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Effect of Different Postharvest Temperatures on the Accumulation of Sugars, Organic Acids, and Amino Acids in the Juice Sacs of Satsuma Mandarin (*Citrus unshiu* Marc.) Fruit

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ABSTRACT: To elucidate the effect of different postharvest temperatures on the accumulation of sugars, organic acids, and amino acids and to determine the best temperature to minimize their postharvest change, their content after harvest was investigated at 5, 10, 20, and 30 °C for 14 days in the juice sacs of Satsuma mandarin (*Citrus unshiu* Marc. cv. Aoshima-unshiu) fruit. In all sugars, the changes were negligible at all temperatures. Organic acids decreased slightly at all temperatures, with the exception of malic acid at 30 °C, which increased slightly. Two amino acids, ornithine and glutamine, increased at 5 °C, but they did not increase at other temperatures. In 11 amino acids (phenylalanine, tryptophan, tyrosine, isoleucine, leucine, valine, threonine, lysine, methionine, histidine, and γ-amino butyric acid), the content was higher at 20 and 30 °C than at other temperatures. Thus, the content of amino acids was more variable than that of sugars and organic acids in response to temperatures. Moreover, amino acids responded to temperature differently: two amino acids were cold responsive, and 11 were heat-responsive. The best temperature to minimize the postharvest changes in amino acid profiles in the juice sacs of Aoshima-unshiu was 10 °C. The responsiveness to temperatures in two cold-responsive (ornithine and glutamine) and five heat-responsive (phenylalanine, tryptophan, valine, lysine, and histidine) amino acids was conserved among three different Satsuma mandarin cultivars, Aoshima-unshiu (late-maturing cultivar), Silverhill (midmaturing cultivar), and Miyagawa-wase (early-maturing cultivar). The metabolic responsiveness to temperature stress was discussed on the basis of the changes in the amino acid profile.

KEYWORDS: *Satsuma mandarin, citrus fruit, postharvest temperature, amino acid, temperature stress*

INTRODUCTION

Postharvest citrus fruit is exposed to various temperatures in a storage room, on a shelf, and during transportation.[1,2] In several citrus cultivars, such as mandarin and grapefruit, postharvest conditioning at high temperatures (e.g., 20 and 30 °C) has been performed because high-temperature treatments before storage are effective to reduce chilling injury during long-term cold storage and to improve the taste and peel color of citrus fruit.[2−8]

Sugars, organic acids, and amino acids are major primary metabolites in the juice sacs of citrus fruit and are important components for internal fruit quality.[1] The contents of sugars and organic acids and their ratios (sugar content/acid content) affect the taste of citrus fruit. Thus, postharvest temperatures affecting the contents of sugars and organic acids have been widely investigated.[1−4,9,10] In contrast, few studies have focused on the effects of temperatures on postharvest changes in the content of amino acids in citrus fruit, although seasonal changes during fruit maturation and varietal differences in the content of amino acid have been investigated in several citrus cultivars, such as orange, lemon, and mandarin.[11,12] Amino acids are major nitrogenous compounds in the juice of citrus fruit and are important for human nutrition.[1,12] Amino acids affect aspects of quality, such as the taste and aroma, in many horticultural crops.[13] Recently, Tietel et al. reported that, in mandarin fruit, some volatiles derived from the lipid and amino acid catabolism, mainly via the leucine and isoleucine degradation pathway, increased during the cold storage of Mor mandarin fruit at 5 °C and proposed that these volatiles are partly responsible for the off-flavor of mandarin fruit after harvest.[14] Their results suggested that some amino acids are a precursor of the off-flavor during the storage of mandarin fruit.[14] Thus, the effects of different postharvest temperatures on the metabolic changes in amino acids, sugars, and organic acids are important for the maintenance of internal quality of postharvest citrus fruit.

Investigations of the changes in amino acid profiles also provide important information with regard to the physiological status of plants.[15,16] The amino acid metabolism is one of the most important biochemical adaptations to many environmental stresses, such as drought and low and high temperatures, before and after harvest.[15,16] In intact plants, such as *Arabidopsis*, it was reported that the content of some amino acids changed remarkably in response to low and high temperatures.[17−20] In postharvest plants, such as asparagus and broccoli, previous reports have shown that some amino acids accumulated after harvest as a result of metabolic changes, such as protein degradation and interconversion of amino acids.[21−24] This amino acid accumulation observed in intact plants and postharvest crops is thought to be one of the metabolic responses to the adaptation of abiotic stresses, such as temperature and harvest stresses caused by the separation of source organs (e.g., drought and nutrient deficiency).[17−24] However, to the best of our knowledge, postharvest changes in

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Table 1. Compound-Dependent MS Parameters Used in the Quantification by LC/MS/MS

analyte	MRM transition (m/z) (precursor−product ion)	collision energy (eV)	internal standard used for quantification	MRM transition (m/z)(precursor−product ion)	collision energy (eV)
fructose	179−89	−10	fructose-$^{13}C_6$	185−92	−10
glucose	179−89	−10	glucose-$^{13}C_6$	185−92	−10
sucrose	341−59	−52	sucrose-$^{13}C_{12}$	353−61	−56
Ala	90−44	17	Ala-2,2,3,3-d_4	94−48	19
Arg	175−70	39	Arg-d_7	182−77	37
Asn	133−74	23	Asn-$^{15}N_2$	135−75	18
Asp	134−74	21	Asp-2,3,3-d_3	137−75	23
Cys	121−76	21	Cys-3,3-d_2	124−61	37
GABA	104−87	15	GABA-2,2,3,3,4,4,-d_6	110−93	17
Gln	147−130	15	Gln-2,3,3,4,4-d_5	152−88	25
Glu	148−84	23	Glu-2,4,4-d_3	151−87	23
Gly	76−30	19	Gly-2,2-d_2	78−32	19
His	156−110	21	His-$^{13}C_6$	162−115	23
Ile	132−86	15	Leu-d_{10}	162−115	23
Leu	132−86	15	Leu-d_{10}	142−96	17
Lys	147−84	23	Lys-4,4,5,5-d_4	151−134	15
Met	150−104	17	Met-methyl-d_3	153−107	15
Orn	133−70	25	Orn-3,3,4,4,5,5-d_6	139−122	15
Phe	166−120	19	Phe-d_8	174−128	21
Pro	116−70	23	Pro-d_7	123−77	23
Ser	106−60	15	Ser-2,3,3-d_3	109−63	17
Thr	102−74	15	Thr-$^{13}C_4$	124−77	17
Trp	205−188	15	Trp-indole-d_5	210−192	17
Tyr	182−136	21	Tyr-phenyl-d_4	186−140	21
Val	118−72	17	Val-d_8	126−80	19
norvaline	118−72	17	Val-d_8	126−80	19
cis-aconitic acid	145−101	−10	citric acid-2,2,4,4-d_4	195−113	−16
citric acid	191−111	−18	citric acid-2,2,4,4-d_4	195−113	−16
fumaric acid	115−71	−10	citric acid-2,2,4,4-d_4	195−113	−16
isocitric acid	191−111	−18	citric acid-2,2,4,4-d_4	195−113	−16
lactic acid	89−43	−20	citric acid-2,2,4,4-d_4	195−113	−16
malic acid	133−115	−14	malic acid-2,3,3-d_3	136−117	−14
2-oxoglutaric acid	145−101	−10	citric acid-2,2,4,4-d_4	195−113	−16
phoephoenol pyruvate	167−79	−54	citric acid-2,2,4,4-d_4	195−113	−16
pyruvate	87−43	−10	citric acid-2,2,4,4-d_4	195−113	−16
succinic acid	117−73	−14	succinic acid-2,2,3,3-d_4	121−77	−14

amino acid profiles in response to temperature stress have scarcely been studied in juice sacs of citrus fruit.

In the present study, to retain the freshness of postharvest mandarin fruit and to estimate temperature stress during storage, the effects of postharvest temperature on the changes in the content of sugars, organic acids, and amino acids were investigated for 14 days in the juice sacs of three different Satsuma mandarin (*Citrus unshiu* Marc.) cultivars, Aoshima-unshiu (late-maturing cultivar), Silverhill (midmaturing cultivar), and Miyagawa-wase (early-maturing cultivar). On the basis of the changes in these metabolites, the best temperature to minimize postharvest changes in these compounds was determined in Aoshima-unshiu fruit. Moreover, metabolic responsiveness to temperature stress in juice sacs of Satsuma mandarin was discussed on the basis of the changes in the amino acid profile.

MATERIALS AND METHODS

Plant Materials and Storage Conditions. Fully ripe fruit of three cultivars of Satsuma mandarin (*Citrus unshiu* Marc.), Aoshima-unshiu (late-maturing cultivar), Silverhill (midmaturing cultivar), and Miyagawa-wase (early-maturing cultivar), were harvested from trees at the National Institute of Fruit Tree Science, Okitsu (Shizuoka, Japan) in early December, late November, and early November, respectively. Fruit uniform in size and color were selected. For Aoshima-unshiu, the fruits were divided into four groups for different temperature treatments (5, 10, 20, and 30 °C) and incubated in the dark at 5, 10, 20, and 30 °C for up to 2 weeks. During temperature treatments, the fruits in each treatment were loosely wrapped in polyethylene film with daily ventilation. At days 0, 7, and 14 after harvest, three fruits for each treatment were collected and subjected to liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis. For Silverhill and Miyagawa-wase, the fruits were divided into two groups for different temperature treatments (5 and 20 °C) and incubated in the dark at 5 and 20 °C for up to 2 weeks in polyethylene film with daily ventilation. At days 0 and 14 after harvest, three fruits for each treatment were collected and subjected to LC/MS/MS analysis. The juice sacs were separated from the sampled fruit, immediately frozen in liquid nitrogen, and stored at −80 °C until use.

Chemicals. Twenty-two amino acids [Gly, Ala, Ser, Pro, Val, Leu, Ile, Thr, Cys, Asn, ornithine (Orn), Asp, Gln, Lys, Glu, Met, His, Phe, Arg, Tyr, Trp, and γ-aminobutyric acid (GABA)], 10 organic acids (citric, malic, succinic, *cis*-aconitic, isocitric, 2-oxoglutaric, lactic, phosphoenolpyruvic, pyruvic, and fumaric acid), three sugars (glucose, fructose, and sucrose), and norvaline as standard compounds were obtained from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan). LC/

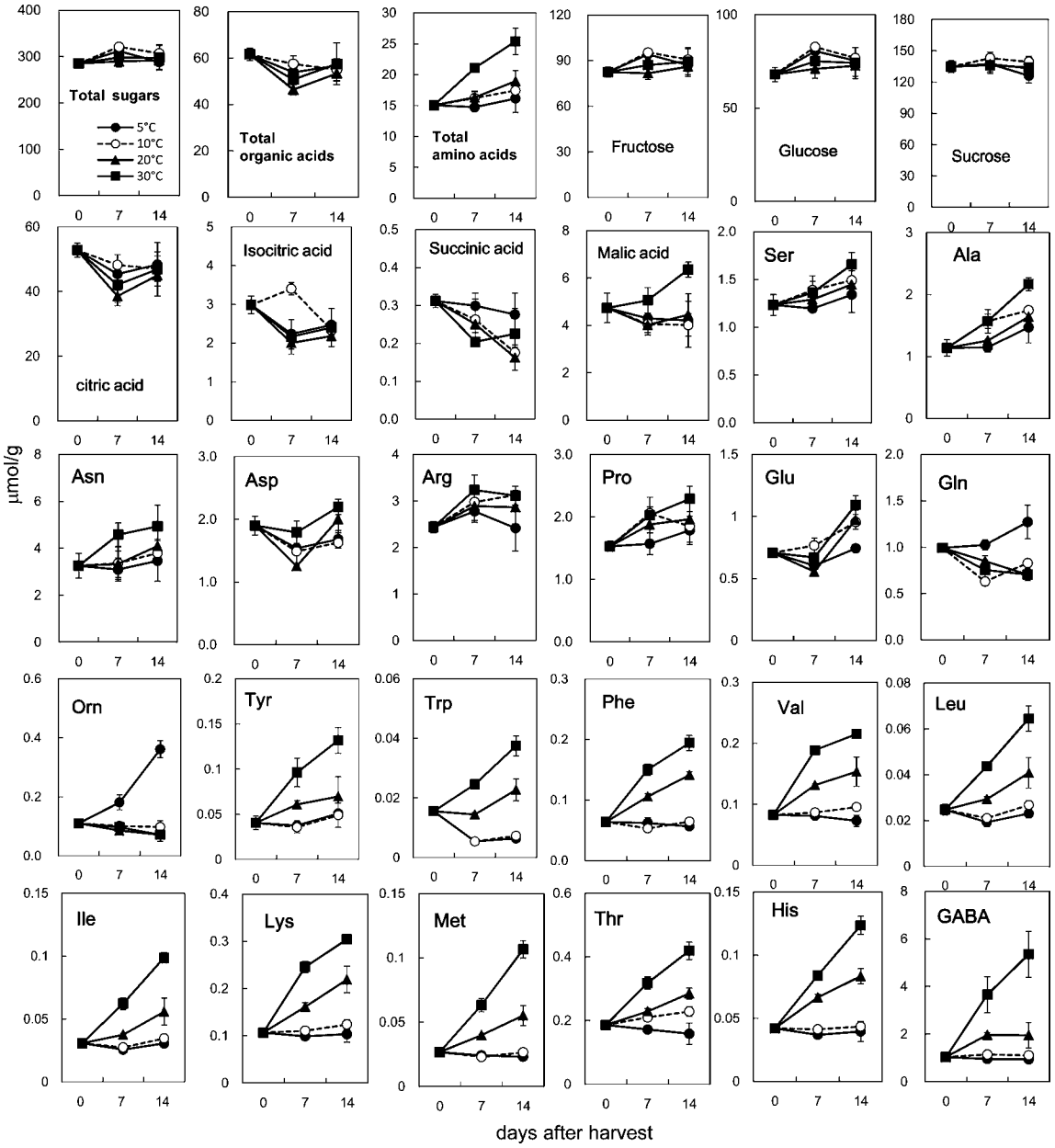
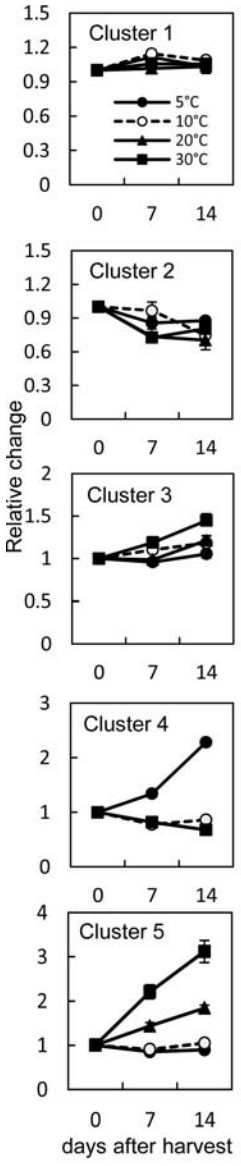


Figure 1. Postharvest change in the metabolite content in the juice sacs of Aoshima-unshiu stored at different temperatures. Fruits were stored at 5 (●), 10 (○), 20 (▲), or 30 °C (■). The content is expressed on a fresh weight basis, μmol/g. The values are the means ± SEs of three fruits.

MS-grade acetonitrile, LC/MS-grade formic acids, and other chemicals were purchased from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan). Stable isotopes for internal standards, Gly (2,2-d_2), Ala (2,2,3,3,-d_4), Ser (2,3,3-d_3), Pro (d_7), Val (d_8), Leu (d_{10}), Thr ($^{13}C_4$), Cys (3,3-d_2), Asn ($^{15}N_2$), Orn (3,3,4,4,5,5-d_6), Asp (2,3,3-d_3), Gln (2,3,3,4,4,-d_5), Lys (4,4,5,5,-d_4), Glu (2,4,4-d_3), Met (methyl-d_3), His ($^{13}C_6$), Phe (d_8), Arg (d_7), Tyr (ring-d_4), and Trp (indole-d_5) were purchased from Cambridge Isotopes (Andover, MA). GABA (2,2,3,3,4,4,-d_6), citric acid (2,2,4,4,-d_4), malic acid (2,3,3-d_3), succinic acid (2,2,3,3,-d_4), glucose ($^{13}C_6$), fructose ($^{13}C_6$), and sucrose ($^{13}C_{12}$) were obtained from Sigma Chemical Co. (St. Louis, MO).

Internal Standards. Stable isotope-labeled compounds were used as internal standards for the analysis of most compounds in which stable isotope-labeled compounds were available (Table 1). For the analysis of Ile, norvaline, and several organic acids, in which stable isotope-labeled compounds were unavailable, structurally similar compounds, Leu (d_{10}), Val (d_8), and citric acid (2,2,4,4,-d_4), were respectively used as an internal standards (Table 1). For organic acid and amino acid analysis, an internal standard mixture solution of three isotope-labeled organic acids (0.8−2.6 mmol/L) and 21 isotope-labeled amino acids (0.026−1.2 mmol/L), shown in Table 1, was prepared in 0.2 mol/L formic acid. For sugar analysis, an internal standard mixture solution of three isotope-labeled sugars (3.8−27 mmol/L), shown in Table 1, was prepared in distilled water. The internal standard mixture solution was dispensed (5 μL) into a 1.5 mL tube by an eVol hand-held automated analytical syringe (SGE Analytical Science Pty, Ltd., Melbourne, Australia) on that day and stored at −30 °C until use.

Extraction and Chromatographic Conditions. The juice sacs (ca. 1 g) were extracted according to a method described previously with slight modifications.[23,25] To determine extraction efficiency, 100 μL of a surrogate standard solution (an aqueous solution of norvaline, 1.875 mg/mL) was added to the juice sacs. The sample was homogenized in ice-cold 95% EtOH using a homogenizer (Polytron homogenizer T-25). The homogenate was adjusted to 100 mL with 80% EtOH in a volumetric flask. After it was shaken vigorously, the extract was filtered through a 0.2 mm filter (Millipore, Bedford, MA).



Cluster No.(profile)	7 days after harvest				14 days after harvest			
Metabolite	5°C	10°C	20°C	30°C	5°C	10°C	20°C	30°C
Cluster 1 (negligible change, small difference among temepratures)								
Fructose	a	a	a	a	a	a	a	a
Glucose	a	a	a	a	a	a	a	a
Sucrose	a	a	a	a	a	a	a	a
Cluster 2 (slight decrease, small difference among temepratures)								
Citric acid	a	a	a	a	a	a	a	a
Isocitric acid	**ab**	**a**	**b**	**b**	a	a	a	a
Succinic acid	a	a	a	a	a	a	a	a
Cluster 3 (slight increase at 30°C, small difference among temepratures)								
Ser	a	a	a	a	a	a	a	a
Ala	a	a	a	a	**b**	**ab**	**ab**	**a**
Asn	a	a	a	a	a	a	a	a
Asp	a	a	a	a	a	a	a	a
Arg	a	a	a	a	a	a	a	a
Pro	a	a	a	a	a	a	a	a
Glu	a	a	a	a	**b**	**ab**	**ab**	**a**
Malic acid	a	a	a	a	a	a	a	a
Cluster 4 (increase at 5°C, large difference among temepratures)								
Gln	**a**	**b**	**ab**	**ab**	**a**	**ab**	**b**	**b**
Ornithine	**a**	**b**	**b**	**b**	**a**	**b**	**b**	**b**
Cluster 5 (increase at 20 and 30°C, large difference among temepratures)								
Tyr	**b**	**b**	**ab**	**a**	**b**	**b**	**ab**	**a**
Trp	**c**	**c**	**b**	**a**	**c**	**c**	**b**	**a**
Phe	**c**	**c**	**b**	**a**	**c**	**c**	**b**	**a**
Val	**c**	**c**	**bc**	**a**	**c**	**bc**	**bc**	**a**
Leu	**c**	**c**	**b**	**a**	**b**	**b**	**b**	**a**
Ile	**b**	**b**	**b**	**a**	**b**	**b**	**b**	**a**
Lys	**c**	**c**	**b**	**a**	**c**	**c**	**b**	**a**
Met	**c**	**c**	**b**	**a**	**c**	**c**	**b**	**a**
Thr	**c**	**bc**	**b**	**a**	**c**	**bc**	**b**	**a**
His	**c**	**c**	**b**	**a**	**c**	**c**	**b**	**a**
γ-Aminobutyric acid	**b**	**b**	**ab**	**a**	**b**	**b**	**b**	**a**

Figure 2. Relative changes in the content of five clusters and statistical differences in the content among the temperatures (5, 10, 20, and 30 °C) at 7 and 14 days after harvest in Aoshima-unshiu. The graphs indicate the means ± SEs of the relative changes of all metabolites in each cluster. The metabolites included in each cluster are shown in the table. Different letters within a row in the table indicate significant differences in the content among temperatures at days 7 and 14 after harvest, respectively (Tukey's HSD test; $P < 0.05$).

For amino acid and organic acid analysis, a 100 μL aliquot of the filtrate was collected with a gastight syringe and mixed with 5 μL of an internal standard mixture solution (21 stable isotope-labeled amino acids and three organic acids, Table 1) in a 1.5 mL tube. The mixtures were evaporated using a centrifugal evaporator to dryness. The dry residue was redissolved in 100 μL of 0.2 mol/L formic acid, sonicated for 5 min, and filtered through a 0.2 mm filter, and 5 μL of the extract was analyzed by LC/MS/MS. Chromatographic separation was performed according to a method described previously.[26] Briefly, the separation of amino acids and organic acids was carried out using a pentafluorophenylpropyl (PFPP)-bonded silica column (Discovery HS-F5, 250 mm × 4.6 mm i.d., 5 μm, Supelco, Bellefonte, PA) and a binary gradient elution with 0.1% formic acid (eluent A) and acetonitrile (eluent B) as a mobile phase at a flow rate of 0.3 mL/min. The column temperature was kept at 40 °C. The linear gradient program was performed as follows: the initial condition was 100% A; 15−35 min, 70% A/30% B; 35−40 min, 50% A/50% B; 40−62 min, 50% A/50% B; and 62−72 min, 100% B. The extraction efficiency calculated on the basis of surrogate recovery (norvaline) was 96 ± 0.7%.

For sugar analysis, a 100 μL aliquot of the filtrate was collected with a gastight syringe and mixed with 5 μL of an internal standard mixture solution (three stable isotope-labeled sugars, Table 1) in a 1.5 mL tube. The mixtures were evaporated using a centrifugal evaporator to dryness. The dry residue was redissolved in 100 μL of water, loaded onto an anion exchange (SAX) cartridge (Discovery DSC-SAX, 1 mL, SUPELCO, Sigma Aldrich, Germany), and eluted with 250 μL of water. The eluent (100 μL) was mixed with 250 μL of acetonitrile, and 5 μL of the extract was analyzed using LC-MS-MS. Chromatographic separation was performed according to a method described previously with slight modifications.[27] Briefly, the extract solution containing three sugars was separated using an aminopropyl bonded phase column (Asahipak NH2P, 150 mm × 4.6 mm i.d., 5 μm, Shodex, Japan) with an isocratic elution of 0.05% formic acid and 75% acetonitrile for 35 min at a flow rate of 0.3 mL/min. The extraction efficiency calculated on the basis of surrogate recovery (norvaline) was 100 ± 0.2%.

LC/MS/MS Conditions. The concentrations of three sugars, 23 amino acids, and 10 organic acids were analyzed by LC/MS/MS. The analysis was performed with an Agilent 1100 series HPLC (Agilent

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Technologies, Palo Alto, CA) consisting of a binary pump, an online degasser, and a temperature-controlled autosampler (set at 4 °C) coupled to an AB SCIEX API2000 triple-stage quadrupole tandem mass spectrometer (AB SCIEX, Foster City, CA) with a TurboIon spray source. The ion spray voltage was used at 5000 V in the positive ion mode and −4500 V in the negative ion mode. The nebulizer gas (air), auxiliary gas (air), curtain gas (nitrogen), and collision gas (nitrogen) were used at 40, 45, 75, and 4 arbitrary units, respectively. The ion source temperature was maintained at 400 °C.

The analyte was detected using electrospray ionization (ESI). Amino acids were detected in the positive ion mode. Organic acids and sugars were detected in the negative ion mode. Peaks of the compounds were identified by comparing multiple reaction monitoring (MRM) transition and retention times with those of the authentic standards. The quantification of the metabolites was performed in a MRM mode using compound-dependent MS parameters (MRM transition and collision energy), which were determined by infusion of each compound dissolved in 50% acetonitrile (Table 1). Data were acquired via Analyst software (version 1.5, AB/MDS Sciex, Concord, Canada). A scheduled MRM algorithm,[28] which makes it possible to monitor MRM transitions of the analytes only around the expected retention time and allows highly sensitive analysis, was used for quantification. The quantification of each metabolite was performed by comparing their peak areas with those of the authentic standards using an internal standard method. The concentrations of the metabolites were determined by reference to standard curves prepared for each compound. The value of total amino acids, total organic acids, and total sugars was the sum of each compound.

Statistical Data Analysis. The statistical significance of the results was analyzed by Tukey's HSD test or Student's *t* test at the 5% level, and hierarchical clustering was performed by the Ward's method using the application software JMP 8 (JMP release 8.0; SAS Institute Inc., Cary, NC). For the classification of metabolites on the basis of the similarity in postharvest changes in content at different temperatures, the content of each metabolite at days 0, 7, and 14 after harvest at all temperatures (5, 10, 20, and 30 °C) was normalized and used for hierarchical cluster analysis. For the observation of optimal temperature to minimize the postharvest changes in amino acid profiles, the profiles of freshly harvested fruit (day 0) were compared with those of fruit stored at different temperatures for 7 or 14 days by hierarchical clustering. With respect to cluster analyses, the contents of metabolites whose difference among the temperatures was significant at each day (Figure 2) were used after normalization.

RESULTS

Characteristics of Postharvest Changes in the Metabolite Content at Different Temperatures. The contents of sugars, organic acids, and amino acids in the juice sacs of Aoshima-unshiu stored at 5, 10, 20, and 30 °C were examined for 14 days. In the present study, LC/MS/MS methods for the quantification of three sugars, 10 organic acids, and 23 amino acids were constructed (Table 1). Figure 1 shows the postharvest changes in the contents of these metabolites at different temperatures. Figure 2 shows the statistical differences among the temperatures (5, 10, 20, and 30 °C) in the contents of each metabolite analyzed at days 7 and 14 after harvest, respectively. In Figure 2, the metabolites were classified into five clusters by hierarchical clustering analysis on the basis of similarity in postharvest changes in the metabolite levels. Relative changes in the metabolite levels of each cluster are also shown in Figure 2.

All sugars, glucose, fructose, and sucrose, were classified into cluster 1 (Figure 2). In this cluster, no distinct changes in the contents of these metabolites were observed at any temperatures during the experimental period (Figures 1 and 2). The difference in the contents among the temperatures was not significant either (Figure 2).

Organic acids were classified into two different clusters, 2 and 3 (Figure 2). Most of the organic acids (citric acid, isocitric acid, and succinic acid) were classified into cluster 2. On the other hand, malic acid was classified into cluster 3. The contents of three organic acids in cluster 2 were slightly decreased at all temperatures after harvest (Figures 1 and 2). The difference in the content among temperatures was not significant at 14 days after harvest (Figure 2). In contrast, the content of malic acid in cluster 3 increased gradually at 30 °C, although the difference in the content among temperatures was not significant (Figures 1 and 2). Thus, the postharvest change in the malic acid content at 30 °C was different from that in other organic acids, citric acid, isocitric acid, and succinic acid.

Amino acids were classified into three different clusters: 3, 4, and 5 (Figure 2). Seven amino acids, Ser, Ala, Asn, Asp, Arg, Pro, and Glu, were classified into cluster 3. At 14 days after harvest, the contents of these metabolites was higher at 30 °C than that at other temperatures (Figures 1 and 2). However, the difference in the contents among temperatures was only significant in Ala and Glu at 14 days after harvest (Figure 2). Two amino acids, Gln and Orn, were classified into cluster 4. Interestingly, the contents of these metabolites were specifically increased at 5 °C but slightly decreased at other temperatures (Figures 1 and 2). In this cluster, the difference in the content between low (5 °C) and high temperatures (20 and 30 °C) was significant at 14 days after harvest (Figure 2). Eleven amino acids, Tyr, Trp, Phe, Val, Leu, Ile, Lys, Met, Thr, His, and GABA, were classified into cluster 5 (Figure 2). The contents of these metabolites at 14 days after harvest were higher at 20 and 30 °C than at 5 and 10 °C (Figures 1 and 2). Especially at 30 °C, the content of these metabolites was significantly higher than that at 5 and 10 °C throughout the experimental periods (Figure 2). In this cluster, the difference in the content among temperatures was large (Figures 1 and 2).

On the basis of these results, in Aoshima-unshiu, the postharvest changes in the contents of sugars, organic acids, and amino acids and their differences among the temperatures (5, 10, 20, and 30 °C) were characterized as follows: sugars, negligible change with small differences among temperatures; organic acids with the exception of malic acid, slight decreases with small differences among temperatures; seven amino acids in cluster 3 and malic acid, slight increases at 30 °C with small differences among temperatures; two amino acids (Gln and Orn) in cluster 4, increases at 5 °C with large differences among temperatures; and 11 amino acids in cluster 5, increases at 20 and 30 °C with large differences among temperatures. Thus, in the juice sacs of Satsuma mandarin (Aoshima-unshiu), the contents were more variable in amino acids in response to the temperature than it was in sugars and organic acids because the differences among the temperatures in the content were larger in amino acids than they were in sugars and organic acids. Amino acids responded to temperature differently: two amino acids were cold responsive (Gln and Orn), and 11 were heat-responsive (Tyr, Trp, Phe, Val, Leu, Ile, Lys, Met, Thr, His, and GABA).

Difference in the Metabolic Profiles between the Fruit before and after Storage at Different Temperatures. On the basis of similarity in the metabolic profiles of Aoshima-unshiu, freshly harvested fruits (day 0) and fruits after storage (day 7 or 14) at different temperatures were classified by hierarchical clustering analysis (Figure 3). At day 7 or 14 after harvest, the metabolites that had significant differences in the contents among temperatures (Figure 2) were selected. For

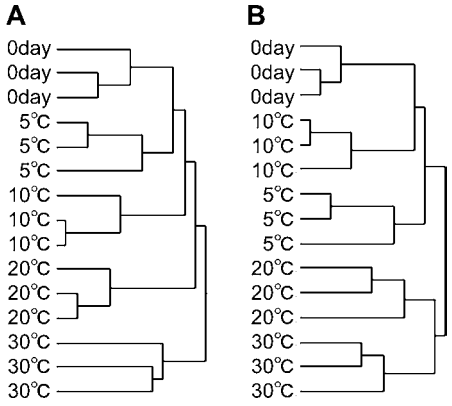

Figure 3. Hierarchical clustering of the Aoshima-unshiu fruit before and after storage for 7 and 14 days at different temperatures. The cluster analysis was performed using the metabolites, which had statistical differences in content among the temperatures at 7 and 14 days after harvest, respectively (Figure 2). (A) Day 7 after harvest. The contents of Trp, Tyr, Phe, Lys, Thr, Met, Ile, Leu, Val, His, Gln, Orn, GABA, and isocitric acid at days 0 and 7 after harvest were normalized and used for the analysis. (B) Day 14 after harvest. The contents of Trp, Tyr, Phe, Lys, Thr, Met, Ile, Leu, Val, His, Glu, Gln, Orn, GABA, and Ala at days 0 and 14 after harvest were normalized and used for the analysis.

clustering of fruits at days 0 and 7, the contents of 14 metabolites (Val, His, Phe, Lys, Thr, Met, Ile, Leu, Trp, Tyr, GABA, Orn, Gln, and isocitric acid) were used (Figure 3A). For clustering of fruits at days 0 and 14, those of 15 metabolites (Val, Lys, Phe, His, Thr, Trp, Met, Ile, Leu, Tyr, GABA, Glu, Ala, Orn, and Gln) were used (Figure 3B). Moreover, the contents of these metabolites at day 0 were compared with those at day 7 or 14 after harvest (Figure 4). Data are shown as the fold-change (content at day 7/content at day 0 and content at day 14/content at day 0) at each temperature. The statistical difference between the content at day 0 and the content at each time point (day 7 or 14 after harvest) was analyzed at each temperature and is shown in Figure 4.

Freshly harvested fruit (day 0) and fruit stored for 7 days at different temperatures were classified by hierarchical clustering (Figure 3A). Freshly harvested fruit (day 0) and fruit stored at 5 °C were classified into the same cluster. The metabolite profile at 5 °C was most similar to that of freshly harvested fruit, and the similarity between fruit stored at 10 °C and freshly harvested fruit was second. In fruit stored at 5 and 10 °C, significant changes were observed in only three amino acids, that is, Trp, Orn, and Gln (Figure 4). The content of Trp decreased (0.35-fold) at both 5 and 10 °C. In Orn, the content increased significantly at 5 °C (1.63-fold) but decreased slightly at other temperatures (Figures 1 and 4). In Gln, the content did not change at 5 °C but decreased at other temperatures (Figures 1 and 4). On the other hand, in fruit stored at 20 and 30 °C, significant changes were observed in most metabolites (Figure 4). The content of most amino acids increased at 20 and 30 °C. The content of Trp, which decreased significantly at 5 and 10 °C, increased significantly at 30 °C. Thus, the similarity of the metabolic profiles to those of freshly harvested

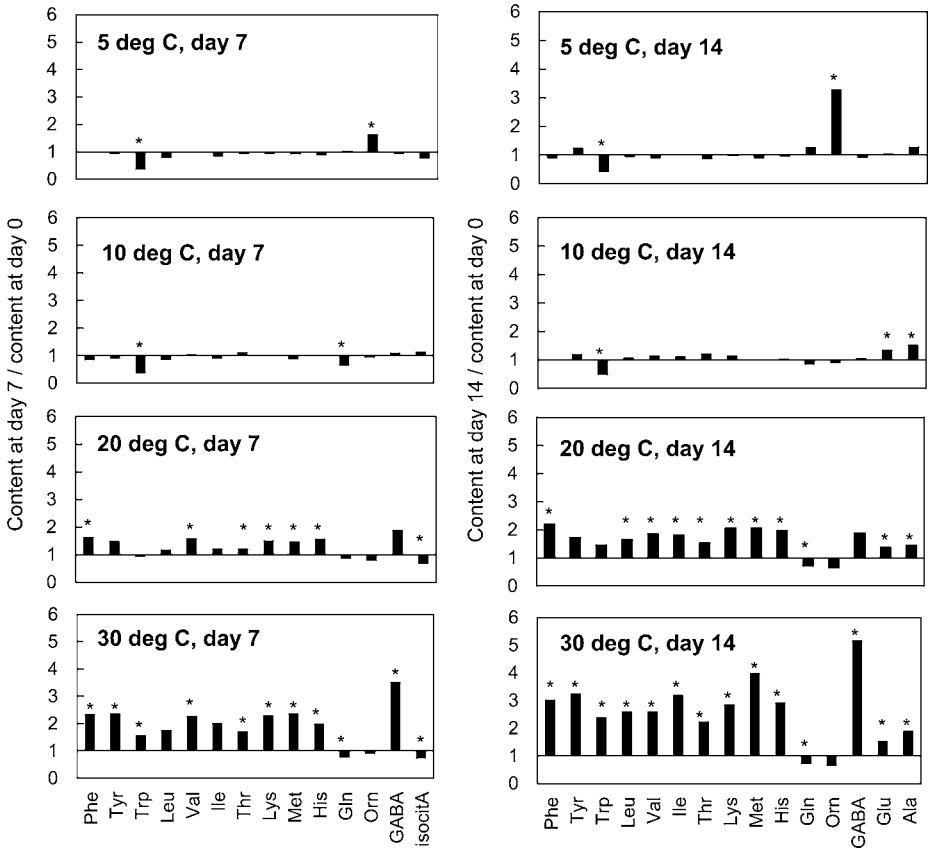

Figure 4. Relative changes in metabolite levels at days 7 and 14 at each temperature in Aoshima-unshiu. The metabolite levels at days 7 and 14 were compared with those at day 0, respectively. Asterisks indicate significant differences in the content between the fruit at day 0 and the fruit at days 7 and 14 after harvest, respectively (Student's t test; $P < 0.05$).

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fruit was lower in the fruit stored at 20 and 30 °C than in those stored at 5 and 10 °C.

Freshly harvested fruit and fruit stored for 14 days at different temperatures were classified by hierarchical clustering (Figure 3B). Freshly harvested fruit (day 0) and fruit stored at 10 °C were classified into the same cluster. Thus, the metabolic profile at 10 °C was most similar to that at day 0 after harvest, and the similarity between fruit at 5 °C and freshly harvested fruit was second. In fruit stored at 5 and 10 °C, significant changes were observed in Orn, Trp, Glu, and Ala (Figure 4). The content of Trp decreased (0.41-fold) at 5 and 10 °C. At 5 °C, the content of Orn remarkably increased (3-fold). At 10 °C, the content of Glu (1.35-fold) and Ala (1.54-fold) increased. Interestingly at 10 °C, the content of Orn, which increased significantly (3-fold) at 5 °C, did not increase. Thus, the similarity of metabolic profiles to those of freshly harvested fruit was lower in the fruit stored at 5 °C than in those stored at 10 °C. The remarkable increase in Orn at 5 °C was responsible for the lower similarity of the fruit stored at 5 °C. On the other hand, in the fruit stored at 20 and 30 °C, the content of all amino acids increased, with the exception of that of Gln and Orn (Figure 4). The content of Trp, which decreased significantly at 5 and 10 °C, increased at 20 and 30 °C. In contrast, the content of Orn and Gln, which increased at 5 °C, decreased at 20 and 30 °C. Thus, the similarity of metabolic profiles to those of freshly harvested fruit was lower in the fruit stored at 20 and 30 °C than in those stored at 5 and 10 °C.

Characteristics of Postharvest Changes at 5 and 20 °C in Different Satsuma Mandarin Cultivars. Postharvest changes in the content of sugars, organic acids, and amino acids after 14 days of storage at 5 and 20 °C were examined in the juice sacs of two Satsuma mandarin cultivars, Silverhill (midmaturing cultivar) and Miyagawa-wase (early-maturing cultivar). The contents of the metabolites at day 0 were compared with those at day 14 after harvest (Figure 5). Data are shown as the fold-change (content at day 14/content at day 0) at 5 and 20 °C. The statistical difference between the content at day 0 and the content at day 14 after harvest was analyzed at 5 and 20 °C, respectively (Figure 5).

In fruit stored at 5 °C, significant changes were observed in Met, Orn, and Gln in Silverhill and in Phe, Tyr, Met, Orn, Gln, and Glu in Miyagawa-wase (Figure 5). As observed in Aoshima-unshiu, the contents of two amino acids of cluster 4 (Figure 2), Orn (4.46-fold in Silverhill and 3.25-fold in Miyagawa-wase) and Gln (1.74-fold in Silverhill and 1.46-fold in Miyagawa-wase), increased significantly. Thus, the accumulation of Orn and Gln in response to 5 °C was common to three Satsuma mandarin cultivars.

In fruit stored at 20 °C, a significant increase in the content was observed in many amino acids, five amino acids (Phe, Trp, Val, Lys, and His) in Silverhill and 10 amino acids (Pro, Phe, Tyr, Trp, Leu, Val, Ile, Lys, His, and Ala) in Miyagawa-wase (Figure 5). Although the accumulation in amino acids in response to high temperatures in Silverhill and Miyagawa-wase was not completely common to that in Aoshima-unshiu (11 amino acids of cluster 5 in Figure 2), significant increases in five amino acids (Phe, Trp, Val, Lys, and His) were common to three Satsuma mandarin cultivars.

In Silverhill, the contents of three organic acids (isocitric acid, succinic acid, and malic acid) decreased slightly regardless of the temperatures. In Miyagawa-wase, the contents of all organic acids also decreased regardless of the temperatures (Figure 5). In succinic acid and malic acid, especially, the contents significantly decreased at 20 °C (Figure 5). In Silverhill and Miyagawa-wase, the changes in the contents of all sugars (sucrose, glucose, and fructose) were negligible regardless of the temperatures (Figure 5).

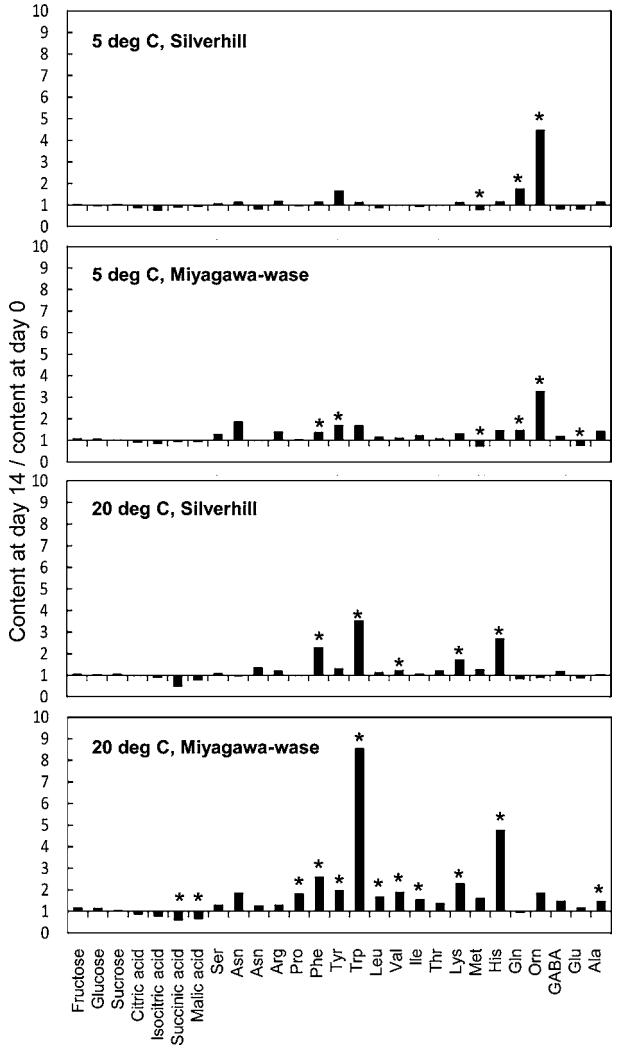

Figure 5. Postharvest relative change in the content in the juice sacs of two Satsuma mandarin cultivars, Silverhill and Miyagawa-wase, at 14 days after harvest at 5 and 20 °C. The metabolite levels at day 14 were compared with those at day 0. Asterisks indicate significant differences in the contents between the fruit at day 0 and the fruit at day 14 after harvest (Student's t test; $P < 0.05$).

DISCUSSION

Sugars, organic acids, and amino acids are major primary metabolites and important components for the internal quality of citrus fruit.[1] To date, the effect of postharvest temperatures on the changes in the contents of sugars and organic acids has been widely studied.[1−4,9,10] However, to the best of our knowledge, little research has been conducted on the effect of postharvest temperature on the changes in amino acid contents in the juice sacs of citrus fruit. In the present study, the effect of different postharvest temperatures on changes in the contents of sugars, organic acids, and amino acids was investigated at different temperatures for 14 days in the juice sacs of three different Satsuma mandarin cultivars, Aoshima unshiu, Silverhill, and Miyagawa-wase. On the basis of the metabolite profiles of the fruit before and after storage at different temperatures,

the best temperature to minimize the postharvest changes in amino acids was determined in Aoshima-unshiu. Moreover, metabolic responsiveness to temperature stress in juice sacs of Satsuma mandarin was discussed on the basis of the changes in the amino acid profile.

Responsiveness to Postharvest Temperature for Changes in the Content of Sugars, Organic Acids, and Amino Acids. The present study showed that in Aoshima-unshiu fruit, the contents of amino acids after harvest varied more than that of sugars and organic acids in response to temperatures (Figures 1, 2, and 5). Moreover, amino acids responded to temperature differently: two amino acids were cold responsive (Gln and Orn), and 11 were heat-responsive (Phe, Tyr, Trp, Leu, Val, Ile, Thr, Lys, Met, His, and GABA). In Aoshima-unshiu, the accumulation of cold-responsive amino acids (Orn and Gln) was enhanced at 5 °C but not at other temperatures (Figures 1 and 4). In other Satsuma mandarin cultivars, Silverhill (midmaturing cultivar) and Miyagawa-wase (early-maturing cultivar), the accumulation of the cold-responsive amino acids, Orn and Gln was also enhanced at 5 °C (Figure 5). These results suggest that the cold-responsive accumulation of Orn and Gln observed in Aoshima-unshiu (late-maturing cultivar) is conserved among three Satsuma mandarin cultivars regardless of their harvesting time. In contrast, in Aoshima-unshiu, the accumulation of 11 heat-responsive amino acids (Phe, Tyr, Trp, Leu, Val, Ile, Thr, Lys, Met, His, and GABA) was enhanced at 20 and 30 °C but not at 5 and 10 °C (Figures 1 and 4). Significant increases in five heat-responsive amino acids (Phe, Trp, Val, Lys, and His) in response to high temperatures above 20 °C were common in three Satsuma mandarin cultivars (Figures 1, 4, and 5). Thus, it seems that heat-responsive accumulation of Phe, Trp, Lys, Val, and His would be conserved among these Satsuma mandarin cultivars.

These results suggest that in the juice sacs of Satsuma mandarin fruit, the amino acid metabolism activated in response to temperature is different between low and high temperatures. At low temperature (5 °C), the metabolism to accumulate cold-responsive amino acids (Orn and Gln) appears to be enhanced (Figures 1, 4, and 5). In contrast, at high temperatures above 20 °C, the metabolism to accumulate heat-responsive amino acids, at least five of them (Phe, Trp, Val, Lys, and His), appears to be enhanced (Figures 1, 4, and 5). These results also suggest that there are threshold temperatures from 5 to 10 °C for the activation of metabolism to accumulate the cold-responsive amino acids and from 10 to 20 °C for the activation of metabolism to accumulate the heat-responsive amino acids.

The Best Temperature To Minimize the Postharvest Changes in Amino Acid Profiles. In citrus fruits, the optimal temperature for storage is usually determined from the viewpoint of maintenance of good appearance, suppression of rot and weight loss, and changes in acidity and total soluble solids.[1,2] In addition, the present study suggests that metabolite profiling (especially amino acid profiling) would be useful to determine the best temperature to keep the internal metabolic profiles of freshly harvested citrus fruits during short-term preservation. In the present study, postharvest changes in the amino acid profiles of Aoshima-unshiu fruits were smaller at 5 and 10 °C than at 20 and 30 °C (Figures 1 and 4). At 14 days after harvest, the amino acid profiles at 10 °C were most similar to those at day 0 after harvest because the fruits stored at 10 °C were classified into the same cluster (Figure 3). These results showed that, in the juice sacs of Aoshima-unshiu, postharvest temperatures of around 10 °C were the best to minimize the postharvest change in amino acid profiles and to maintain the internal metabolic profile of freshly harvested fruit.

At 20 and 30 °C, the similarity to freshly harvested fruit was lower than it was at 5 and 10 °C during the experimental period. At 20 and 30 °C, the accumulation of heat-responsive amino acids was responsible for the low similarity to freshly harvested fruit (Figures 3 and 4). At 5 °C, the metabolite profiles were most similar to those of freshly harvested fruit at 7 days after harvest, but the similarity decreased at 14 days after harvest (Figure 3). At 5 °C, a cold-responsive remarkable accumulation of Orn was primarily responsible for the decrease in the similarity to freshly harvested fruit (Figures 3 and 4). Recently, it was also reported that low-temperature storage (below 5 °C) resulted in the induction of the cold-responsive metabolism and the decrease in the sensory quality of mandarin fruit.[14,29,30] These studies reported that in some mandarin varieties, W. Murcott and Odem mandarin, the flavor and sensory quality of the fruits stored at 8 °C were better than those of fruits stored at temperatures lower than 5 °C.[29,30] Tietel et al. also reported that, in Odem mandarin, the massive accumulation of terpenes during the storage at low temperatures (2 and 5 °C) was most likely responsible for the decrease in flavor acceptability.[29] Thus, in several mandarins such as Satsuma mandarin and Odem mandarin, temperatures below 5 °C seem to be low enough to induce the cold-responsive metabolism in juice sacs.

Physiological Function of Amino Acid Accumulation at Different Temperatures. At 5 °C, the cold-responsive accumulation of Orn and Gln was conserved among three different Satsuma mandarin cultivars (Figures 1, 4, and 5). The accumulation of Orn and Gln in response to low temperature has also been reported in other plants.[16−19] Cook et al. reported that Orn, Gln, and citrulline accumulated in *Arabidopsis* at low temperature and suggested that the up-regulation of the urea cycle would occur as a result of low temperature.[19] It has also been reported that transgenic *Arabidopsis*, which accumulates a high content of Orn, showed a higher tolerance to salt and drought stress than the wild type because Orn is required for the synthesis of polyamine, which acts as a stress-tolerant mechanism.[31] Thus, it seems that the accumulation of Orn and Gln at 5 °C observed in the present study would result from the up-regulation of the urea cycle by low temperature. Moreover, the accumulation of these amino acids would be a metabolic response to low-temperature stress in the juice sacs of Satsuma mandarin fruit.

Like other subtropical crops, citrus fruits are chilling-sensitive plants.[1] Thus, exposure to low temperature causes various physiological alterations in citrus fruits, which sometimes cause chilling injury symptoms, such as necrosis in the peel.[1,2,32] Although chilling injury symptoms were not observed externally in the present study, a physiological alternation in the amino acid metabolism as a result of low-temperature stress was confirmed in the juice sacs of Satsuma mandarin fruits in the present study. Therefore, the present results suggest that the accumulation of Orn and Gln (cold-responsive amino acids) might be a useful indicator to detect a low-temperature stress response in the juice sacs of Satsuma mandarin without external chilling injury symptoms.

At high temperatures (20 and 30 °C), the heat-responsive accumulation of Phe, Trp, Val, Lys and His was conserved among three different Satsuma mandarin cultivars (Figures 1, 4,

and 5). In many intact plants, such as *Arabidopsis* and mature kernel, similar accumulations of amino acids were observed in response to high temperatures to enhance defense mechanisms against stresses, such as pathogen attack and physical injury, because these amino acids serve as precursors for various secondary metabolites, such as flavonoids, lignins, and phytoalexins.[17,20] In postharvest crops, proteolysis generally occurs in early storage with a concomitant increase in free amino acids.[21−24] In harvested asparagus and broccoli, aromatic amino acids (Tyr and Phe) and branched-chain amino acids (Ile, Leu, and Val) were reported to accumulate during the storage at 20 °C.[22−24] In harvested jute leaves, the accumulation of amino acids was larger at high temperature than at low temperature.[21] In these postharvest crops, it was suggested that proteolysis led to the accumulation of amino acids such as aromatic and branched-chain amino acids. Thus, it was thought that the accumulation of heat-responsive amino acids observed in the present study would be a result of the proteolysis caused by high temperatures in the juice sacs of Satsuma mandarin fruit.

In citrus fruits, such as Satsuma mandarin and grapefruit, postharvest high-temperature conditioning at around 20−30 °C has been used to reduce adverse phenomena (e.g., rot, peel disorder, and chilling injury) during storage at low temperature.[2−7] In the peel of grapefruit, prestorage heat conditioning induced physiological alteration, such as enhanced expression of various stress-related genes.[33−35] The present results suggest that high temperatures of approximately 20−30 °C would also affect the physiological alteration in the juice sacs of citrus fruits. Moreover, the present results suggest that postharvest temperatures of 20 and 30 °C would be high enough to induce a high-temperature stress response in the juice sacs of Satsuma mandarin fruit and that the accumulation of heat-responsive amino acids might be a useful indicator of the high-temperature stress response in juice sacs of Satsuma mandarin.

Effect of Postharvest Temperature on the Accumulation of Sugars and Organic Acids. In the present study, the changes in the contents of three sugars (sucrose, glucose, and fructose) were negligible during storage for 14 days at all temperatures in three Satsuma mandarin cultivars (Figures 1 and 5). Regarding the sugar contents in postharvest mandarin fruits, previous studies showed that the contents in juice sacs were approximately constant during storage for 2 months, although slight changes were observed.[9,29,30,36] On the other hand, after long-term storage of *Citrus reticulata* (Ponkan) for 90 days at 4 °C, Yun et al. observed a decrease in the contents of sucrose, glucose, and sucrose in the juice sacs and suggested that this might be due to the activation of the pentose phosphate pathway on the basis of proteomic analysis.[37] The negligible change in the sugar contents in the present study would be due to short-term (14 days) storage.

In organic acids, with the exception of malic acid in Aoshima-unshiu, the contents decreased slightly or were unchanged at all temperatures in three Satsuma mandarin cultivars (Figures 1 and 5). In contrast, the contents of malic acid in Aoshima-unshiu increased at 30 °C but decreased at other temperatures (Figure 1). Previous studies have reported that in the juice of Satsuma mandarin fruit, the titratable acidity and contents of citric acid and malic acid gradually decreased during storage.[38] Burdon et al. suggested that the reduction in acidity may be due to a rapid turnover of citric acid or the continuation of acid metabolism from pre- to postharvest.[38] In *Citrus reticulata* (Ponkan), Yun et al. also observed a significant decrease in the contents of citric acid and malic acid after long-term storage for 90 days at 4 °C and suggested that this might be due to the activation of aconitase and malate dehydrogenase, which catalyze the metabolism of citric acid and malic acid, on the basis of proteomic analysis.[37] Interestingly, in Aoshima-unshiu, the contents of malic acid increased at 30 °C but decreased at other temperatures (Figure 1). In many plants, the accumulations of TCA intermediates (e.g., malic acid) in response to high-temperature stresses have been reported.[16,17,20] For example, in some plants, such as mature kernel and asparagus, the accumulation of malic acids was observed under high temperatures.[20,39] In Satsuma mandarin, Burdon et al. reported that postharvest temperature treatment at 30 °C increased the contents of malic acid but decreased those of citric acid in the juice and suggested that the acid metabolism was affected by high-temperature treatment at 30 °C.[38] Thus, the accumulation of malic acid observed at 30 °C would be a metabolic response to adapt to temperature stress in Satsuma mandarin fruit. Moreover, our results suggest that the threshold temperatures for the activation of metabolism to accumulate malic acid in the juice sacs of Satsuma mandarin fruits are from 20 to 30 °C because the accumulation of malic acid occurred at 30 °C but did not at 20 °C in the present study (Figure 1).

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Notes

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