Original article

A combination of hot water and abscisic acid (ABA) biosynthesis inhibitor regulates ripening of Japanese apricot (*Prunus mume*) fruit

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Summary

Japanese apricot fruit are generally harvested at the mature green stage and have a short storage life. Low-temperature storage is rarely used for Japanese apricot due to its susceptibility to chilling injury. Therefore, effective storage techniques that can regulate fruit ripening are required. Japanese apricot is categorized as a climacteric fruit. Ethylene and abscisic acid (ABA) are ripening-related phytohormones in this fruit. Both hot water (HW) immersion and nordihydroguaiaretic acid (NDGA), an inhibitor of ABA biosynthesis, are effective at delaying the ripening of some fruits and vegetables. We examined the synergistic effect of the combination of HW and NDGA treatment in Japanese apricot. Japanese apricot 'Inazumi' fruit were harvested from an open field at the mature green stage. The effects of HW treatment (40°C, 5 min), NDGA (100 µM), and a combination of HW and NDGA (HW+NDGA) on fruit firmness, hue angle, chlorophyll concentration, ethylene production, ABA concentration, and expression of ethylene-related and ABA-related genes were investigated. Fruit firmness, hue angle, and total chlorophyll concentration in HW+NDGA-treated fruit were highest at 3 and 6 days after treatment. HW+NDGA treatment most effectively retarded ethylene production, expression of ethylene-related genes (*PmACS1*, *PmACO1*, *PmETR1*, *PmERS1*, and *PmCTR1*), endogenous ABA concentration, and expression of ABA-related genes (*PmNCED1* and *PmPP2C37-like*). These results suggested that a combination of hot water and NDGA treatment demonstrated a potential to delay fruit ripening effectively in Japanese apricot.

Keywords

ethylene receptor, nordihydroguaiaretic acid (NDGA), 9-cis-epoxycarotenoid dioxygenase (NCED), type 2C protein phosphatases (PP2Cs), postharvest treatment

Significance of this study

What is already known on this subject?

- Japanese apricot has a short storage life. Ethylene and abscisic acid (ABA) are ripening-related phytohormones in Japanese apricot.
- Heat treatment can decrease ethylene production and delay ripening in some fruits and vegetables.
- Application of nordihydroguaiaretic acid (NDGA), an inhibitor of ABA biosynthesis, can reduce endogenous ABA concentrations in fruits.

What are the new findings?

- Combination treatment of hot water immersion at 40°C for 5 min and NDGA influenced ethylene and ABA signal transduction as well as ethylene and ABA concentrations in Japanese apricot.

What is the expected impact on horticulture?

- To extend the storage period of Japanese apricot.

age techniques should be developed. Japanese apricot is categorized as a climacteric fruit that produces ethylene during storage. Ethylene is perceived by membrane-localized receptor genes such as ethylene response 1 (*ETR1*) and ethylene response sensor 1 (*ERS1*), and these receptors then transmit a signal to the downstream kinase constitutive triple response 1 (*CTR1*) (Ju and Chang, 2015). The expressions of these ethylene receptors and *CTR1* genes were upregulated during ripening, which ethylene production also increased (El-Sharkawy et al., 2007).

Although the phytohormone abscisic acid (ABA) has been mainly regarded to function in the maturation of non-climacteric fruits (Wheeler et al., 2009; Jia et al., 2011), some reports indicate its regulatory role in the ripening of climacteric fruits (Setha et al., 2004; Mou et al., 2016; Wang et al., 2019). The key step in ABA biosynthesis is catalyzed by 9-cis-epoxycarotenoid dioxygenase (NCED), an enzyme that converts the epoxy carotenoids to xanthoxin, which is a precursor of ABA. ABA signaling is repressed by clade A type 2C protein phosphatases (PP2Cs), and then ABA binds to the ABA receptor (PYR/PYL/RCAR) protein family, which in turn inhibits PP2Cs activity and activates signaling (Umezawa et al., 2009). Recently, PP2Cs were identified and characterized in various plant species. For instance, two PP2Cs, *AtABI1* and *AtPP2CA*, are repressors of ABA signaling, which interacts with the sucrose nonfermenting 1 (SNF1)-related protein kinase 2 (SnRK2) in *Arabidopsis thaliana* (Krzywinska et al., 2016). In grape (*Vitis vinifera* L.), *VvPP2C4* and *VvPP2C9* were

Introduction

Japanese apricot (*Prunus mume* Sieb. et Zucc.) fruit are generally harvested at the mature green stage and then processed as a beverage, liquor, or pickle. It is well known that Japanese apricot has a short storage life. Low-temperature storage is well documented to effectively extend the storage life of fruits and vegetables. However, Japanese apricot is extremely susceptible to chilling injury (Imahori et al., 2008; Endo et al., 2019). Therefore, effective Japanese apricot stor-

the major binding partners of the ABA receptor (Boneh et al., 2012). Nordihydroguaiaretic acid (NDGA) inhibits ABA biosynthesis by blocking the NCED (Creelman et al., 1992). Several reports have demonstrated the effect of NDGA treatment on ripening in climacteric fruit. For instance, exogenous application of NDGA repressed ethylene production in cherry tomato (*Lycopersicon esculentum*) (Mou et al., 2016) and mango (*Mangifera indica* L.) (Zaharah and Singh, 2012).

Heat treatment has been reported to influence ethylene production and respiration rate in some fruits. For instance, postharvest hot-air treatment delayed the climacteric peak of ethylene production and respiration rate in plum (*Prunus salicina* Lindl.) fruit (Luo et al., 2010). Moreover, postharvest hot water treatment reduced both respiration and ethylene production in lime (*Citrus aurantifolia* Swingle) fruit (Kaewsuksaeng et al., 2015) and apricot (*Prunus armeniaca* L.) fruit (Wu et al., 2015). On the other hand, heat treatment induced ABA accumulation in tobacco (*Nicotiana tabacum* L.) and grape (*Vitis vinifera* L.) (Teplova et al., 2000; Abass and Rajashekar, 1993). Therefore, a combination of heat treatment and NDGA may delay fruit ripening by suppressing ethylene and inhibiting ABA biosynthesis.

This study investigated the synergistic roles of postharvest hot water and NDGA treatment on fruit ripening in Japanese apricot.

Materials and methods

Plant materials and treatment

Fruit from the outer part of the canopy was harvested at the pre-climacteric stage (122 days after flowering, before the onset of ethylene production: see Figure 3A) from two 30-year-old Japanese apricot 'Inazumi' (*Prunus mume* Sieb. et Zucc.) trees growing in an open field at Chiba University (35°N, 140°E) in 2019. From the harvest, 336 fruit of uniform size and peel color were selected and divided into four groups (84 fruit per treatment). In the first group, the fruit were dipped in distilled hot water at 40°C, a temperature that completely inhibited ethylene production in pear (*Pyrus communis* L.) fruit (Hansen, 1942) and that inhibited ethylene production of Japanese apricot in the preliminary experiment in 2018, for 5 min and then immediately dipped in distilled water at 22°C for 5 min to cool. In the second group, the fruit were dipped in 100 µM NDGA, which was the effective concentration for the inhibition of ABA biosynthesis in the preliminary experiment in 2018, at 22°C for 5 min. The third group was a combination of hot water and NDGA treatment; the fruit were dipped into hot water as previously described for the first group and then cooled using 100 µM NDGA at 22°C for 5 min. The fourth group was the untreated control; fruit dipped in distilled water at 22°C for 5 min. Seven fruit per replicate were packed in a low-density polyethylene bag (0.03 mm thickness, 18 cm × 27 cm, with four 5-mm holes). Each treatment contained three bags, representing three replications. All bags were stored at 25°C with 95% relative humidity under the dark in a controlled room. Twenty-one fruit (three replications of seven fruit) were sampled at 0, 1, 3, and 6 days after treatment (DAT). After measurement of fruit firmness, peel color, and ethylene production, the peels were frozen by liquid nitrogen and stored at -80°C until analysis of chlorophyll, ABA, and gene expression. The peels were withdrawn for analysis of chlorophyll, ABA, and gene expression at 0, 1, 3, and 6 DAT. The fruit firmness, peel color, and ethylene were not measured at 1 DAT.

Fruit firmness, peel color, and total chlorophyll measurement

The firmness of the Japanese apricot (three replications of seven fruit) was measured using a CR-100 Rheometer (Sun Scientific, Tokyo, Japan) with a 3-mm-diameter plunger. The measurement was performed at the two opposite ends along the fruit equator after peel removal. The hue angle value of the peel (three replications of seven fruit) at three equatorial positions was measured by a CM-2002 Spectrophotometer (Konica Minolta, Tokyo, Japan). The hue angles at approximately 120° and 60° represent green and yellow colors, respectively (McLellan et al., 1995). Total chlorophyll concentrations were determined using the method of Porra et al. (1989). Briefly, 0.5 g of the frozen peels were immersed in 3 mL of *N,N*-dimethylformamide and kept at 4°C in the dark for 24 h. The absorbance was measured at 647 nm and 664 nm using a U-2910 spectrophotometer (Hitachi, Tokyo, Japan). The total chlorophyll concentration was calculated according to Porra et al. (1989).

Analysis of ethylene production

Ethylene production of fresh fruit was analyzed according to Nimitkeatkai et al. (2011). From each treatment, 21 fruit (three replications of seven fruit) were sealed in 1.7-L plastic containers and kept at 25°C for 1 h. Thereafter, 1 mL of the headspace gas containing ethylene was withdrawn and analysed, using a gas chromatograph with a flame ionization detector (GC-2014, Shimadzu, Kyoto, Japan) equipped with a Porapak Q column (2.2 mm i.d. × 2.0 m, Waters, Milford, MA, U.S.A.). The column temperature was 50°C, and the carrier gas was helium at a flow rate of 30 mL min⁻¹.

Quantitative analysis of endogenous ABA concentrations

ABA was extracted from peels as described by Kondo et al. (2014) with slight modifications. Then, 0.5 g of frozen peels (three replications of seven fruit) were homogenized in pre-cooled 80% (v/v) methanol containing 0.1% butylated hydroxytoluene, 0.1% L (+) ascorbic acid, and 0.5 g of polyvinylpyrrolidone (PVPP) with 200 ng [²H₆]-ABA as an internal standard. The homogenate was concentrated to an aqueous phase and extracted by ethyl acetate. The extract was then dried completely and re-dissolved in 1 mL 100% (v/v) methanol. Analysis and quantification of the solution containing ABA was analyzed and quantified using liquid chromatography-mass spectrometry (LC-MS). Analyses were performed on an LC-2010 C (Shimadzu) HPLC system consisting of a temperature-controlled autosampler (set at 4°C) coupled with an LCMS-2010 EV mass spectrometer (Shimadzu). The injection volume was 3 µL. Separation was achieved by a Mightysil RP-18 octadecylsilyl column (5 µm, 2 mm i.d. × 150 mm; Kanto Chemical, Tokyo, Japan). ABA was eluted isocratically with 80% (v/v) methanol containing 20 mM formic acid at a flow rate of 0.3 mL min⁻¹. The conditions of the LC-MS analysis were as follows: detector pressure 1.5 kV, nebulizer gas (N₂) flow rate 1.5 L min⁻¹, electrospray ionization (ESI) voltage 4.5 kV, block heater temperature 250°C. ABA was quantified in the selected ion monitoring (SIM) mode. ABA was analyzed in the negative ion mode. The ABA concentration was calculated from the ratio of peak areas for *m/z* 263 (ABA)/269 ([²H₆]-ABA). Data collection and peak analysis were carried out using Labsolutions v. 3 (Shimadzu).

TABLE 1. Primer sequences used in RT-qPCR analysis.

Gene name	Forward/Reverse primer (5'–3')		Accession No.
<i>PmACS1</i>	(F)	TCCCAGTTTCAGAAAGGCTGT	NM_001293270
	(R)	ACCAGCCCCGAACTTGACAT	
<i>PmACO1</i>	(F)	GGGCTTCTTTGAGCTTGAGT	NM_001293254
	(R)	TGGGCAATCACTCTGTGCTC	
<i>PmETR1</i>	(F)	TAGTGCAAGGCAATGGCATGTC	XM_008220708
	(R)	TTGCAGCATGTGACAGAGCAAC	
<i>PmERS1</i>	(F)	ATCTCATTGCGCACAGACCAAC	XM_008236910
	(R)	ACGACCATCGGCTTTCAAACAC	
<i>PmCTR1</i>	(F)	AGCAAGACTTTCATGCCGAACG	XM_008243072
	(R)	TTTGGATGCCGCGAGTCGTTTC	
<i>PmNCED1</i>	(F)	CCCCCGTGATCTACGACAAG	XM_008228017
	(R)	ACAACGACTTCGTCCGTCTC	
<i>PmPP2C37-like</i>	(F)	TTCGGACGCCTCCATTTTGTG	XM_008235259
	(R)	AAAACGACGCCGATTAGCAC	
<i>PmUbiquitin</i>	(F)	AAGGCGAAGATCCAGGACAAGGAG	Pm009747
	(R)	CCACGGAGACGAAGCACCAAA	
<i>PmActin</i>	(F)	CCCTAAGGTAACAGAGAAAAGA	Pm005252
	(R)	CAGCAAGGTCCAGACGAAGAAT	

RNA extraction and cDNA synthesis

Total RNA from 0.5 g of frozen peels (three replications of seven fruit) was extracted using cetyltrimethylammonium bromide (CTAB)-based methods (Li et al., 2008; Henderson and Hammond, 2013). The extracted RNA was purified by a MicroElute Clean-Up Column (Favorgen Biotech, Tokyo, Japan) and Recombinant DNase I (Takara Bio, Tokyo, Japan). cDNA was synthesized from 500 ng of the extracted RNA using the ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) according to the manufacturer's instructions.

Gene expression analysis by reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR was performed on the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, U.S.A.) using the KAPA SYBR FAST qPCR Master Mix ABI Prism (Kapa Biosystems, Wilmington, MA, U.S.A.). Gene-specific primers for each gene were designed by Primer 3 Plus (Table 1). The expression levels of target genes were determined by the comparative cycle threshold ($2^{-\Delta\Delta C_t}$) values and normalized to the C_t values of *PmActin* and *PmUbiquitin* (Wang et al., 2014).

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and means separated by Tukey's HSD test at $P < 0.05$ using SPSS Statistics v. 26 (IBM, Chicago, IL, U.S.A.).

Results and discussion

Fruit firmness, hue angle, and total chlorophyll concentrations

The visual peel color changes are illustrated in Figure 1. Fruit firmness declined sharply at 3 DAT (Figure 2A). The firmness in hot water (HW) + NDGA-treated fruit was significantly higher at 3 DAT. The hue angle of the peels decreased during storage (Figure 2B). In the untreated control, the hue angle generally declined sharply throughout the storage period. Meanwhile, the hue angles of both the hot water- and NDGA-treated peel were significantly higher than those in the untreated control at 3 and 6 DAT. In the HW + NDGA-treated

fruit, the hue angle was remarkably higher than in the other treatments at 3 and 6 DAT. The total chlorophyll concentration in the peels decreased as storage time increased (Figure 2C). At 3 and 6 DAT, the total chlorophyll concentration in HW + NDGA-treated fruit was significantly higher than in the untreated control. Lurie (1998) suggested that the suppression of fruit softening was associated with inactivation of cell wall degradation-related enzymes. In tomato (*Lycopersicon esculentum* L.) fruit, heat treatment drastically decreased expression levels of the polygalacturonase gene (Lurie et al., 1996). The loss of commercial value in Japanese apricot begins with the yellowing of the peel (Zhang et al., 1991). Postharvest yellowing is a result of chlorophyll degradation in Japanese apricot (Wang et al., 2019). In lime (*Citrus aurantifolia* Swingle) fruit, HW treatment delayed the destruction of chlorophyll and reduced the levels of some chlorophyll degradation products such as pheophorbide *a* and pyropheophorbide *a* (Opio et al., 2017; Kaewsuksaeng et al.,

FIGURE 1. Visual peel color changes in Japanese apricot fruit in untreated control (first row), HW (second row), NDGA (third row) and HW + NDGA (fourth row).

FIGURE 2. Changes in fruit firmness (A), hue angle (B), and total chlorophyll concentrations (C) in the untreated control and in HW-treated, NDGA-treated, and HW + NDGA-treated fruit. Data are means $\pm$ SE (three replications of seven fruit) and separated by Tukey's HSD test at $P < 0.05$; ns: not significant.

FIGURE 3. Changes in ethylene production (A) and gene expressions of *PmACS1* (B) and *PmACO1* (C) in the untreated control and in the HW-treated, NDGA-treated, and HW + NDGA-treated fruit. Data are means $\pm$ SE (three replications of seven fruit) and separated by Tukey's HSD test at $P < 0.05$; ns: not significant.

FIGURE 4. Gene expressions of *PmETR1* (A), *PmERS1* (B), and *PmCTR1* (C) in the untreated control and in HW-treated, NDGA-treated, and HW+NDGA-treated fruit. Data are means $\pm$ SE (three replications of seven fruits) and separated by Tukey's HSD test at $P < 0.05$; ns: not significant.

2019). Moreover, pre-harvest NDGA treatment suppressed chlorophyll breakdown with downregulated gene expression of chlorophyllase, which catalyzes the degradation of chlorophyll, in Japanese apricot (Wang et al., 2019). These results suggested that the combination treatment of HW and NDGA was more effective at suppressing fruit softening and yellowing through inactivation of the cell wall and chlorophyll degradation enzymes.

Ethylene production and expression levels of ethylene-related genes

Ethylene production was not observed at 0 DAT (Figure 3A). Ethylene production in the untreated control, HW- and NDGA-treated fruit peaked at 3 DAT and then decreased until 6 DAT. The ethylene levels were significantly lower in the HW- and NDGA-treated fruit than in the untreated control at 3 DAT. Meanwhile, in the HW + NDGA-treated fruit, the peak of ethylene production was delayed and observed at 6 DAT, and ethylene levels were lowest at 3 DAT. Expression levels of ethylene biosynthesis genes, *PmACS1* and *PmACO1* were drastically increased at 3 DAT (Figure 3B and C). Expression levels of both *PmACS1* and *PmACO1* were significantly lower in HW-treated fruit than in untreated control at 1 DAT. Expression levels of *PmACS1* were significantly lower in NDGA-treated fruit than in untreated control at 1 and 3 DAT. Expression levels of both *PmACS1* and *PmACO1* were significantly lower in HW+NDGA-treated fruit than in the untreated control fruit at 1 and 3 DAT. Expression levels of ethylene receptor and signaling genes, *PmETR1*, *PmERS1*, and *PmCTR1*, respectively, were significantly lower in HW-treated fruit than in the untreated control at 1 DAT (Figure 4A–C). Expression levels of *PmETR1* and *PmCTR1* were significantly lower in NDGA-treated fruit at 1 and 3 DAT than in the untreated control,

and *PmERS1* levels were lower at 1 DAT than in the untreated control. Expression levels of *PmETR1*, *PmERS1*, and *PmCTR1* were significantly lower in HW+NDGA treated-fruit than in the untreated control at 1 and 3 DAT. Postharvest HW treatment is an effective technique for regulating ethylene production in some fruits (Opio et al., 2017). Ketsa et al. (1999) suggested that the inhibition of ethylene production by heat treatment in mango (*Mangifera indica* L.) fruit was due to the suppression of both ACS and ACO enzyme activities. Our results supported those previous results. In addition, the effect of NDGA treatment on ethylene production in our results agrees with those in previous reports on Japanese apricot (Wang et al., 2019). With regard to the ethylene receptors and the downstream component of the pathway, expressions of *PmETR1*, *PmERS1*, and *PmCTR1* were downregulated by both the HW and NDGA treatments. Mou et al. (2016) demonstrated that NDGA application in cherry tomato (*Lycopersicon esculentum*) fruit suppressed the ethylene production and expressions of *LeACS2*, *LeACO1*, and *LeACO3* but did not affect the expressions of *LeETR1*, *LeERS1*, and *LeCTR1*. The responses of *ETR1*, *ERS1*, and *CTR1* genes to chemical growth regulators may vary between fruit species. For instance, Dal Cin et al. (2006) reported that the expression levels of *MdETR1*, *MdERS1*, and *MdCTR1* in apple (*Malus × domestica* Borkh.) fruit were downregulated by 1-methylcyclopene (1-MCP), which is an inhibitor of ethylene perception; in contrast, the expressions of *PpETR1*, *PpERS1*, and *PpCTR1* in peach (*Prunus persica* L.) were not affected. Our results suggest that *ETR1*, *ERS1*, and *CTR1* genes may be more responsive to NDGA treatment in Japanese apricot than in cherry tomato, as previously reported. These results imply that HW+NDGA treatment was more effective at suppressing ethylene biosynthesis and signal transduction than either HW or NDGA treatment.

Endogenous ABA concentrations and expression levels of ABA-related genes

Endogenous ABA concentrations in the untreated control increased until peaking at 3 DAT and then decreased at 6 DAT (Figure 5A). Endogenous ABA levels were significantly high-

er in HW-treated fruit than in the untreated control at 1 DAT. At 3 DAT, the endogenous ABA concentration was significantly lower in HW+NDGA-treated fruit than in fruit receiving the other treatments. Both HW- and NDGA-treated fruit also showed lower ABA concentrations compared to the untreated

FIGURE 5. Changes in endogenous ABA concentration (A) and gene expressions of *PmNCED1* (B) and *PmPP2C37-like* (C) in the untreated control and in HW-treated, NDGA-treated, and HW + NDGA-treated fruit. Data are means $\pm$ SE (three replications of seven fruits) and separated by Tukey's HSD test at $P < 0.05$; ns: not significant.

FIGURE 6. Schematic diagram of the regulation of fruit ripening by combination treatment of hot water + NDGA. Dark arrows indicate upregulation. Blank arrows indicate downregulation.

control at 3 DAT. The expression patterns of *PmNCED1* were similar to those of endogenous ABA concentrations (Figure 5B). Expression levels of *PmNCED1* were significantly higher in HW-treated fruit than in the untreated control at 1 DAT. However, at 3 DAT, the expression levels of *PmNCED1* in HW-, NDGA-, and HW + NDGA-treated fruit were significantly lower compared to those in the untreated control. On the other hand, expression patterns of the *PmPP2C37-like* gene showed the opposite tendency compared to both ABA concentrations and *PmNCED1* expressions (Figure 5C). Expression of the *PmPP2C37-like* gene was significantly lower in HW-treated fruit than in the untreated control at 1 DAT. In HW + NDGA-treated fruit, expression levels of the *PmPP2C37-like* gene were significantly higher compared to the untreated control at 3 DAT. In HW treatment, endogenous ABA concentrations at 1 DAT were higher with the downregulation of *PmPP2C37-like* gene expressions; nevertheless, the ABA levels at 3 DAT were lower and similar to those receiving the NDGA treatment. ABA has been well characterized against abiotic stress (Sah et al., 2016). Several studies have reported on the role of ABA in heat stress response. For instance, endogenous ABA concentration increased temporarily by heat treatment in reed (*Phragmites communis* Trin) and grape (*Vitis vinifera* L.) to enhance thermotolerance (Ding et al., 2010; Wang et al., 2005). In maize (*Zea mays*) and creeping bentgrass (*Agrostis stolonifera*), exogenous application of ABA significantly increased its thermotolerance (Gong et al., 1998; Larkindale and Huang, 2005). Moreover, in tobacco (*Nicotiana tabacum*), expression of *NtPP2C1*, a negative regulator in the ABA signaling pathway, was repressed rapidly by heat treatment (Vranova et al., 2000). Therefore, the temporal increase of ABA in HW-treated fruit in the present study may be a response to heat stress. Our study showed that endogenous ABA concentrations were lowest in HW + NDGA-treated fruit and that expression of the *PmPP2C37-like* gene was highest at 3 DAT. These results suggested that the combination treatment of HW and NDGA effectively inhibited ABA biosynthesis and signaling.

Conclusion

The present study revealed that a combination of post-harvest hot water treatment at 40°C for 5 min and treatment with 100 µM of NDGA in Japanese apricot effectively suppressed ethylene production and endogenous ABA concentration by regulating the ethylene-related genes (*PmACS1*, *PmACO1*, *PmETR1*, *PmERS1*, and *PmCTR1*) and the ABA-related genes (*PmNCED1* and *PmPP2C37-like*), resulting in delayed chlorophyll degradation, yellowing, and ultimately ripening (Figure 6). These results suggested that the combination of postharvest heat treatment and ABA biosynthesis inhibitor treatment may be effective for extending the storage life of Japanese apricot.

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