treatments of IAA and NAA. The mRNA levels were analyzed by TaqMan real-time RT-PCR, and the expression of 18S ribosomal RNA (rRNA) was used as a control to normalize the raw data. Columns and bars represent the means and SE (*n*=3), respectively. Different letters (a, b, and c) indicate significant differences at the 5 % level by Tukey's test.

Fig. 7. Changes of the ethylene production in the GA and PDJ-treated citrus fruit on the 3rd and 6th days after postharvest treatments of IAA and NAA. Columns and bars represent the means and SE (*n*=3), respectively. Different letters (a, b, and c) indicate significant differences at the 5 % level by Tukey's test. N.D., not detectable.

Fig. 8. Changes of the ethylene biosynthetic genes expression in the GA and PDJ-treated citrus fruit on the 6th day after postharvest treatments of IAA and NAA. Columns and bars represent the means and SE (*n*=3), respectively. Different letters (a, b, and c) indicate significant differences at the 5 % level by Tukey's test.
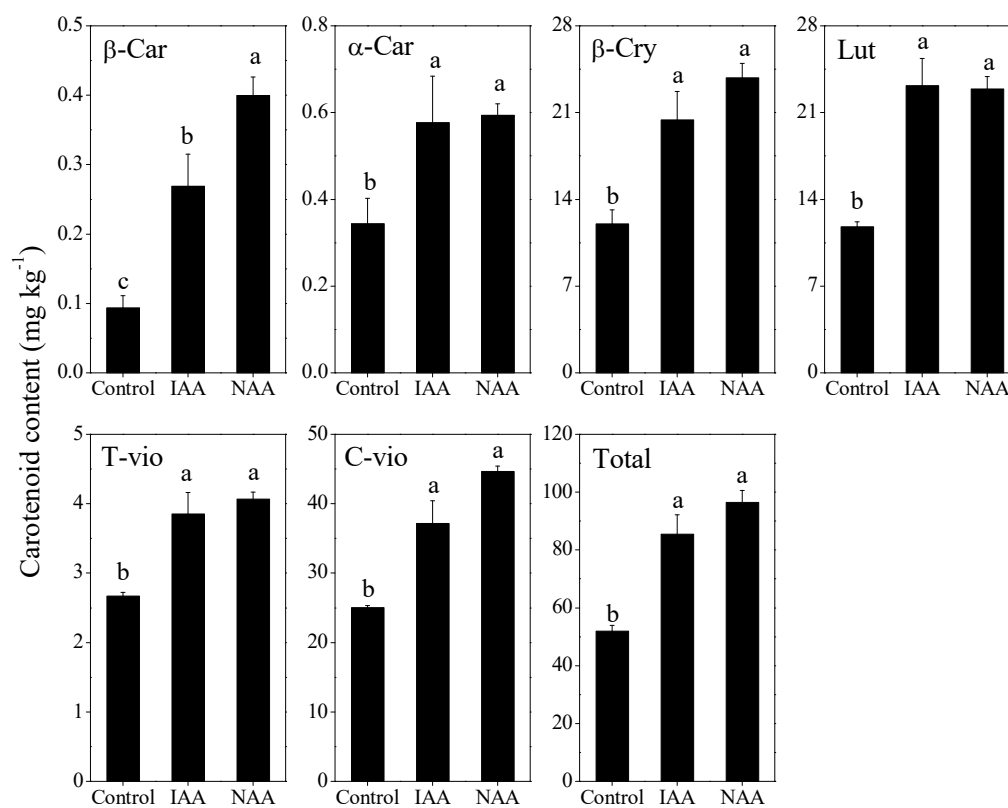
Figure 1
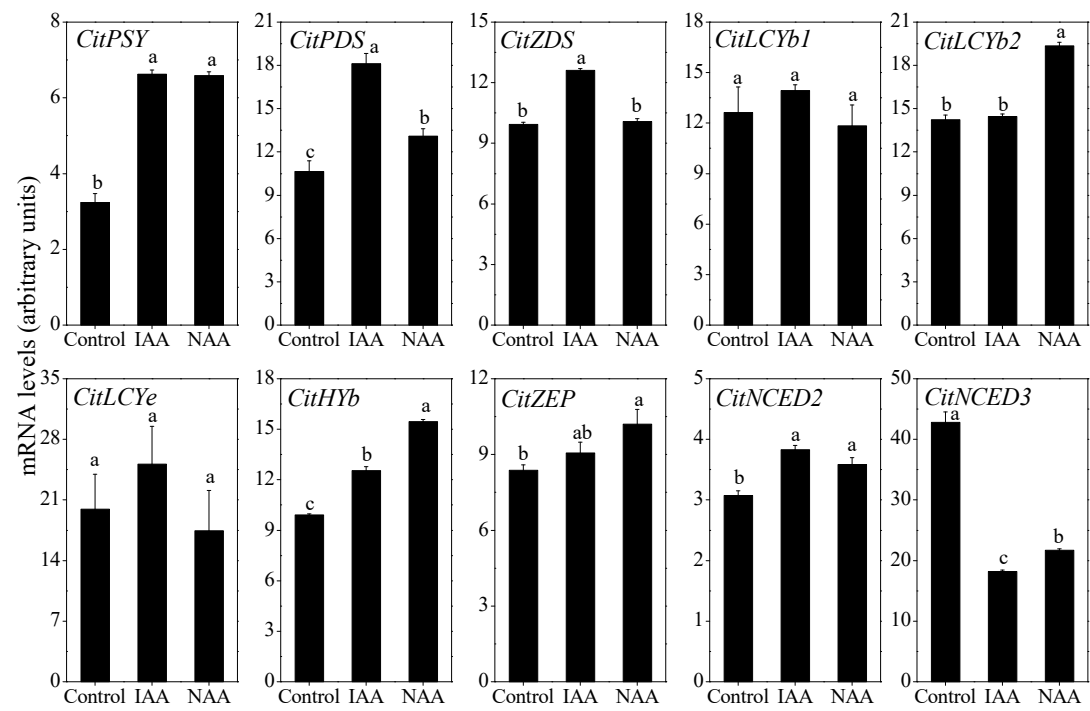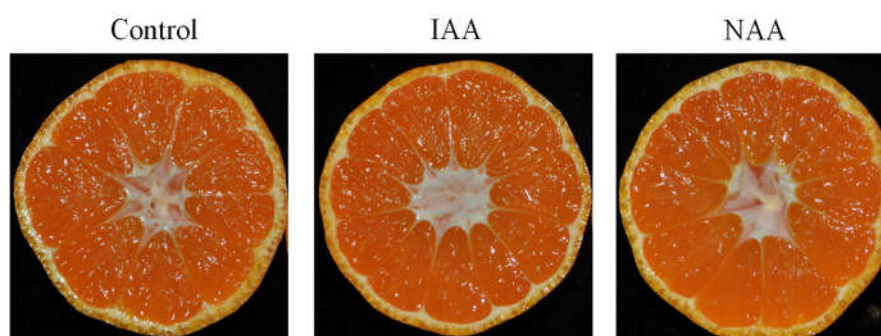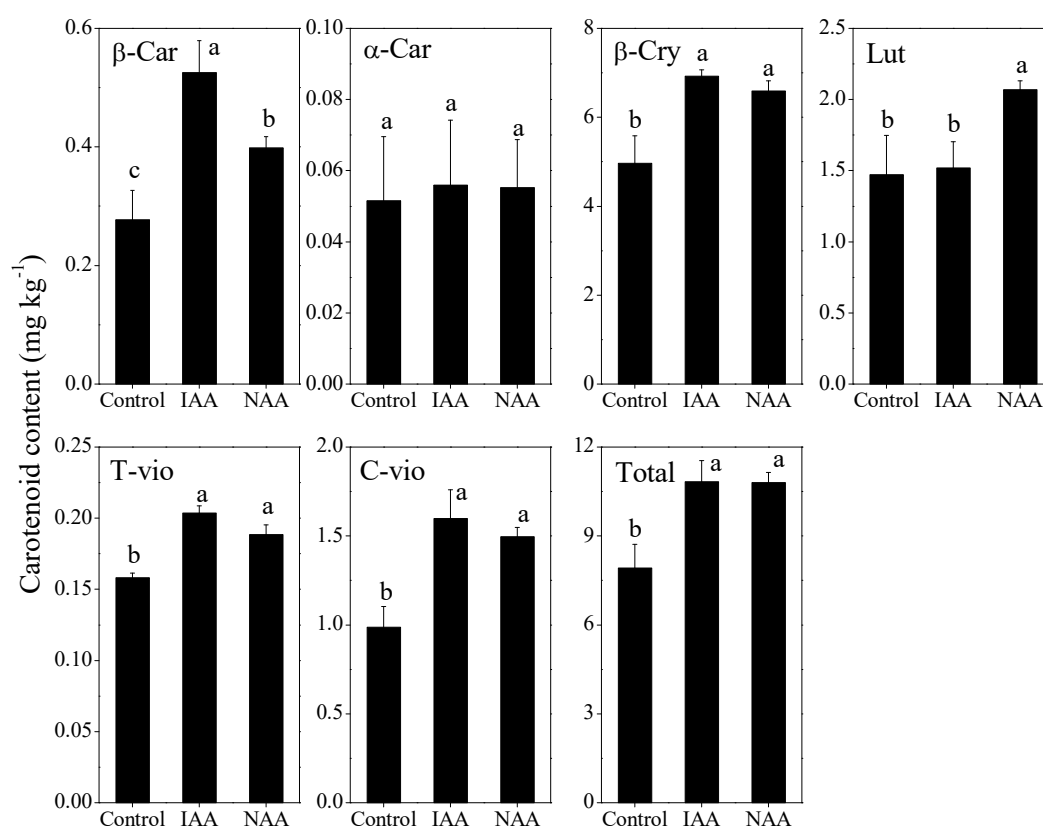
Figure 2

Figure 3

A
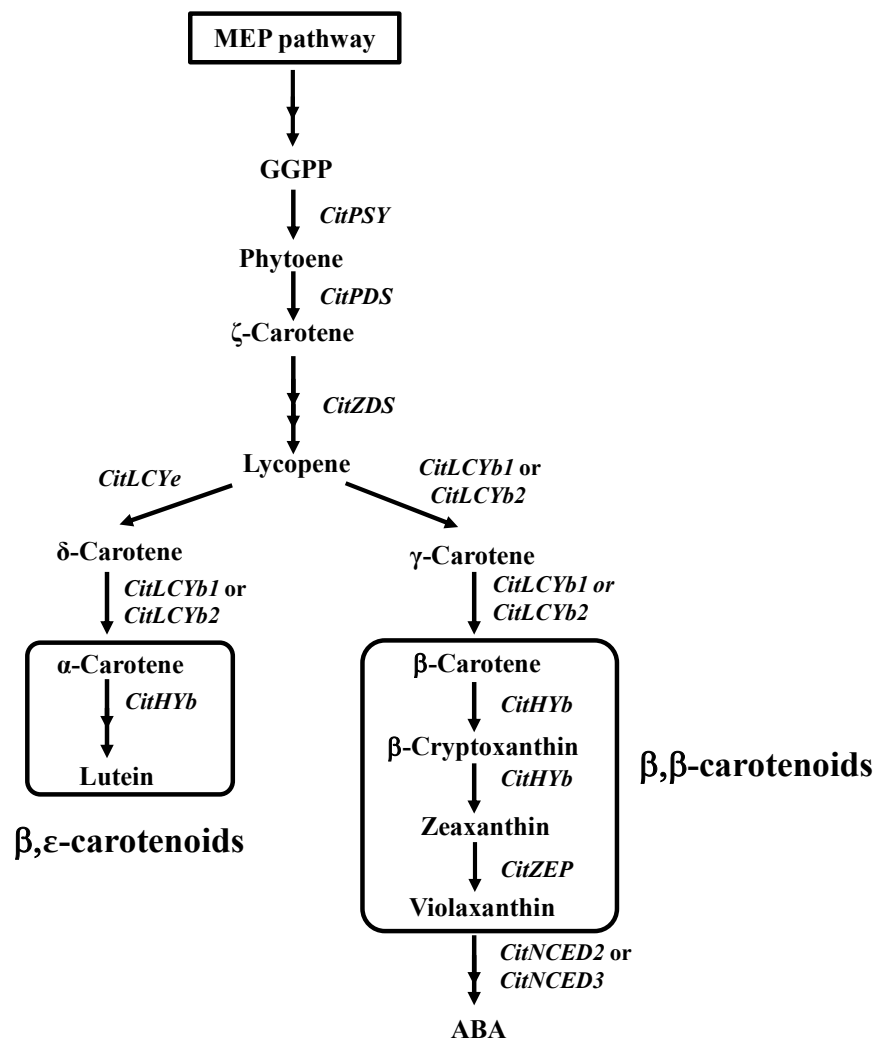
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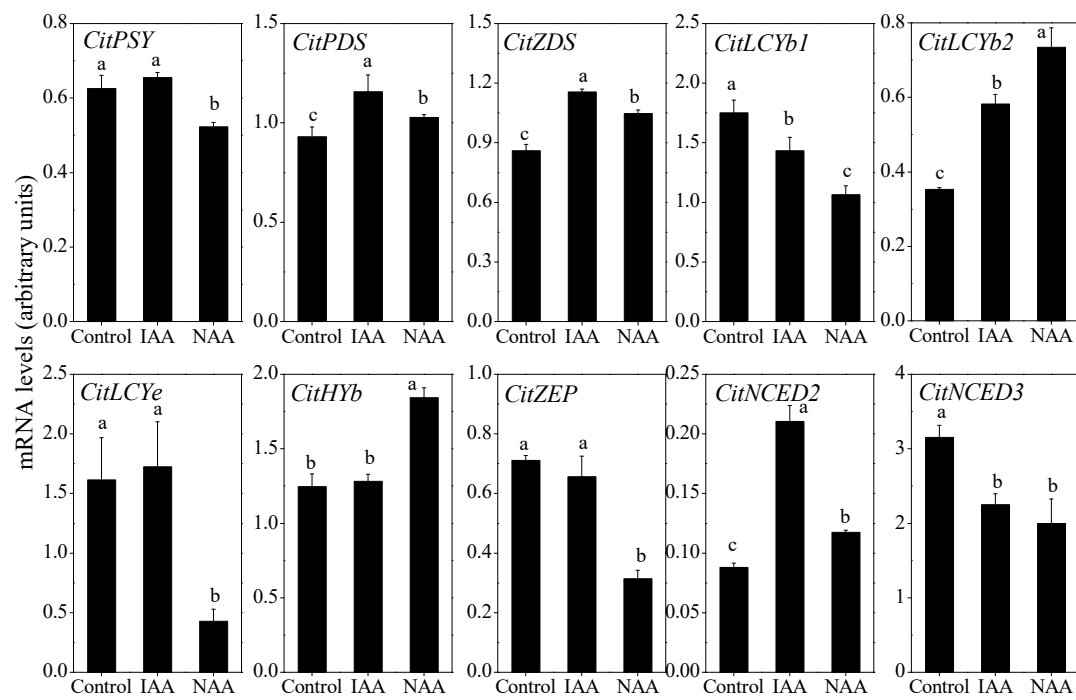
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Figure 4
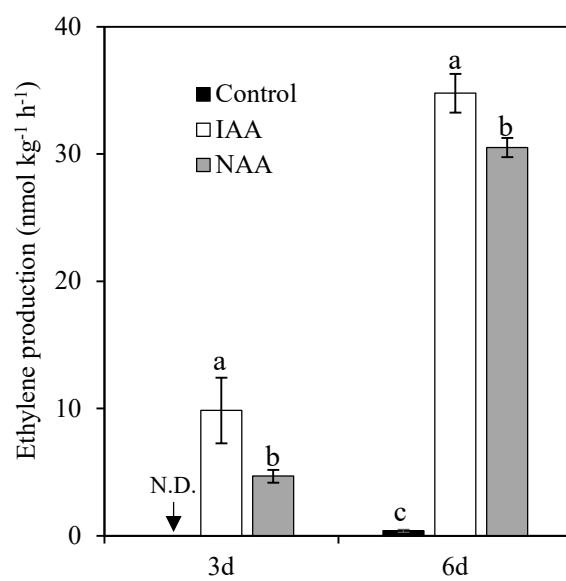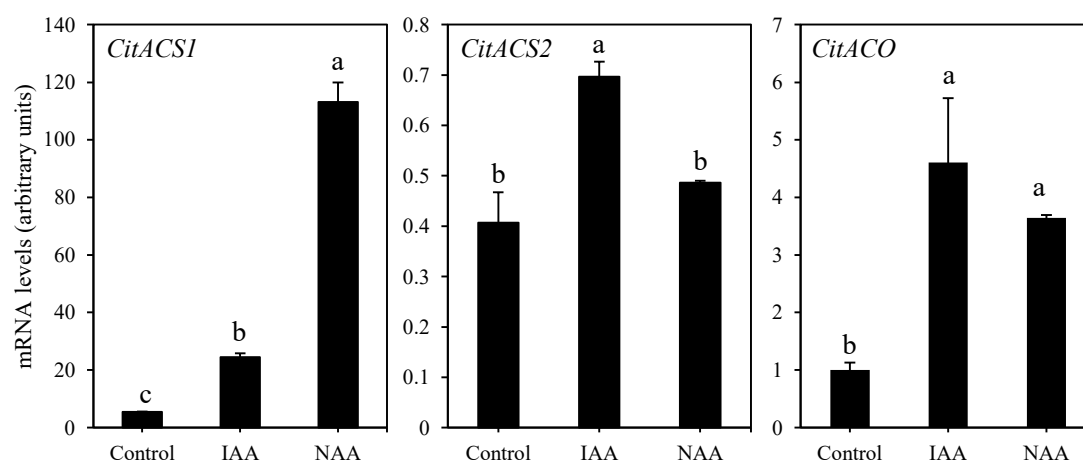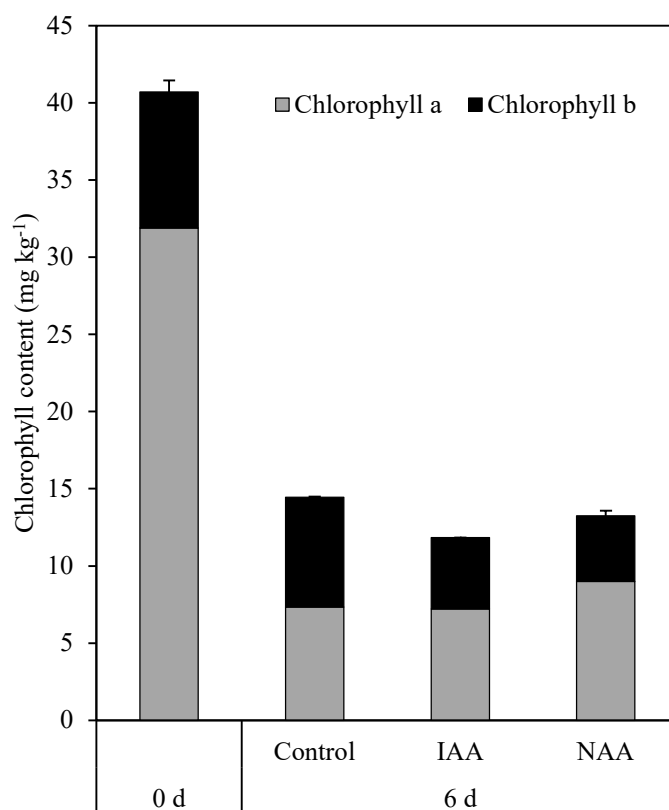
Figure 5

A

B

Figure 6

Figure 7

Figure 8

Fig. S1. Changes of the chlorophylls contents in the flavedos of GA and PDJ-treated citrus fruit after postharvest treatments of IAA and NAA. Columns and bars represent the means and SE (*n*=3), respectively.