Effects of GA₃ and Ethephon on the Level of ABA and Peroxidase Activity in the Peel of Satsuma Mandarin (*Citrus unshiu* Marc.)

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Summary

GA₃ and ethephon were applied to satsuma mandarin (*Citrus unshiu* Marc.) fruit at a concentration of 100 ppm 35 days before harvest. GA₃ depressed the level of ABA in the flavedo during fruit maturation and senescence. On the contrary, ethephon raised the ABA level. Peroxidase activity in the flavedo decreased rapidly during fruit maturation and then leveled off towards senescence. This rapid decrease in enzyme activity was inhibited by GA₃ application and accelerated by ethephon. Although there were 15 isozymes of peroxidase in the flavedo of immature fruit, some of the isozymes disappeared during fruit maturation and only 9 isozymes were finally found at harvest time. GA₃ application decreased the reduction in number of peroxidase isozymes though it conversely increased the reduction in some isozymes. The activity of 2 isozymes was increased and other 2 isozymes disappeared by ethephon treatment. In general, very low activity and fewer isozymes of peroxidase were observed in the albedo as compared to those in the flavedo.

Introduction

Development of puffiness or creasing of the peel was alleviated when GA₃ was applied at color break to satsuma mandarins (15) and Valencia oranges (19). However, GA₃ application caused a delay in degradation of chlorophylls and formation of carotenoid pigments. It is postulated that aging of the citrus peel tissue is delayed by application of GA₃.

It is accepted nowadays that peroxidase activities are closely related to maturation and senescence of fruits. In fact, several investigators have confirmed a positive relation between increase in peroxidase activity and advance in fruit maturity (7, 12, 14, 22). Other reports pointed out an inhibitory and a promotive effect of GA₃ and ethylene, respectively, on maturation and senescence of Navel oranges (16) and mango fruits (17). However, the interrelationship is not well understood between activity of the enzymes concerned and action of plant growth regulators applied at fruit

maturation stage.

The purpose of this study was to determine the relationship between ABA level and peroxidase activity and also to reveal peroxidase isozyme patterns in the peel of satsuma mandarin as affected by GA₃ and ethephon treatments during fruit maturation and senescence.

Materials and Methods

1. GA₃ and ethephon treatments and sampling of fruits.

The present experiment was conducted with fruit of 'Ikeda' satsuma mandarin trees grown in an experimental orchard at Ehime University located in Matsuyama. Fruit were collected at 2-week intervals from August 21, 1975, to February 7, 1976. GA₃ was applied to the fruit by the dipping method at concentrations of 0 and 100 ppm on October 25. Ethephon was also applied to the fruit by the same method and at the same concentrations as those of GA₃. Age of fruit on October 25 was of color break; i.e., most part of the rind surface generally became light green and had yellow spots on the styler end. A half of the treated and the control fruits each was har-

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vested on November 25, 1975, and then stored at room temperature. Another half of the fruits remained on the tree until February 2, 1976. The fruits collected at either time were peeled and the flavedo was separated from the albedo. Both albedo and flavedo tissues were used for determination of abscisic acid (ABA), peroxidase activity and its isozyme patterns.

2. Determination of ABA.

The tissues were extracted with 80% methanol and the extracts were fractionated by the method of Badr et al. (2), methylated with diazomethane (25), and determined by gas liquid chromatography as described in the previous paper (15).

3. Extraction and purification of enzyme.

Using a modified procedure of Shannon *et al.* (26), peroxidases in the peel were extracted and partially purified. Twenty grams of fresh peels were homogenized with redistilled water in a Waring blender. The homogenates were filtered through cheesecloth, and ammonium sulfate was added to 35% saturation. After standing overnight at 4°C, the liquid was centrifuged at 8000 g for 30 min. The supernatant liquid was made up to 95% ammonium sulfate saturation. After standing overnight at 4°C, the liquid was centrifuged. The residues were dissolved in 6 ml of 0.005 M Tris buffer, pH 7.0. After centrifugation, the supernatant liquid was dialyzed against 0.005 M Tris buffer, pH 8.0, containing 0.1 M KCl. The dialyzate was centrifuged. The supernatant was again dialyzed against 0.005 M acetic acid buffer, pH 4.4, and centrifuged. The supernatant liquid was used as crude enzyme extracts.

4. Enzyme assay.

Peroxidase activity in the crude enzyme extracts was determined by the method using pyrogallol as described by Sumner and Gjesing (27). One ml of the extracts was assayed for peroxidase activity at pH 6.0 in 4 ml 0.5 % sodium phosphate solution containing 5% pyrogallol as hydrogen donor and 0.01 M H₂O₂ as oxidant. Purpurogallin produced by the oxidation of pyrogallol was extracted with ether and then absorbance was measured at 460 nm with a Hitachi EPU-photoelectric spectrophotometer.

5. Gel electrofocusing.

The crude enzyme extracts were dissolved in 7.5% acrylamide gel containing 1% ampholine (pH 3-10) as described by Davis (5), Momotani and Nakae (18), and Yamamoto and Momotani (29). Electrofocusing was carried out for 3.5 hr at 4°C using 200 V per tube (5 mm diameter, 10 cm length). The peroxidase bands were determined by dipping the gel pieces into 0.2 M acetate buffer (pH 4.3) containing 0.1% *o*-dianisidin for 10 min and by transferring them further into 0.2 M acetate buffer (pH 6.5) containing 0.06% H₂O₂.

Results

1. Seasonal changes of ABA.

During the fruit growth, ABA content in the untreated flavedo tissue increased gradually until late October, and subsequently continued to increase rapidly reaching a very high value in early December. ABA content in the GA₃-treated flavedo increased not so high as that of the untreated. In the ethephon-treated flavedo ABA increased more rapidly than in the untreated (Fig. 1).

2. Seasonal changes of peroxidase activity.

Assays for peroxidase activity in the flavedo and albedo of the peel revealed that the activity was higher in the former tissue than in the latter (Fig. 2). The activity in the flavedo was kept high from late August to late October, decreased rapidly until late November,

Fig. 1. Effects of GA₃ and ethephon treatments on the level of free ABA in satsuma mandarin peels. Fruits attached to the tree: ○-control, □-GA₃, △-ethephon. Fruits detached from the tree: ●-control, ■-GA₃, ▲-ethephon.

Fig. 2. Effects of GA₃ and ethephon treatments on the peroxidase activity of satsuma mandarin peels. Activity in flavedo: ○-control, □-GA₃, △-ethephon. Activity in albedo: ●-control, ■-GA₃, ▲-ethephon.

and then leveled off towards February. Although seasonal changes in peroxidase activity of the GA₃- and ethephon-treated flavedo tissues were similar to those of the untreated flavedo, the enzyme activity was higher in the

GA₃-treated and lower in the ethephon-treated flavedo than that of the untreated.

The peroxidase activity in the albedo decreased gradually from late August until late December, while the activity in the fruit treated with GA₃ slightly increased in late November. In late December, the peroxidase activity of both untreated and ethephon-treated peels decreased to such a low level as undetectable.

3. Isozymes of peroxidase.

Fig. 3 and 4 show the isozyme patterns of peroxidase in both flavedo and albedo at different stages of fruit growth. Fifteen isozymes of peroxidase in the flavedo and 9 isozymes in the albedo were observed at the peak of fruit growth, August 21. The staining of peroxidase isozymes in the flavedo, however, was weakened in the enzyme bands (No. 1, 2, 5, and 7) and bands of 3, 6, 10, 11, 12, 13 and 14 disappeared during fruit maturation. In the albedo, on the other hand, peroxidase

Fig. 3. Acrylamide gel zymograms of peroxidase extracted from the flavedo of satsuma mandarin fruit at different growth stages. GA₃ and ethephon were applied 35 days before harvest.

Fig. 4. Acrylamide gel zymograms of peroxidase extracted from the albedo of satsuma mandarin fruit at different growth stages. GA_3 and ethephon were applied 35 days before harvest.

activity disappeared in all of the isozymes as the fruit became mature.

In the fruit treated with GA_3 , not only all the enzyme bands detected formerly but a new enzyme band (No. 16) were observed at color break, October 25. The same tendency in a change of isozyme constitution as in the untreated fruit was observed in the peel that was treated with ethephon at the stage of color break.

These isozymes were mainly anionic and included the major bands 1, 3, 4, 7, and 8. Although the cationic isozymes were found at early stages of fruit growth, their staining became less intense as the fruit became mature.

Discussion

In the previous paper (15) we have shown that the percentage of puffy fruit was decreased by the GA_3 applied at a month before harvest. This treatment also increased GA -like substances and reduced the rapid increase in ABA of the flavedo of satsuma mandarin fruit. In this experiment the data obtained showed that ABA concentration in the flavedo was rapidly increased by ethephon application and decreased by GA_3 as compared to that of controls.

Numerous investigators (3, 4, 6, 10, 11, 16, 23) reported that senescence of plant tissues is delayed by GA_3 application and promoted by ethylene. A report (9) has shown that in the flavedo of Shamouti orange the concentration of ABA-like growth inhibitors increased mark-

edly while GA -like substances decreased and finally almost disappeared during the first 48 hr after harvest. Treatment with ethephon, which is known to release ethylene, causes an increase in the concentration of ABA and a decrease in the concentration of GA -like substances. The above results show clearly that senescence of the flavedo of satsuma mandarin fruit is delayed by GA_3 and accelerated by ethephon.

Peroxidases catalyze the oxidation of substrates such as phenolic substances, cytochrome c, ascorbic acid, leuco-dyes and indoles in the presence of H_2O_2 (24). The enzymes are widely distributed among higher plants and are implicated in many phenomena involving the synthesis of ethylene (24).

Most recent report (7, 12, 14, 22) concerned have shown that peroxidase activity increased during fruit maturation. Vines and Oberbacher (28), however, reported that peroxidase activity was greatest in small immature fruits and the decrease in enzyme activity, expressed on the basis of fresh weight, was consistent with increase in fruit size. Similar decreases in peroxidase activity were observed in our experiment (Fig. 2).

Aharoni (1) and Murata and Miyashita (21) have shown that ethylene production of citrus fruit was greatest at immature stage and decreased during fruit maturation. Ku et al. (13) and Young (30, 31, 32) have demonstrated that peroxidases catalyze ethylene production *in vitro*. They have proposed that if these *in vitro* peroxidase systems actually participate in the biosynthesis of ethylene *in vivo*, it is reasonable to suggest that production of ethylene by plant tissues is regulated by the activity of peroxidases or their cofactors. Although Gahagan et al. (8) and Morgan and Fowlar (20) have reported that ethylene increased peroxidase activity in the extract from the plant tissues, it seems quite reasonable to consider that ethylene production can conversely be controlled by peroxidases in the peel of satsuma mandarin fruit, which is assumed from the results obtained by Ku et al., Young, Aharoni, Murata and Miyashita, and our experiment.

Since peroxidase activity is closely related to changes in peroxidase isozyme constitution, effects of GA_3 and ethephon on changes of

peroxidase activity during fruit maturation can be explained by changes in peroxidase isozyme constitutions which are affected by GA₃ and ethephon applications. As the fruit became mature, several isozymes in the peel disappeared in both the control and ethephon-treated fruits in which peroxidase activity decreased rapidly. In the GA₃-treated fruit, however, no isozymes weaken their stainability and a new isozyme is formed during the fruit development. Based on the results of our experiment, no implications can be given on the role of peroxidase isozymes in ethylene production of the peel.

Since the peel of satsuma mandarin, a non climacteric fruit, generally contains low levels of endogenous ethylene, it is assumed that ethylene application may exert marked effects on fruit maturation, in contrast to the fact that climacteric fruits produce much endogenous ethylene before the onset of climacteric rise.

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GA₃ およびエセホンが温州ミカン果皮内 ABA 含量ならびに ペルオキシダーゼ活性に及ぼす影響

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摘 要

温州ミカンの着色始期にジベレリン (GA₃) ならびにエセホン各 100ppm を用いて果実を浸漬処理し、果皮内アブサイシン酸 (ABA) 含量およびペルオキシダーゼ活性とそのアイソザイムについて分析を行なった。

その結果、果皮フラベド内 ABA 含量は、着色始期以後急激に増加し、とくに、収穫直後の増加量は著しく大となり、それ以後貯蔵期間中は減少の傾向を示した。一方、収穫期以後も樹上に残した果実においても、収穫果と同様な傾向を示したが、ABA 増加の程度は収穫果に比べて著しく低かった。GA₃ 処理果は ABA の増加が著しく抑制され、エセホン処理果は処理直後から ABA 含量の急激な増加がみられた。

フラベド内ペルオキシダーゼ活性は着色始期から急激に減少し、収穫期以後にはほぼ一定となった。GA₃ 処理は活性の減少を抑制し、エセホンは促進する傾向を示した。果実発育最盛期には 15 のバンドが検出されたペルオキシダーゼアイソザイムは、果実の成熟に伴って染色度は低下し、数も減少した。しかし、GA₃ 処理果では新しいアイソザイムが現われ、収穫期における染色度も発育最盛期のものと変らなかった。エセホン処理果では無処理果よりも染色が薄かった。

アルベドはフラベドに比べてペルオキシダーゼ活性も低く、アイソザイム数も少なく、収穫期以後には全部消失した。