Changes in carotenoid levels of novel berries produced by interspecific hybridization between *Lonicera gracilipes* var. *glandulosa* (miyama-uguisukagura) and *Lonicera caerulea* ssp. *edulis* (haskap)

（ミヤマウグイスカグラ（*Lonicera gracilipes* var. *glandulosa*）とハスカップ（*Lonicera caerulea* ssp. *edulis*）の種間交雑によって作出された新規果実におけるカロテノイドの成分の変化に関する研究）

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Contents

Abbreviations

APCI	Atmospheric pressure chemical ionization
CN	Cyanopropyl
cps	counts per seconds
CrtZ	Beta-carotene 3-hydroxylase
DAD	Diode array detector
DUIS	Dual ion source of ESI and APCI
DW	Dry weight
ESI	Electronic spray ionization
EtOH	Ethanol
FW	Fresh weight
HPLC	High performance liquid chromatography
LC-MS/MS	Liquid chromatograph-tandem mass spectrometer
LC-MS/MS	Liquid chromatography/tandem mass spectrometry
LUT2	Lycopene beta/epsilon cyclase
LUT5	Beta-ring hydroxylase
LYC	Lycopene cyclase
MeOH	Methanol
MRM	Multi reaction monitoring
MS/MS	Tandem mass spectrometry
ODS	Octadecylsilyl (C18)
PC	Principal component
PCA	Principal component analysis
QC	Quality control
TBME	*tert*-butyl methyl ether
UV-Vis	Ultraviolet-visible

Chapter 1 General introduction

1.1 Background

Plant domestication for crop production occurred approximately 13,000 years ago (Diamond, 2002). This process marks the commencement of plant breeding, brought about by the deliberate selection of favorable wild species by humans. Plant breeding is an essential part of agriculture and has developed through the selection of strains with desirable traits (crop quality, yield, disease resistance, and ease of cultivation, etc.) for humans. In recent years, the various breeding goals have been as follows: climate responsiveness, crop health functionality, crop color diversity, and stress tolerance. To achieve these goals, various breeding methods are used: hybridization, polyploid breeding, mutation breeding, the molecular breeding, etc. The most popular and traditional strategy for plant breeding is hybridization coupled with selection, the method humans used to domesticate crops.

1.2 Interspecific hybrid trait prediction

Interspecific hybridization is one of the techniques for plant breeding. In fruits, interspecific hybridization is used in plants such as *Citrus* (Singh et al., 2023; Al-Naggar et al., 2009), *Vitis* (Koyama et al., 2022; Alexandrov, 2016), *Vaccinium* (Redpath et al., 2022; Miyashita et al., 2019), *Rubus* (Toshima et al., 2021), etc. It enables the introduction of new species traits into the original species. However, it is difficult to predict the traits of hybrids, especially in interspecific hybridization. Table 1 summarizes previous studies that is focused on metabolite level changes by interspecific hybridization in fruits. Interspecific hybrid compound levels usually show intermediate traits of their parents, with their sometimes increasing and decreasing compared to those in the parents. Moreover, the hybrids rarely newly produce the compounds that are not present in their parents or lack compounds existed in their parents (Gomez et al., 1993; Gancel et al., 2002; Schuster et al., 2013; Shigyou et al., 2014; Zhu et al., 2021). Although the prediction of the hybrid traits has not yet been archived recently, genomic selection has been used to select superior strains in the early stages of cultivation by predicting traits based on individual genome structure (Heffner et al., 2009; Heslot et al., 2014; Minamikawa et al., 2017). However, it is difficult to use genomic selection and predict the

hybrid traits in interspecific hybridization for the following reasons: obtaining sufficiently large datasets is challenging because interspecific hybridization is more difficult than intraspecific hybridization; interspecific hybrids possess both parents' genomes, which causes lower accuracy prediction because of different genomic architecture and epistasis (Yong and Reif 2015; Olatoye et al. 2020). Thus, it is difficult to predict what will be produced until it is actually produced and grown. The reasons include interaction among the genes derived from different genomes (Landry et al., 2007); genome structure changes by transposon activation by hybridization between distantly related strains (Fukai et al., 2022), and gene expression changes caused by epigenomic changes by hybridization (Shiraki et al., 2023).

To estimate the traits in interspecific hybrids, information on fundamental trait of parents and progeny plants is needed. Using artificially produced interspecific hybrids and investigating their traits may provide information for predicting the traits of interspecific hybrids. Among the various traits, fruit color is one of the important traits in fruits. Because fruits color is easily recognized visually and the color pigments can be quantified, it is a suitable for trait to provide information for predictions.

Moreover, color pigments are produced by biosynthesis in plants. A color pigment can be a precursor to another color pigment and can also be degraded. Because spatio-temporal gene expression is involved in the prediction of traits, analysis of pigment components during maturation stages improves the prediction accuracy. Few reports have described the measurement of components between interspecific hybrid and their parents during fruit development (Toyama et al. 2021). In the present study, the accumulation pattern was measured in the newly produced interspecific hybrids and their parents.

1.3 Production and evaluation of interspecific hybrids between miyama-uguisukagura and haskap

A novel interspecific hybrid between miyama-uguisukagura and haskap was produced (Fig. 1) as described by a previous study for producing interspecific hybrids between uguisukagura and haskap (Miyashita and Hoshino, 2010). Haskap, blue honeysuckle or honey berry, belongs to *Lonicera*, Capriforiaceae and is distributed in the cold regions in Eurasia, America, and Japan (Miyashita et al.,

2011; Plekhanova, 2000). It produces blue-purple, oval berries (Fig. 1b) that taste sweet, sour, bitter, and harsh (Takada et al., 2003). Miyama-uguisukagura, related species to haskap, belongs to *Lonicera*, Capriforiaceae and is distributed in the south of Honshu in Japan. It produces red oval berries (Fig. 1a) that taste sweet and watery. Interspecific hybrid fruits between these species showed red-purple twin-shaped berries (Fig. 1c).

The main color pigment in fruits of haskap is cyanidin 3-glucoside, an anthocyanin (Terahara et al., 1993; Chaovanalikit et al., 2004; Jordheim et al., 2007; Svarcova et al., 2007; Wojdyło et al., 2013; Celli et al., 2015; Zhao et al., 2015; Wang et al., 2016). The cause of color changes by the hybridization between miyama-uguiskagura and haskap was estimated in previous studies (Fujita et al. 2020a; Fujita et al. 2020b). The anthocyanin concentration and distribution in fruits of haskap, miyama-uguisukagura, and interspecific hybrids have been described (Fujita et al., 2020a; Fujita et al. 2020b). In these studies, anthocyanins were distributed mainly in the fruit skin, and the distribution patterns were slightly different among species. The concentration of cyanidin 3-glucoside is high in haskap and low in miyama-uguisukagura, and intermediate in an interspecific hybrid, whereas the concentration of cyanidin 3,5-diglucoside in the fruits of an interspecific hybrid was higher than that of parents. The color from anthocyanin is also affected by pH, aglycone, glycone, and co-pigmentation (Harborne, 1958; Yabuya et al., 1997; Heredia et al., 1998; Takeda et al., 2010). Although fruits pH was also investigated (Fujita et al., 2020a), the color changes could not be explained by anthocyanin and pH data.

The red color in miyama-uguisukagura fruits supposed to be derived from carotenoids (Watanabe et al., 2018; Royer et al., 2020). The fruits in uguisukagura (*Lonicera gracilips* var. *glabra*), a species related to miyama-uguisukagura, contain several types of carotenoids (Watanabe et al., 2018), and the carotenoid composition of *Lonicera* fruits vary by strains (Royer et al., 2020). To investigate the color changes by interspecific hybridization, the carotenoid composition of interspecific hybrids and their parents, miyama-uguisukagura and haskap was investigated.

1.4 Approaches for carotenoid measurements in interspecific hybrid

Detailed compounds were analyzed by LC/MS/MS to clarify carotenoid contents and provide

fundamental information on the changes in the carotenoid levels by interspecific hybridization. Interspecific hybridization in fruit trees has been frequently studied in *Vitis* spp., *Citrus* spp., etc. (Al-Naggar et al., 2009; Alexandrov, 2016; Koyama et al., 2022; Singh et al., 2023). Because haskap belongs to the Pinaceae in asterids, information on which is relatively limited compared that on rosids (Fig. 2), the present study also provides important common knowledge for the hybridization in fruits. This study also investigated the accumulation pattern of components in interspecific hybrids by measuring the amount of components at each developmental stage of the fruit; the relationship with carotenoid biosynthesis is discussed.

1.5 Aims and flow of this study

The aims of this study are to establish a carotenoid quantitative method in *Lonicera* fruits and to quantify carotenoid accumulation in fruits of miyama-uguisukagura, haskap, and their interspecific hybrid. These results are expected to provide fundamental information on fruit quality estimation in plant breeding.

In Chapter 2, the method that was established for carotenoid quantification in fruits of *Lonicera* by LC/MS/MS has been described. In this chapter, sample and standard concentration for extraction, chromatographic conditions, and mass spectrometric conditions were optimized to measure carotenoid amount in frutis of miyama-uguisukagura, haskap, and their interspecific hybrid.

Chapter 3 details the measurements of the carotenoid contents were measured in maturing fruits of miyama-uguisukagura, haskap, and their interspecific hybrid using the method established in Chapter 2. Four carotenoids were compared during development among strains and the changes induced by interspecific hybridization are discussed.

In Chapter 4, the changes and prediction of compounds by interspecific hybridization are discussed based on fundamental data obtained in this study.

Table 1. Previous studies comparing fruit components of interspecific hybrids and their parents.

genus	parents	compounds	number of compounds	number and ratio of compound chnages by hybridization					reference
				higher than parents	lower than parents	intermediate	newly produced	diminished	
Lonciera	*L.gracilipes* × *L. caerulea*	anthocyanin	6	2 (33%)		4 (67%)			Fujita et al. 2020a
Vitis	*V. murensis* × *V. vinifera*	anthocyanin	47	17 (36%)		26 (55%)	4		Zhu et al. 2021
Prunus	*P. cerasus* × *P. maackii*	anthocyanins	20	4 (20%)		7 (35%)	1 (5%)	8 (40%)	Schuster et al. 2013
Vaccinium	*V. corymbosum* × *V. bracteatum*	anthocyanins	15	6 (40%)	1 (7%)	8 (53%)			Toyama et al. 2021
Vaccinium	*V. uliginosum* × *V. corymbosum*	anthocyanins	13	8 (62%)	1 (8%)	3 (23%)	1 (8%)		Shigyou et al. 2014
Prunus	*P.domestica* × *P. cerasifera*	anthocyanins	4			4 (100%)			Treutter et al. 2012
Prunus	*P.domestica* × *P. spinosa*	anthocyanins	4		1 (25%)	3 (75%)			Treutter et al. 2012
Rubus	*R. idaeus* × *R. parvifolius*	anthocyanins	4	2 (50%)	1 (25%)	1 (25%)			Toshima et al. 2017
Citrus	*C. nobilis* × *C. tangerina*	carotenoid	8		6 (75%)	2 (25%)			Nonaka et al. 2012
Citrus	*C. nobilis* × *C deliciosa*	carotenoid	8	1 (13%)	1 (13%)	6 (75%)			Nonaka et al. 2012
Prunus	*P.domestica* × *P. cerasifera*	catechins	2	1 (50%)		1 (50%)			Treutter et al. 2012
Prunus	*P.domestica* × *P. spinosa*	catechins	2		1 (50%)	1 (50%)			Treutter et al. 2012
Citrus	*C. nobilis* × *C. kinokuni*	flavonoid	10		2 (20%)	8 (80%)			Kawaii et al. 2001
Citrus	*C. sinensis* × *C. limon*	flavonols	10		2 (20%)	8 (80%)			Tusa et al. 2007
Prunus	*P.domestica* × *P. cerasifera*	flavonols	7	3 (43%)		4 (57%)			Treutter et al. 2012

Table 1 (contiunued). Previous studies comparing fruit components of interspecific hybrids and their parents.

genus	parents	compounds	number of compounds	number and ratio of compound chnages by hybridization					reference
				higher than parents	lower than parents	intermediate	newly produced	diminished	
Prunus	*P.domestica* × *P. spinosa*	flavonols	7	2 (29%)	1 (14%)	4 (57%)			Treutter et al. 2012
Prunus	*P.domestica* × *P. cerasifera*	organic acids	3			3 (100%)			Treutter et al. 2012
Prunus	*P.domestica* × *P. spinosa*	organic acids	3	1 (33%)		2 (67%)			Treutter et al. 2012
Citrus	*C. maxima* × *C. sinensis*	minerals	10	1 (10%)	5 (50%)	4 (40%)			Singh et al. 2023
Rubus	*R. idaeus* × *R. parvifolius*	organic acids	3		2 (67%)	1 (33%)			Toshima et al. 2017
Vaccinium	*V. uliginosum* × *V. corymbosum*	organic acids	3	1 (33%)		2 (67%)			Shigyou et al. 2014
Citrus	*C. sinensis* × *C. limon*	organic acids	4		1 (25%)	3 (75%)			Tusa et al. 2007
Prunus	*P. davidiana* × *P. persica*	organic acids	9	2 (22%)	1 (11%)	6 (67%)			Moing et al. 2003
Vaccinium	*V. corymbosum* × *V. darrowii*	organic acids	4	2 (50%)		2 (50%)			Herniter et al. 2023
Vaccinium	*V. corymbosum* × *V. bracteatum*	polyphenols	5	1 (20%)		4 (80%)			Toyama et al. 2021
Rubus	*R. idaeus* × *R. parvifolius*	sugars	3	2 (67%)	1 (33%)				Toshima et al. 2017
Vaccinium	*V. uliginosum* × *V. corymbosum*	sugars	3		1 (33%)	2 (67%)			Shigyou et al. 2014
Prunus	*P. davidiana* × *P. persica*	sugars	4	2 (50%)		2 (50%)			Moing et al. 2003
Elaeis	*E. oleifera* × *E. guineensis*	tryacylglicerols	13	2 (15%)		11 (85%)			Cadena et al .2012
Prunus	*P. armeniaca* × *P. salicina*	volatile compounds	115	27 (23%)	2 (2%)	58 (50%)	13 (11%)	15 (15%)	Gomez et al, 1993

Table 1 (contiunued). Previous studies comparing fruit components of interspecific hybrids and their parents.

genus	parents	compounds	number of compounds	number and ratio of compound chnages by hybridization					reference
				higher than parents	lower than parents	intermediate	newly produced	diminished	
Citrus	*C. aurantifolia* × *C. paradisi*	volatile compounds	55	20 (36%)	3 (5%)	23 (42%)		9 (16%)	Gancel et al. 2002

Fig. 1. Fully-ripened fruits appearance of miyama-uguisukagura, haskap, and their interspecific hybrid

(a) miyama-uguisukagura (b) haskap (c) interspecific hybrid between miyama-uguisukagura and haskap

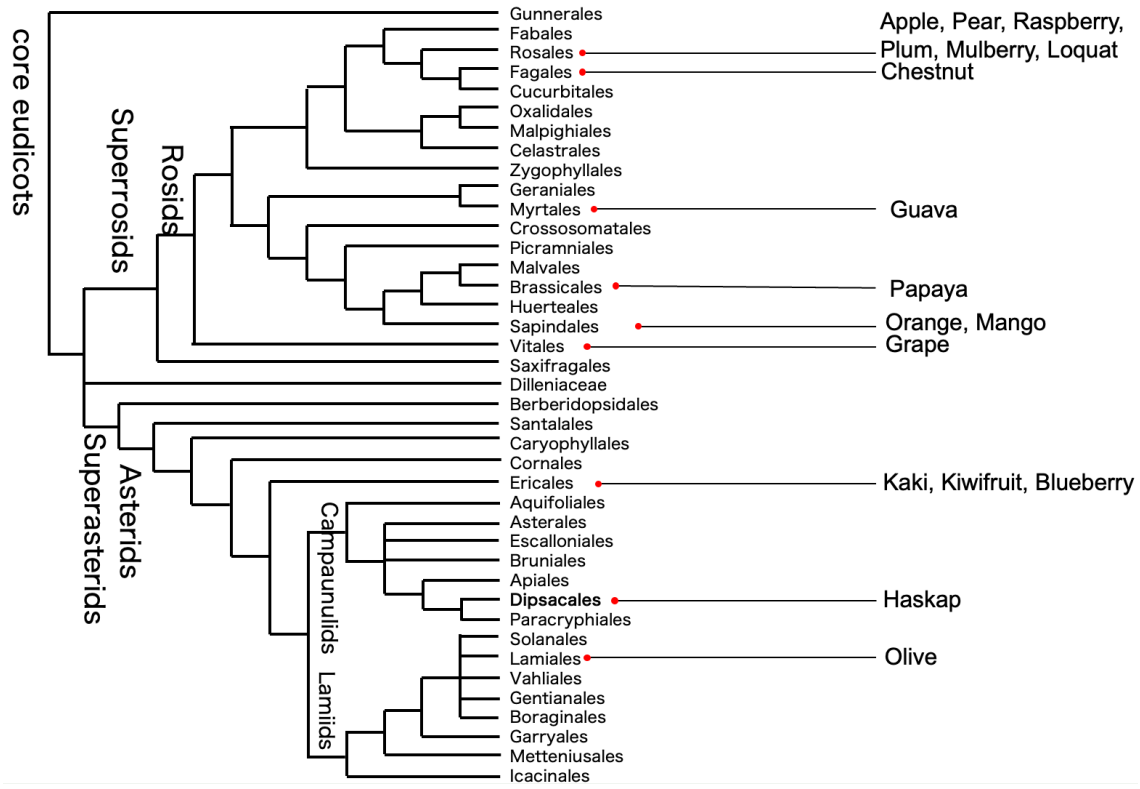

Fig. 2. Phylogenetic tree of core eudicots and representative fruit order

Chapter 2 Optimization of carotenoid quantification by LC/MS/MS

2.1 Introduction

Chromatography was invented by Tswett (1906), who reported that chlorophylls and carotenoids are separated from plant pigments by calcium carbonate and petrol ether. Horvarth and Lipsky (1967) invented HPLC (called "fast liquid chromatography" in this article), which is the origin of today's instruments that can be used for separation and quantification. At that time, no instruments could separate and quantify compounds at the same time except gas chromatography, which cannot be used for compounds that are not stable at high temperature or that do not gasify. Because HPLC overcomes these disadvantages in gas chromatography, it is widely used recently.

For carotenoid quantification, HPLC coupled with a diode array detector (DAD) or ultraviolet-visible (UV-Vis) absorption spectroscopy detector is frequently used (Arvayo-Enríquez et al., 2013). HPLC with mass spectrometry becomes frequently used for carotenoid quantification because when using DAD or UV-Vis detectors, the compounds have similar absorption spectra overlap with the target compounds, and unknown compounds cannot be qualified. Mass spectrometry enables to distinguish these compounds by the compound-specific spectrum. Moreover, tandem mass spectrometry (MS/MS) distinguish multiple compounds by product ion spectrum, that improves the accuracy of identification and quantification of compounds. By using LC/MS/MS, compound mixture is separated by three-step strategy. However, carotenoids are hardly ionized by electronic spray ionization (ESI) which is frequently used in LC/MS/MS because they are low-polarity compounds. The most frequently used method for carotenoid ionization is atmospheric pressure chemical ionization (APCI) which is suitable for no- or low-polarity compounds. ESI is the softer ionization method and commonly used whereas APCI is efficient method for low-polarity compounds and causes more frequent fragmentation than ESI because APCI is harder ionization method. However, switching ESI and APCI ion sources is time-consuming. Furthermore, APCI ion source might not be available in laboratories. DUIS, an ion source system developed by Shimadzu, includes both ESI and APCI, which is realized by adding an APCI probe to the ESI ion source. DUIS can be used for compounds with a wide range of polarity. Compared to an APCI ion source alone, it is cost-effective and time-efficient. There are no studies that report

carotenoid ionization by DUIS. In this chapter, the method for carotenoid DUIS ionization is established.

In liquid chromatography, one of the most frequently used methods is reversed-phase chromatograph with a C18 (ODS) column. C18 column is frequently the first-choice method for a standard application, c.f. metabolome analysis. Reversed-phase chromatography was invented by Schmit et al. (1971) and is frequently used for high-polarity compound separation with a low-polarity stationary phase and a high-polarity mobile phase. In contrast, a C30 column is frequently used in carotenoid analysis because carotenoid polarity is not high (Amorim-Carrilho et al., 2014). Although the column stationary phases are the same, separation patterns are changed by the bounded style, modification, and manufacture of columns and mobile phase conditions.

This chapter describes the chromatographic conditions that were optimized for the quantification of carotenoid concentration in frutis of haskap, miyma-uguisukagura, and their interspecific hybrid.

2.2 Materials and methods

2.2.1 Plant materials

One strain of miyama-uguisukagura (*Lonicera gracilipes* Miq. var. *glandulosa*) (Lg1), one strain of haskap (*L. caerulea* L. subsp. *edulis* (Turcz. ex Herder) Hultén) (Lc27), and one strain of interspecific hybrid (M27-1) were used. M27-1 was one of interspecific hybrids between Lg1 and Lc27. Lg1 is a line that was propagated from cuttings from the Botanic Garden, Hokkaido University and planted on Experiment Farm in Hokkaido University and Lc27 is planted from the site in Yufutsu managed by Hokkaido Electronic Power Co., Inc to Experiment Farm in Hokakido University. Fully ripened fruits were harvested in June 2021. After harvesting and measuring the fresh weight (FW), every 10 fruits considered one sample, and the fruits were immediately stored at -80 °C until analysis.

2.2.3 Chemicals

HPLC grade organic solvent were used for all organic solvent. Methanol (MeOH) was purchased from Kanto Chemical Co. Ltd. (Tokyo, Japan). 0.1% formic acid-water, 0.1% formic acid-

acetonitrile, and *tert*-butyl methyl ether (TBME) were purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan).

Reference standards were prepared according to previous studies which reported carotenoid in haskap fruits. α-Carotene, β-carotene, and lutein were purchased from Wako Pure Chemical Industries (Osaka, Japan). β-Cryptoxanthin was purchased from Ehime Beverage (Matsuyama, Ehime, Japan). Trans-β-apo-8′-carotenal (as an internal standard) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Hessen, Germany).

2.2.4 Carotenoid extraction

The fruit samples were freeze-dried with FDU-2200 (Tokyo Rikakikai, Tokyo, Japan) until vacuum gauge was constant for two days. After freeze-drying, the dry weight (DW) was measured and then dried fruits were milled using motor and pestle. Samples were extracted according to Salem et al. (2017) with modification. Samples (10 mg or 50 mg) were suspended in TBME/MeOH (3:1, v/v) containing 0.1 or 1 μg/mL trans-β-apo-8'-carotenal as an internal standard. The mixture was incubated at 4 °C and 800 rpm for 45 min with Thermomixer comfort (Eppendorf, Hamburg, Germany). Then, the mixture was sonicated in ice water for 15 min, 650 μL of ice-cold MeOH/water (3:1, v/v) was added, and the mixture was stirred for 1 min. After stirring, the sample was centrifuged at 16,100 × g at 4 °C for 5 min. The resulting mixture was divided into an ether-based phase, a water-based phase, and a precipitate. The ether-based phase was filtered with a 0.20 μm filter, DISMIC-25CS (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). Extracts were stored at -80°C until HPLC analysis.

For the standard, 1 mg/mL samples in 100% ethanol (EtOH) were prepared as stock solution. To optimize the concentration of standard, 2.5 μg/mL samples were used first and then adjusted to the optimal concentration. The final concentrations (and number of points) to make calibration curves of α-carotene, β-carotene, lutein, and β-cryptoxanthin were 5-1,000 ng/ml (seven points), 10-4000 ng/ml (nine points), 100-10,000 ng/ml (six points), and 50-20,000 ng/ml (nine points), respectively.

2.2.5 HPLC conditions

HPLC was peformed with a Nexera XR system (Shimadzu, Kyoto, Japan). According to

previous studies, major HPLC columns for carotenoid separation are the cyanopropyl (CN) and C30 columns. Both protocols were conducted for chromatographic optimization.

First, a CN column was used with reversed-phase mode using acetonitrile according to Abate-Pella et al. (2017) with a few modifications. A YMC-Pack CN column (100 mm × 2.0 mm i.d., 3 μm; YMC, Kyoto, Japan) was used and column temperature was set at 25 °C. The mobile phase consisted of 0.1% formic acid-water (A) and 0.1% formic acid-acetonitrile. The gradient program was as follows: 0–70 min, 65–95% B; 70–90 min, 95% B; 90–90.01 min, 100-0% B; 90.01–100 min, 65% B (equivalation time). Flow rate was set to 0.4 mL/min.

Second, a C30 column was used with reversed phase mode using ether. A Navi C30-5 column (150 mm × 2.0 mm i.d., 5 μm; Wako Pure Chemical, Osaka, Japan) was used and the column temperature was set at 25 °C. The mobile phase consisted of MeOH/TBME/water (81:15:4, v/v/v) (A) and MeOH/TBME/water (7:90:3, v/v/v) (B). The gradient was from 0 to 100 % B linearly with a 0.2 mL/min flow rate for different time (30 and 90 min).

2.2.6 Mass spectrometry conditions

Mass spectrometry was conducted with LCMS-8045 (Shimadzu, Kyoto, Japan). Ionization mode was conducted with only the ESI mode or the DUIS mode in positive ion mode. The parameter conditions were as follows: nebulizer gas (nitrogen) flow rate, 3.0 L/min; drying gas flow rate, 3.0 L/min; heating gas flow rate, 10.0 L/ min; interface voltage, 4.0 kV; interface temperature, 400 °C; corona needle voltage (only in DUIS mode), 4 kV; desolvation line (DL) temperature, 250 °C; heat block temperature, 400 °C; conversion dynode, 10 kV; collision-induced dissociation (CID) gas (argon), 230 kPa. Reference standards were used for the optimization of the Q1 and Q3 rod bias and collision energy. To detect carotenoids except when used as reference, the multi reaction monitoring (MRM) mode was used. By comparing retention time and mass spectra with previous studies, carotenoids without reference were identified.

2.2.7 Data analysis

LC-MS/MS raw data were processed with LabSolutions (version 5.109) (Shimadzu, Kyoto,

Japan). Calibration curves were drawn with Microsoft Excel (ver 16.78).

2.3 Results

2.3.1 Optimization for chromatograph condition

First, the CN column method was performed with an analytical standard. The MRM chromatogram of β-carotene is shown in Fig. 3. Using a CN column with reversed-phase mode, the peak that should include the β-carotene showed an early retention time (2.4-3.4 min) and a broad peak. β-Carotene polarity is relatively lower than that of other carotenoids, which suggests that it is difficult to separate carotenoids using this method.

Then C30 column method was then performed with an analytical standard. The MRM chromatogram is shown in Fig. 4. All carotenoid peaks were almost separated: the retention times of α-carotene, β-carotene, lutein, and β-cryptoxanthin were 33.3 min, 35.8 min, 9.25 min, and 21.5 min, respectively (Table 2). These peaks were sharp, whereas α- and β-carotene peaks were not separated in the 30 min method, although they showed specific retention times (Fig. 5).

2.3.2 Optimization of mass spectrometry condition

To optimize the Q1 and Q3 pre-rod bias and the collision energy and select the product ion for quantification, 5 µL of 1 µg/mL standard in TBME/MeOH (1:1, v/v) was injected using the C30 column 90 min method. Table 2 shows the most effective values of mass spectrometry conditions.

To explore the other carotenoids, the same sample extracts were used. Based on previous studies and column manufacture instructions, nine carotenoids which are highly related to α-carotene, β-carotene, lutein, and β-cryptoxanthin in carotenoid biosynthesis were estimated by retention time and mass spectrum. However, no matched these carotenoids were observed in the fruits of Lg1, M27-1, and Lc27.

2.3.3 Optimization of sample and standard concentration

To optimize sample and standard concentration, the 10 mg sample method and 2.5 µg/mL standards except the internal standard (1 µg/mL) were tried first. The intensity of each carotenoid and

each sample are listed in Table 3. Because internal standard intensity was 1.6×10^5 cps, which was higher than the average of the other standards, the internal standard concentration should be decreased. Some carotenoid intensities in were under 1×10^4 cps, which was a little low for stable quantification. Sample concentration should be increased fivefold; in other words, 50 mg samples were recommended for extraction. Calibration curves showed good linearity in all carotenoid analytical standards (Fig. 6).

2.4 Discussion

This study showed that the C30 column was more suitable for the separation of carotenoids, which is consistent with previous studies (Amorim-Carrilho et al., 2014; Rivela et al. 2012). In the experiments with CN column, carotenoids were not retained. The CN column is a cyanopropyl-bonded silica sorbent column and is frequently used for hydrophobic analytes in reversed-phase mode (Jandera and Hájek, 2019). The C30 column includes triacontane (low polarity) and retains analytes more strongly than the C18 column, the most frequently used column known as ODS column (Vyňuchalová and Jandera, 2015). Carotenoids are low-polarity, hydrophobic compounds. The CN column has been used for carotenoid separation in a previous study (Abate-Pella et al. 2017), whereas carotenoids were not retained in the present study with the CN column. That may be caused by differences in manufacture and variety of columns. Stationary phases of columns are often modified: i.e., the hydroxy group is replaced by a methyl group; the length of carbon chain is different. The C30 column showed a higher retaining ability than the CN column in the present study.

Carotenoids were separated using only the 90 min protocol. In this protocol, the most strongly retained carotenoid was β-carotene, with a retention time of 35.8 min. To save running time, latter half gradient can be shortened. It suggests that the following gradient is the best for the separation of these carotenoids: 0–45 min, 0–50% B (same gradient as 90 min protocol); 45–50 min, 50–100% B (linear gradient); 50–55 min, 100% B; 55–55.01 min, 100–0% B; 55.01–60 min, 0% B (equivalation time).

The DUIS method was used for carotenoid ionization, which enables ESI and APCI at the same time. Usually ESI is used for high-polarity compounds and APCI is used for low- to middle-polarity compounds. In most studies, carotenoid is ionized with the APCI method (Amorim-Carrilho et al., 2014) because of carotenoid's low-polarity traits. There are some reports that carotenoid is ionized by

the ESI method, despite carotenoids being low-polarity compounds (Mertz et al., 2010; Vallverdú-Queralt et al., 2012). Taken together, carotenoids are ionized by both the ESI and APCI methods, which indicates that the DUIS method is suitable for carotenoid ionization. Based on the preliminary experiments, carotenoids are difficult to detect using only the ESI mode (data not shown).

In this chapter, the detection of four carotenoids has been described, and no other carotenoids were detected in miyama-uguisukgura, haskap, or their interspecific hybrid fruits. Palíková et al. (2008) reported that haskap fruits contained six carotenoids, carotene α, carotene, β, xanthophyll, lycopene, cryptoxanthin, and zeaxanthin. Watanabe et al. (2018) also reported that haskap fruits include two carotenoids, lutein and β-carotene. These reports are inconsistent with the present study, which may be caused by difference in extraction and detection methods, strains and cultivation environment. Carotenoid were detected by UV/Vis detector in these studies (Palíkova et al. 2008; Watanabe et al. 2018), whereas mass spectrometer is used in the current study. León-Chan et al. (2017) reported that the carotenoid concentration was increased at low temperatures and ultraviolet radiation stress in bell pepper (*Capsicum annuum*).

The LC/MS/MS methods were optimized to separate, ionize, and detect carotenoids. This achievement allowed the measurement of the carotenoid concentration of fruits in the present study (miyama-uguisukagura, haskap, and their interspecific hybrid). The methods are expected to be applicable to the quantification of carotenoids in other fruits.

2.5 Conclusion

A carotenoid quantification method was optimized for fruits of haskap and related species fruits. A C30 column with a reversed-phase mode efficiently separated the compounds, and the DUIS probe improved the ionization efficiency of carotenoids compared to the ESI probe. This is the first study for using DUIS probe to ionize carotenoids. For the separation of α-carotene, β-carotene, lutein, β-cryptoxanthin, and trans-β-apo-8′-carotenal, the following gradient was suitable: a linear gradient of 0-45 min of ether rich elution, then washing and equivalation. This method is expected to be applied for carotenoid quantification in various horticultural crops as well as *Lonicera* fruits and provides the insights for the use of DUIS to detect carotenoids.

Table 2. Retention time and MRM (multi reaction monitoring) settings for carotenoid detection.

Analyte	Retention time (min)	MS (*m/z*)	MS^2 (*m/z*)	Q1 pre-rod bias (V)	Collision energy (eV)	Q3 pre-rod bias (V)
trans-β-apo-8'-carotenal	15.3	417.60	105.10	-12.0	-9.0	-17.0
α-carotene	33.3	537.30	123.10	-20.0	-27.0	-24.0
β-carotene	35.8	537.30	445.40	-28.0	-16.0	-23.0
lutein	9.25	553.40	119.20	-20.0	-37.0	-13.0
β-cryptoxanthin	21.5	553.40	461.35	-20.0	-16.0	-25.0

Table 3. The list of the intensity of carotenoids in the standard mixture (2.5 μg/ml each carotenoid and 1.0 μg/ml internal standard) and each sample (10 mg/ml at first extraction)

solution	trans-β-apo-8'-carotenal	α-carotene	β-carotene	lutein	β-cryptoxanthin
standard mixture	1.6×10^5	1.4×10^5	7.5×10^4	2.9×10^4	2.3×10^4
Lg1 extract	-	1.0×10^4	2.2×10^4	3.5×10^3	2.0×10^4
M27-1 extract	-	3.0×10^3	3.0×10^4	3.5×10^3	1.0×10^4
Lc27 extract	-	nd	2.2×10^4	6.0×10^3	6.0×10^3

nd: not detected
-: no data

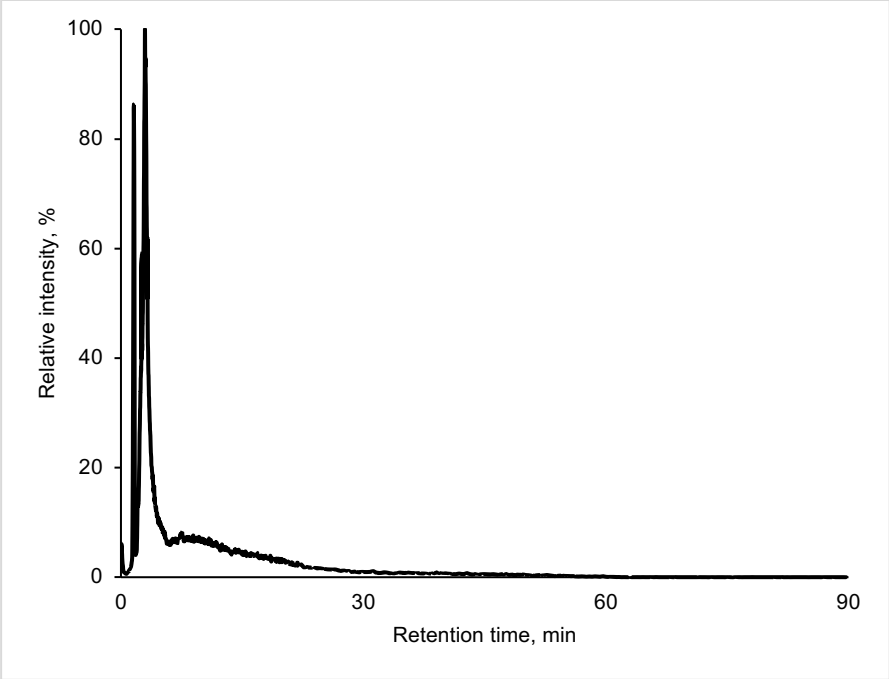

Fig. 3. UHPLC/MS/MS single reaction monitoring chromatogram of β-carotene analytical standard

Column, YMC-Pack CN column (2.0 mm × 100 mm i.d., 3 μm; YMC, Kyoto, Japan)

Gradient time ,90 min.

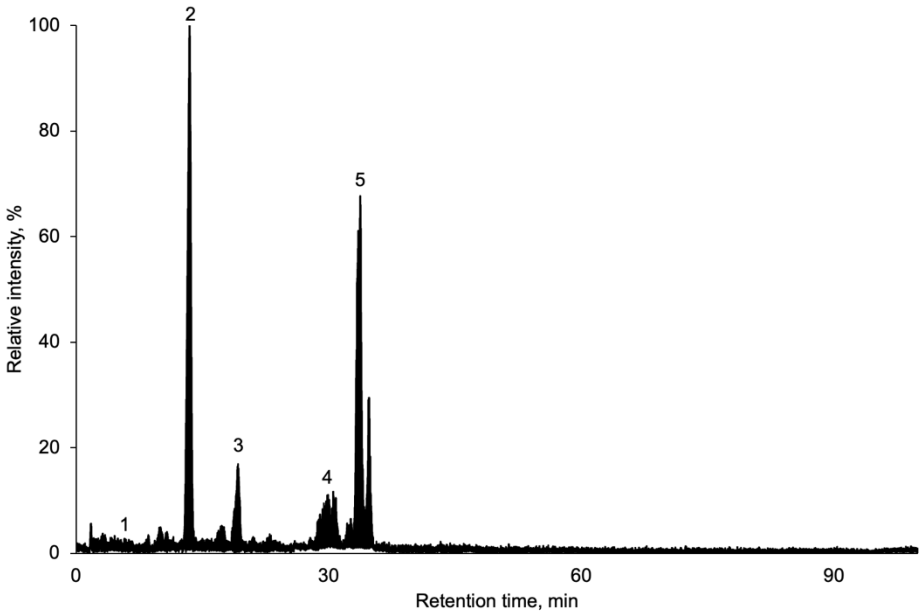

Fig. 4. UHPLC/MS/MS total ion current chromatogram of analytical standard with the 90 min method

Column, Navi C30-5 column (2.0 mm × 150 mm i.d., 5 μm; Wako Pure Chemical, Osaka, Japan); Gradient time ,90 min.

Peak numbers correspond to 1, lutein; 2, trans-β-apo-8'-carotenal (internal standard); 3, β-cryptoxanthin; 4, α-carotene; 5, β-carotene.

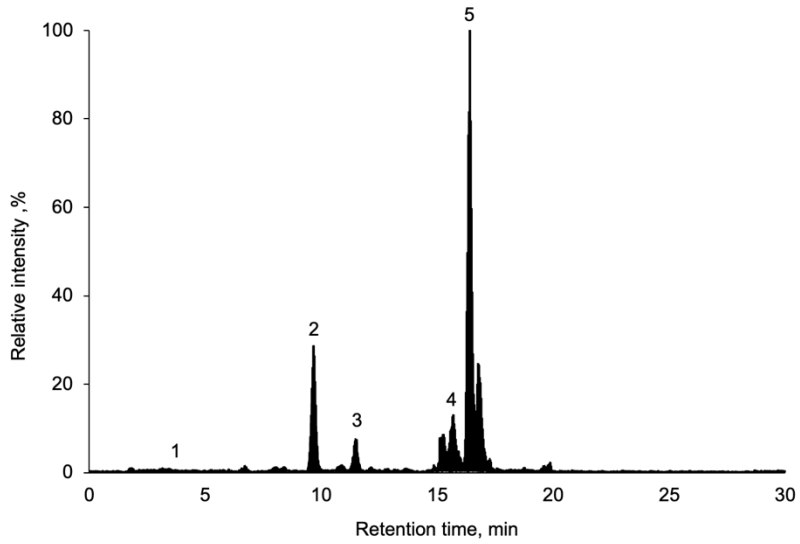

Fig. 5. UHPLC/MS/MS total ion current chromatogram of analytical standard with the 30 min method

Column, Navi C30-5 column (2.0 mm × 150 mm i.d., 5 μm; Wako Pure Chemical, Osaka, Japan); Gradient time ,30 min.

Peak numbers correspond to 1, lutein; 2, trans-β-apo-8'-carotenal (internal standard); 3, β-cryptoxanthin; 4, α-carotene; 5, β-carotene.

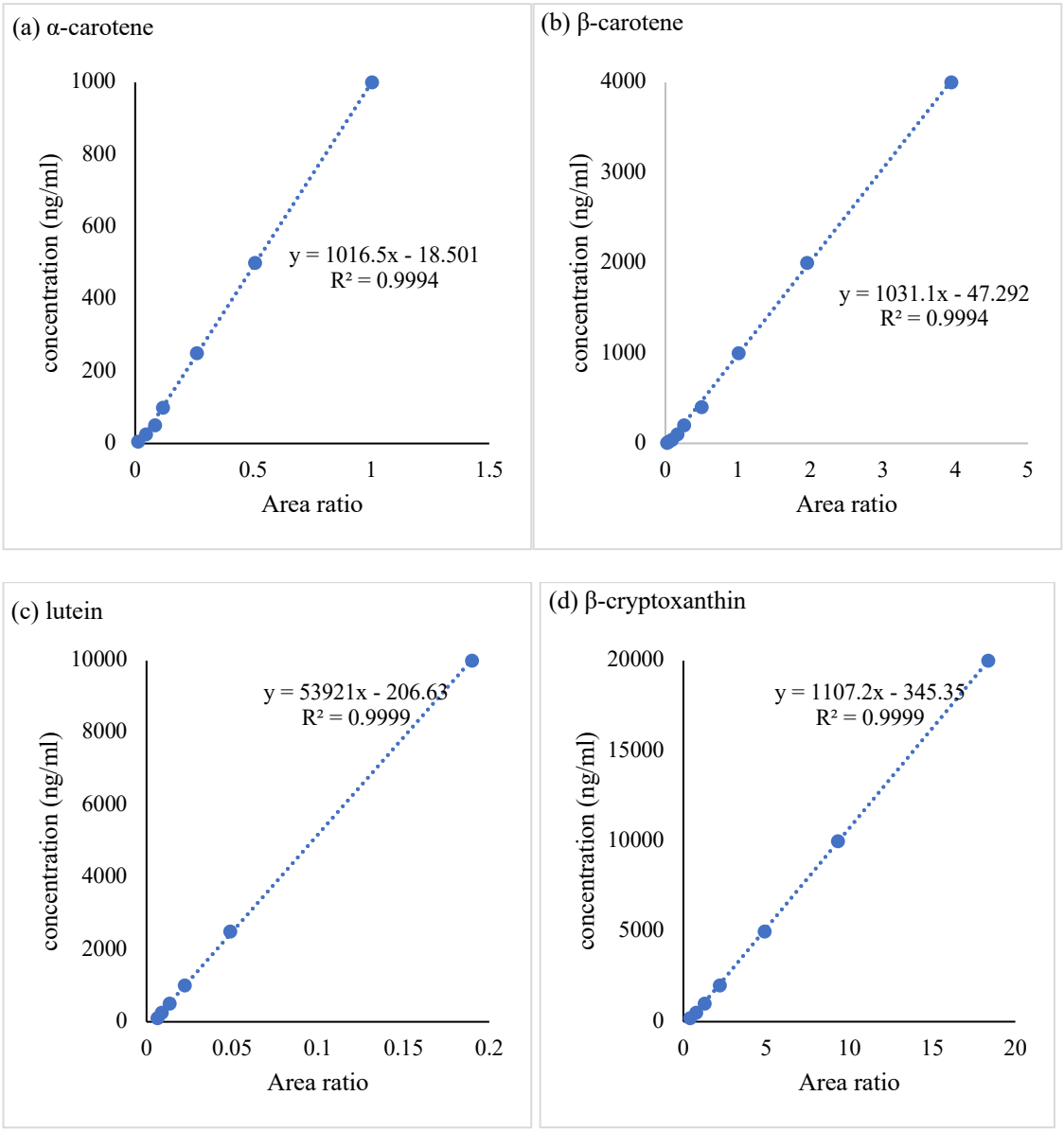

Fig. 6. Calibration curve of four carotenoids.

(a) α-carotene (b) β-carotene (c) lutein (d) β-cryptoxanthin

Chapter 3 Carotenoid concentrations in fruits of miyama-uguisukagura, haskap, and their interspecific hybrid

This chapter is rewritten based on the research article "Novel production of β-cryptoxanthin in haskap (Lonicera caerulea subsp. edulis) hybrids: Improvement of carotenoid biosynthesis by interspecific hybridization" (https://doi.org/10.1016/j.scienta.2022.111547) published in Scientia Horticulturae 308:111547 (2023).

3.1 Introduction

Carotenoids are healthy functional compounds and β-cryptoxanthin, one of the carotenoids, is registered as an ingredient of "Foods with Functional Compounds" in Japan. The following beneficial functions to human health of carotenoids have been reported: protective compounds in cancer (Nishino et al. 2009), diabetes mellitus (Roohbakhsh et al., 2017), and non-alcoholic fatty liver disease (Xiao et al., 2019). Dietary intake of these compounds is important.

Previous studies revealed the concentration of each carotenoid in fruits (Table 4). Representative fruits such as papaya (0.16–0.494 mg/100 g FW), cherry (0.10–0.16 mg/100 g FW), and apricot (0.0021–1.19 mg/100 g FW) contain β-cryptoxanthin (Dias et al., 2009; Schweiggert et al., 2012; Zhou et al., 2020). Carotenoid concentrations also differ by species in the same genus; Satsuma mandarin (*Citrus unshiu*) contains a high β-cryptoxanthin concentration (1.50–1.75 mg/100 g FW) (Ernawita et al., 2016) whereas indigenous *Citrus* (*Citrus* spp.) contain less β-cryptoxanthin (0–0.4 mg/100 g FW) (Matsumoto et al., 2019).

Few reports describe the carotenoid concentration in *Lonicera* spp. fruits. One of these reports claims that 0.01 mg/100 g FW β-cryptoxanthin is included in haskap berries (Palíková et al., 2008). The other study reports that uguisukagura, a related variety of miyama-uguisukagura, includes β-cryptoxanthin whereas haskap does not (Watanabe et al., 2018).

Interspecific hybrids between miyama-uguisukagura and haskap have been investigated (Fujita et al., 2020a; Fujita et al., 2020b). These strains should be good plant materials for haskap breeding because the hybrids are expected to inherit superior traits (high concentration of β-cryptoxanthin) from miyama-uguisukagura. However, there is no information on the carotenoid composition of

interspecific hybrids of miyama-uguisukagura and haskap.

Hybridization is one of the popular strategies for plant breeding. When genetically distantly related strains are hybridized, hybrid vigor sometimes occurs. Hybrid vigor is a phenomenon in which the progeny shows traits superior to those of its parents, and it is a frequently used method for producing vegetable cultivars: e.g., starch content, protein content, and grain yield increased in *Zea mays* (Khan et al. 2014); yield, quality disease resistance, and tolerance increased in *Brassica oleracea* (Ji et al. 2020); productivity, vitality, resistance, and uniformity are increased in *Solanum melongena* (Kumar et al. 2020). The hybrid vigor also occurs in the case of interspecific hybridization: e.g., seed cotton yield increased in *Gossypium* spp. (Gohli et al. 2017); seed yield increased in *Brassica* spp. (Gupta et al. 2018); drought stress resistance increased in *Fraxinus* spp. (Zeng et al. 2014). In the case of interspecific hybridization between miyama-uguisukagura and haskap, a previous study reported that hybrid fruits of some lines contained more cyanidin 3,5-diglucoside and peonidin 3,5-diglucoside than its parents. Thus, it is expected that the carotenoid composition is also dramatically changed by interspecific hybridization.

The aim of this study was to quantify these carotenoid accumulations in the fruits of miyama-uguisukagura, haskap, and their interspecific hybrid during maturation. The optimized LC/MS/MS method with the C30 column (described in Chapter 2) was used to separate and detect the compounds. It provides a basis for the hybrid vigor mechanism in different side from the previous study, which corresponds to bioactive compounds, polyploidy, and a novel interspecific hybrid.

3.2 Materials and methods

3.2.1 Plant materials

One strain of miyama-uguisukagura (*Lonicera gracilipes* Miq. var. *glandulosa*) (Lg1), three strains of haskap (*L. caerulea* L. subsp. *edulis* (Turcz. ex Herder) Hultén) (Lc23, Lc27, and Lc37), and four strains of interspecific hybrids (M23-1, M27-1, M27-4, and M37-2) were used. Lg1 is a line that was propagated from cuttings from the Botanic Garden, Hokkaido University and planted on Experiment Farm in Hokkaido University. Lc23 and Lc27 are planted from the site in Yufutsu managed by Hokkaido Electrinc Power to Experiment Farm in Hokakido University and Lc37 are planted from

Kashiwahara in Yuhutsu Mire. M23-1, M27-1, M27-4, and M37-2 were interspecific hybrid between Lg1 and Lc23, Lc27, Lc27, and Lc37, respectively. These interspecific hybrid plants were produced as described by Miyashita and Hoshino (2010). Briefly, seeds were obtained by crossing between miyama-uguisukagura (2n = 36; supposed to be tetraploid), which was used as a seed parent, and haskap (2n = 36; tetraploid), which was used as a pollen parent. The seeds were grown in sterile media and transplanted to the field. The obtained interspecific hybrid was allotetraploid (amphidiploid). Fruits were harvested from May to June in 2021 in five stages (I, young fruit; II, green grown fruit; III, half-veraison fruit; IV, veraison fruit; V, fully ripened) (Fig. 7). After harvesting and measuring the FW, every 10 fruits were considered one sample, and the fruits were immediately stored at -80 °C until analysis.

3.2.2 Chemicals

The used chemicals were almost the same as those described in Chapter 2. HPLC-grade MeOH was purchased from Kanto Chemical (Tokyo, Japan). HPLC-grade *tert*-butyl methyl ether were purchased from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). α-Carotene, β-carotene, and lutein were purchased from Wako Pure Chemical Industries (Osaka, Japan). β-Cryptoxanthin was purchased from Ehime Beverage (Matsuyama, Ehime, Japan). Trans-β-apo-8′-carotenal (as an internal standard) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Hessen, Germany).

3.2.3 Sample extraction

The sample extraction procedure was almost as described in Chapter 2. Dry samples (50 mg) were used to extract carotenoid. All samples (120 samples: eight strains with five stages with three replicates) were pooled with equal amounts as quality control (QC). QC was used to normalize the drifts of mass spectrometry signal (called LOWESS normalization).

3.2.4 Chromatography

HPLC was performed with a Nexera XR system (Shimadzu, Kyoto, Japan). A Navi C30-5 column (150 mm × 2.0 mm i.d., 5 μm; Wako Pure Chemical, Osaka, Japan) was used and the column

temperature was set at 25°C. The mobile phase consisted of MeOH/TBME/water (81:15:4, v/v/v) (A) and MeOH/TBME/water (7:90:3, v/v/v) (B). The gradient program was as follows: 0–45 min, 0–50% B; 45–50 min, 50–100% B; 50–55 min, 100% B; 55–55.01 min, 100–0% B; 55.01–60 min, 0% B (equivalation time). Flow rate was set to 0.2 mL/min. Experiments were performed as three replicates for each strain and stages.

3.2.5 Mass spectrometry

Mass spectrometry was performed with an LCMS-8045 (Shimadzu, Kyoto, Japan). Ionization mode was performed with a DUIS mode in positive ion mode. The parameter conditions were as described in Chapter 2: nebulizer gas (nitrogen) flow rate, 3.0 L/min; drying gas flow rate, 3.0 L/min; heating gas flow rate, 10.0 L/ min; interface voltage, 4.0 kV; interface temperature, 400 °C; corona needle voltage (only in DUIS mode), 4 kV; desolvation line (DL) temperature, 250 °C; heat block temperature, 400 °C; conversion dynode, 10 kV; collision-induced dissociation (CID) gas (argon), 230 kPa. Q1 and Q3 pre-rod bias and the collision energy are listed in Table 2.

3.2.6 Data analysis

LC-MS/MS raw data were processed with LabSolutions (version 5.109) (Shimadzu, Kyoto, Japan). Area data of the four carotenoids and the internal standard were manually obtained. Extracted data were normalized with the LOWESS method, and the area ratio was calculated by as follows: (target carotenoid area)/(internal standard area) with Microsoft Excel (ver 16.78). The obtained data were analyzed and visualized using Microsoft Excel (ver 16.78) and Python version 3.9.7, with the following libraries: pandas (version 1.3.4), numpy (version 1.20.3), sklearn (version 0.24.2), statsmodels (version 0.12.2), and matplotlib (version 3.4.3). Tukey's honestly significant difference (HSD) test was performed between growth stages in each strain and between strains in the stage V (fully ripened). Principal component analysis (PCA) was performed using all the data and only the stage V (fully ripened) data.

3.3 Results

3.3.1 Comparison of fully ripened fruit carotenoid concentration comparison among miyama-uguisukagura, haksap, and their interspecific hybrid

Four carotenoids, α-carotene, β-carotene, lutein, and β-cryptoxanthin, were commonly detected in fully ripened fruits of all strains (Fig. 8). However, in the haskap strains, Lc23, Lc27, and Lc37, the concentration of β-cryptoxanthin was lower than the quantification limit (trace). Fig. 9 and Table 5 shows each carotenoid concentration per 100 g of FW fruit among the eight strains.

α-Carotene was more abundant in the miyama-uguisukagura strain than in the interspecific hybrid. Interspecific hybrid strains showed intermediated values and there were significant differences between interspecific hybrid strains and their parents except M23-1 vs. Lc23.

β-Carotene was most abundant in interspecific hybrids. M23-1 and M37-2 contained significantly high concentrations of β-carotene among all strains and all interspecific hybrid strains tended to contain higher concentrations of β-carotene than their parents. The β-carotene concentration in haskap strains was almost the same as that in miyama-uguisukagura.

Lutein was most abundant in Lc27, a haskap strain. However, no significant differences were observed in lutein concentration between an interspecific hybrid and its parent haskap.

β-Cryptoxanthin was most abundant in Lg1. As mentioned before, the β-cryptoxanthin concentrations of haskap were below the limit of quantification. Hereinafter, a concentration below the limit of quantification is regarded as zero. Interspecific hybrids showed intermediated values significantly higher than those of haskap and lower than those of miyama-uguisukagura except M37-2.

PCA was performed using the data shown in Table 6 and Fig. 10. The first and second principal components (PC1 and PC2) accounted for 59.0% and 27.4% of total variance, respectively. The samples of each strain were almost clustered, and especially, miyama-uguisukagura samples were distantly clustered from other strains. Interspecific hybrid samples were not plotted on an intermediate region; M23-1 and M37-2 showed higher values of PC2. The vector directions of α-carotene and β-cryptoxanthin were similar and the ones of β-carotene and lutein were almost opposite.

3.3.2 Comparison of carotenoid concentration in all fruit stages in each strain

Fig. 11 and Table 7 show the fruit carotenoid concentration changes during fruits ripening. Generally, the levels of each carotenoid did not increase or decrease linearly and most of carotenoid concentration remained unchanged during ripening with a few exceptions. Lutein levels commonly decreased in the earlier stage among all strains. Although β-cryptoxanthin levels tended to increase, the accumulation pattern differed among strains; in Lg1, β-cryptoxanthin was accumulated gradually, whereas in M27-4, which contains the highest β-cryptoxanthin concentration among all strains except Lg1, it was significantly accumulated from stage IV (veraison) to stage V (fully ripened).

By using these 120 datasets (8 strains × 5 stages × 3 replicates), PCA was performed (Table 8 and Fig. 12). The first and the second principal components (PC1 and PC2) accounted for 45.4% and 36.8% of total variance, respectively. Lg1 was almost classified with any other strains through all stages. The interspecific group was spread widely and included the haskap group. The vectors of α-carotene and β-cryptoxanthin pointed in the same direction as in Fig. 10, whereas the vectors of β-carotene and lutein pointed in a same direction different from that in Fig. 10.

Generally, compound concentrations in fruits were expressed as mg/100 g FW or mg/100 g DW. However, FW and DW of fruits increased during maturing (Table 9). Carotenoid concentrations are shown as mg/fruit in Fig. 13 and Table 10. The trends of accumulation pattern were similar to Fig. 11 and Table 7 except for the stage I (young) and stage V (fully ripened). As shown in Table 9, FW and DW in stage I (young) and V (fully ripened) were quite different from stage II (green grown) to stage IV (veraison).

3.3.3 Correlation analysis

Table 11 shows a correlation coefficient between each carotenoid concentration in stage V (fully ripened) and each carotenoid concentration in all stages. α-Carotene and β-cryptoxanthin in stage V were highly correlated with α-carotene and β-cryptoxanthin in all stage. Interestingly, lutein concentration in stage I (young fruit) was highly correlated with β-cryptoxanthin concentration in stage V (fully ripened).

3.4 Discussion

It was revealed that all strains had four carotenoids; however, haskap possessed small amounts of β-cryptoxanthin. Haskap contains four carotenoids: Palíková et al. (2008) reported that blue honeysuckle contains <0.05 mg/100 g FW of α-carotene, 0.72 mg/100 g FW of β-carotene, and 0.01 mg/100 g FW of β-cryptoxanthin; Mech-Nowak et al. (2014) reported that lutein concentration in *L. caerulea* var. *kamtschatica* ranged from 0.0238 to 0.4289 mg/100 g FW. These values were slightly different from those of the current study, which may be caused by differences in strains or varieties.

In the present study, β-cryptoxanthin was quantificated in interspecific hybrid, although it could not be quantified in haskap. This suggests that interspecific hybridization induces the β-cryptoxanthin synthesis ability of haskap. As described in the introduction of this chapter, there are few fruit species that contain high concentrations of β-cryptoxanthin. These experiments revealed that β-cryptoxanthin concentration in miyama-uguisukagura and interspecific hybrid fruits was comparable to that of cherry, papaya, apricot, etc. Interspecific hybridization may be an effective breeding method to induce or increase functional compound synthesis ability.

Interspecific hybrids tended to contain higher concentrations of β-carotene than their parents. This phenomenon, the increasing in some components by interspecific hybridization, has been previously reported (Mikulic-Petkovsek et al., 2014; Stanys et al., 2019; Storsberg et al., 2004). In miyama-uguisukagura (Lg1), β-carotene levels decreased from stage II to stage III, and in haskap, they decreased or tended to decrease at the same time, whereas in interspecific hybrids, β-carotene levels remained unchanged during ripening. β-Carotene is synthesized from δ-carotene and is a precursor of β-cryptoxanthin. The results suggest that β-carotene's synthesis and metabolic rates are equilibrated during ripening in interspecific hybrids, whereas the metabolic rate exceeds the synthesis rate in miyama-uguisukaguara and haskap. This suggestion is supported by the results that β-cryptoxanthin levels increased during ripening in miyama-uguisukagura. However, β-cryptoxanthin was not quantificated in haskap. In haskap, β-cryptoxanthin synthesis may be rate-controlled by downstream biosynthesis or LUT5 or CrtZ are not present at certain periods when β-carotene is present.

Fig. 14 shows the common carotenoid biosynthesis pathway in plants with the final concentrations of carotenoids detected in this study. The four carotenoids detected are mainly divided into two groups: α-carotene and lutein (δ-carotene pathway), and β-carotene and β-cryptoxanthin (γ-carotene pathway). The concentration ratio of δ-carotene pathway compounds/γ-carotene pathway compounds were higher in haskap (0.60-1.04). Interspecific hybrids showed intermediate or lower ratios (0.13-0.48) than miyama-uguisukagura (0.22). This suggests that during carotenoid biosynthesis, the gene expression ratio of LUT2/LYC in haskap is greater than that in miyama-uguisukagura or interspecific hybrid, indicating that biosynthesis is modified by interspecific hybridization.

The accumulation pattern of β-cryptoxanthin was different between Lg1 and M27-1 (Fig. 11). In Lg1, β-cryptoxanthin increased linearly during development, whereas in M27-1, it increased rapidly in the latter developmental stages. As mentioned above, β-cryptoxanthin is synthesized by LUT5 or CrtZ from β-carotene. β-carotene was present in the fruits of all stages, suggesting that the timing of LUT5 or CrtZ expression was different between these strains. In Lg1, LUT5 or CrtZ thought to be expressed constatly during development, whereas in M27-1, these genes were expressed in the latter stages. Different timing of carotenoid biosynthesis-related gene expression leads to different patterns of carotenoid accumulation using squash strains (Nakkanong et al. 2012), which is consistent with the hypothesis mentioned above.

In most species and for most compounds, hybrids show intermediate traits of their parents (Storsberg et al., 2004; Hallgren et al., 2003; Bajpai et al., 2019). The present study and a previous study (Fujita et al., 2020a; Fujita et al., 2020b) showed that some compounds are increased by interspecific hybridization. This phenomenon can be defined as "vigor" phenomenon. Shimizu-Inatsugi et al. (2009) and Paape et al. (2016) also reported that *Arabidopsis kamchatica*, an interspecific hybrid between *A. lyrata* and *A. harelli*, distributed in broader habitats than its parent species and possesed the zinc accumulation ability from *A. harelli*, which indicated that the hybrid inherited traits from both parents and obtained robustness regarding various climates and environments. The hybrid vigor mechanism remains unknown, although some hypotheses have been proposed. Three of the most prevalent theories are "dominance," "over-dominance," and "epistatic interactions" (Botet et al., 2020). Epigenetic (retrotransposon) changes are also different between

intraspecific hybridization and interspecific hybridization in *Lotus* spp. (Fukai et al., 2022). This suggests that some genes (isoforms) are newly expressed, or some are lost by interspecific hybridization. In the current study, tetraploid plants were used, which means that there are usually four copies of the same alleles and the expression levels of these copies should be different. Transposable elements insertion or deletion in genes or promoter regions leads to changes in expression levels (Fig. 15). It is also reported that DNA methylation is also involved in carotenoid accumulation changes during the flowering stage in *Lonicera japonica* (Yu et al. 2023), which suggests that DNA methylation may also be altered during fruit maturation. These correspond to the hypotheses of the hybrid vigor mechanism from the epigenetic view. The mechanism underlying the increase in the levels of some compounds in the present study cannot be revealed because genetic analysis was not conducted. In most studies focusing on hybrid vigor mechanisms, intraspecific hybrids and diploid species are used (Li et al., 2016; Semel et al., 2006; Wang et al., 2015). The present study provides a setting to study the hybrid vigor phenomenon from another direction, interspecific hybridization and tetraploid species.

3.5 Conclusion

The present study revealed that β-carotene content increased and β-cryptoxanthin synthetic ability was induced to haskap by interspecific hybridization. The interspecific hybrids are expected to be used as an origin for haskap breeding. Developmental analysis revealed that accumulation patterns differed by strain and species and provided the basic information for carotenoid accumulation changes in berries by interspecific hybridization. These results provide an important basis for the prediction of hybrid traits and for revealing the hybrid vigor mechanisms. Combined with further multiomics data, the results of this study are expected to help solve these issues.

Table 4. Carotenoid concentration in representative fruits reported in previous studies.

Plant species	α-carotene	β-carotene	lutein	β-cryptoxanthin	References
Carica papaya	-	0.2-0.554	0.16-0.494	-	Schweiggert et al. 2012
Citrus spp.	n.d.	n.d.	0-0.5	0-0.4	Ernawita et al. 2016
Citrus unshiu	-	0.2-0.25	0.05-0.065	1.5-1.75	Matsumoto et al. 2019
Diospyros kaki	0.05-0.15	12.5	0.05-0.1	0.02-0.05	Qi et al. 2019
Eriobotrya japonica	-	0.78	-	0.48	Godoy et al. 1995
Hippophae rhamnoides	0.437	4.291	0.141	n.d.	Pop et al. 2015
Malpighia emarginata	0.0078-0.0593	0.2655-1.6694	0.0376-0.1007	0.0163-0.0565	De Rosso and Mercadante. 2005
Passiflora edulis	nd	0.156	nd	0.027	Yano et al. 2005
Prunus armeniaca	0.0002-0.0567	0.0686-3.6	0.0043-0.142	0.0002-1.19	Zhou et al. 2020
Prunus avium	0.023-0.037	0.078-0.082	0.018-0.023	0.10-0.16	Dias et al. 2009
Prunus domestica	-	0.04-0.188	0.003-0.013	-	Gil et al. 2002
Rubus idaeus	0.06-0.109	0.46-1.22	1.06-1.23	-	Ponder and Hallmann. 2019
Rubus idaeus	0.15-0.33	0.15-0.38	0.2-0.52	-	Bradesh et al. 2015
Vitis vinifera	-	0.0232-0.05932	0.04709-0.0855	-	Bunea et al. 2012

Data were shown in mg/100 g FW in whole fruit or fruit pulp with minimum to maximum range.
nd: not detected
-: no data

Table 5. Carotenoid concentration in mature fruits of miyama-uguisukagura, haskap, and their interspecific hybrid.

	carotenoids (mg/100 g FW)																				
sample name	α-carotene				β-carotene				lutein				β-cryptoxanthin				total				
Lg1	0.096	±	0.0084	a	0.375	±	0.0384	bc	0.032	±	0.0080	d	0.209	±	0.0085	a	0.711	±	0.0610	abc
M23-1	0.010	±	0.0030	cd	0.766	±	0.0699	a	0.095	±	0.0468	cd	0.029	±	0.0034	c	0.900	±	0.1447	ab
M27-1	0.029	±	0.0046	b	0.602	±	0.0674	ab	0.250	±	0.0607	ab	0.080	±	0.0081	b	0.961	±	0.1252	a
M27-4	0.028	±	0.0025	b	0.492	±	0.0906	abc	0.226	±	0.0695	abc	0.034	±	0.0057	c	0.780	±	0.0593	ab
M37-2	0.025	±	0.0066	bc	0.684	±	0.0736	a	0.102	±	0.0398	cd	0.009	±	0.0060	d	0.821	±	0.1528	ab
Lc23	0.003	±	0.0016	d	0.329	±	0.1164	bc	0.269	±	0.0141	ab			tr	d	0.601	±	0.1588	bc
Lc27	0.006	±	0.0022	d	0.354	±	0.0872	bc	0.360	±	0.0296	a			tr	d	0.721	±	0.0913	abc
Lc37	0.007	±	0.0016	d	0.254	±	0.0704	c	0.147	±	0.0151	bcd			tr	d	0.408	±	0.0898	c

Data are shown as mg/100 g FW.
The values are shown as mean ± SD (standard deviations).
Different alphabetical letter besides means they are different among strains at the 5% level by Tukey's HSD test.
tr: trace (less than limit of quantification)

Table 6. Rotation of principal component using four carotenoid concentration data on stage V (fully ripened fruit).

	PC1	PC2	PC3	PC4
α-carotene	0.622	-0.232	-0.201	-0.720
β-carotene	0.155	0.866	-0.476	-0.013
lutein	-0.467	-0.360	-0.805	-0.062
β-cryptoxanthin	0.609	-0.259	-0.291	0.691

Table 7. Carotenoid concentration in fruits during maturing in each strain

carotenoid	sample name development stage	Lg1				M23-1				M27-1				M27-4			
α-carotene	I (young)	0.076	±	0.0142	bA	0.015	±	0.0024	aB	0.035	±	0.0052	aB	0.021	±	0.0073	aB
	II (green grown)	0.229	±	0.0705	aA	0.032	±	0.0008	aB	0.032	±	0.0091	aB	0.041	±	0.0028	aB
	III (half-veraison)	0.124	±	0.0175	abA	0.034	±	0.0154	aBC	0.035	±	0.0097	aBC	0.044	±	0.0175	aB
	IV (veraison)	0.129	±	0.0297	abA	0.034	±	0.0102	aB	0.046	±	0.0149	aB	0.038	±	0.0023	aB
	V (fully ripened)	0.096	±	0.0084	bA	0.010	±	0.0030	aCD	0.029	±	0.0046	aB	0.028	±	0.0025	aB
β-carotene	I (young)	0.806	±	0.1343	aA	0.543	±	0.0212	aAB	0.613	±	0.0456	aAB	0.581	±	0.0712	aAB
	II (green grown)	0.834	±	0.1306	aA	0.615	±	0.1034	aAB	0.547	±	0.0368	aB	0.567	±	0.0278	aAB
	III (half-veraison)	0.289	±	0.0319	bA	0.591	±	0.3704	aA	0.407	±	0.0957	aA	0.480	±	0.1631	aA
	IV (veraison)	0.343	±	0.0156	bA	0.390	±	0.0953	aA	0.499	±	0.1011	aA	0.484	±	0.0461	aA
	V (fully ripened)	0.375	±	0.0384	bBC	0.766	±	0.0699	aA	0.602	±	0.0674	aAB	0.492	±	0.0906	aABC
lutein	I (young)	1.599	±	0.3116	aA	0.856	±	0.1420	aBC	1.208	±	0.0571	aAB	0.887	±	0.1175	aBC
	II (green grown)	1.124	±	0.2210	aA	0.800	±	0.1252	aABC	1.004	±	0.0477	aAB	0.706	±	0.0300	abBC
	III (half-veraison)	0.180	±	0.0282	bC	0.451	±	0.2077	abABC	0.648	±	0.1151	bA	0.542	±	0.1261	bAB
	IV (veraison)	0.094	±	0.0132	bC	0.253	±	0.0732	bB	0.513	±	0.0370	bcA	0.488	±	0.0639	bcA
	V (fully ripened)	0.032	±	0.0080	bD	0.095	±	0.0468	bCD	0.250	±	0.0607	cAB	0.226	±	0.0695	cABC
β-cryptoxanthin	I (young)	0.011	±	0.0051	dAB	0.012	±	0.0175	aAB	0.007	±	0.0097	bAB	0.040	±	0.0198	aA
	II (green grown)	0.051	±	0.0147	cdA	0.006	±	0.0047	aB			tr	bB	0.016	±	0.0078	aB
	III (half-veraison)	0.092	±	0.0231	bcA	0.013	±	0.0136	aB	0.020	±	0.0128	bB	0.033	±	0.0098	aB
	IV (veraison)	0.121	±	0.0104	bcA	0.005	±	0.0010	aBC	0.033	±	0.0165	bB	0.025	±	0.0090	aBC
	V (fully ripened)	0.209	±	0.0085	aA	0.029	±	0.0034	aC	0.080	±	0.0081	aB	0.034	±	0.0057	aC
total	I (young)	2.491	±	0.5605	aA	1.427	±	0.2116	aBC	1.863	±	0.1323	aAB	1.530	±	0.1718	aBC
	II (green grown)	2.238	±	0.3446	aA	1.453	±	0.2711	aBC	1.584	±	0.0736	aB	1.330	±	0.0781	aBC
	III (half-veraison)	0.685	±	0.1063	bA	1.090	±	0.7421	aA	1.110	±	0.2270	bA	1.098	±	0.3859	abA
	IV (veraison)	0.686	±	0.0190	bAB	0.683	±	0.2188	aAB	1.092	±	0.2019	bA	1.035	±	0.0166	abA
	V (fully ripened)	0.711	±	0.0610	bABC	0.900	±	0.1447	aAB	0.961	±	0.1252	bA	0.780	±	0.0593	bAB

Data are shown as mg/100 g FW.
The values are shown as mean ± SD (standard deviations).
Different alphabetical letters besides mean they are different among the stages in the same strains (small letters) and among the strains in the same stages (capital letters) at the 5% level by Tukey's HSD test.
tr: trace (less than limit of quantification)

Table 7 (continued). Carotenoid concentration in fruits during maturing in each strain

carotenoid	sample name development stage	M37-2				Lc23				Lc27				Lc37			
α-carotene	I (young)	0.031	±	0.0139	aB	0.009	±	0.0057	aB	0.020	±	0.0014	aB	0.009	±	0.0013	aB
	II (green grown)	0.021	±	0.0082	aB	0.017	±	0.0106	aB	0.011	±	0.0032	aB	0.006	±	0.0018	aB
	III (half-veraison)	0.023	±	0.0021	aBC	0.006	±	0.0017	aC	0.011	±	0.0023	aBC	0.005	±	0.0016	aC
	IV (veraison)	0.034	±	0.0137	aB	0.008	±	0.0032	aB	0.011	±	0.0052	aB	0.004	±	0.0026	aB
	V (fully ripened)	0.025	±	0.0066	aBC	0.003	±	0.0016	aD	0.006	±	0.0022	aD	0.007	±	0.0016	aD
β-carotene	I (young)	0.556	±	0.1552	aAB	0.487	±	0.1186	aB	0.540	±	0.0351	aAB	0.431	±	0.0537	aB
	II (green grown)	0.425	±	0.0299	aB	0.532	±	0.0892	aB	0.433	±	0.0549	abB	0.418	±	0.0856	aB
	III (half-veraison)	0.312	±	0.0561	aA	0.381	±	0.1179	aA	0.297	±	0.0529	bA	0.218	±	0.0937	aA
	IV (veraison)	0.392	±	0.1248	aA	0.366	±	0.1520	aA	0.248	±	0.0609	bA	0.238	±	0.0560	aA
	V (fully ripened)	0.684	±	0.0736	aA	0.329	±	0.1164	aBC	0.354	±	0.0872	abBC	0.254	±	0.0704	aC
lutein	I (young)	0.735	±	0.0869	aBC	0.691	±	0.0975	aC	0.918	±	0.1355	aBC	0.528	±	0.0590	aC
	II (green grown)	0.508	±	0.0942	bCD	0.580	±	0.1536	abC	0.455	±	0.0743	bCD	0.163	±	0.0454	bD
	III (half-veraison)	0.320	±	0.0303	bcABC	0.309	±	0.0200	bcABC	0.238	±	0.0877	bcBC	0.113	±	0.0113	bC
	IV (veraison)	0.219	±	0.0441	cdBC	0.149	±	0.0355	cBC	0.180	±	0.0275	cBC	0.120	±	0.0276	bBC
	V (fully ripened)	0.102	±	0.0398	dCD	0.269	±	0.0141	cAB	0.360	±	0.0296	bcA	0.147	±	0.0151	bBCD
β-cryptoxanthin	I (young)			tr	aB			tr	aB			tr	aB			tr	aB
	II (green grown)			tr	aB			tr	aB			tr	aB			tr	aB
	III (half-veraison)	0.008	±	0.0048	aB			tr	aB			tr	aB			tr	aB
	IV (veraison)	0.014	±	0.0088	aBC			tr	aC			tr	aC			tr	aC
	V (fully ripened)	0.009	±	0.0060	aD			tr	aD			tr	aD			tr	aD
total	I (young)	1.322	±	0.2698	aBC	1.186	±	0.2462	aBC	1.478	±	0.1216	aBC	0.969	±	0.0281	aC
	II (green grown)	0.953	±	0.1072	abCD	1.130	±	0.2866	abBCD	0.899	±	0.1234	bCD	0.587	±	0.1608	bD
	III (half-veraison)	0.663	±	0.0926	bA	0.696	±	0.1520	abcA	0.547	±	0.0915	cA	0.335	±	0.1185	bA
	IV (veraison)	0.660	±	0.2336	bAB	0.522	±	0.1928	cB	0.439	±	0.1008	cB	0.362	±	0.0859	bB
	V (fully ripened)	0.821	±	0.1528	bAB	0.601	±	0.1588	bcBC	0.721	±	0.0913	bcABC	0.408	±	0.0898	bC

Data are shown as mg/100 g FW.
The values are shown as mean ± SD (standard deviations).
Different alphabetical letters besides mean they are different among the stages in the same strains (small letters) and among the strains in the same stages (capital letters) at the 5% level by Tukey's HSD test.
tr: trace (less than limit of quantification)

Table 8. Rotation of principal component using four carotenoid concentration data on stage V (fully ripened fruit).

	PC1	PC2	PC3	PC4
α-carotene	-0.566	-0.403	0.480	0.536
β-carotene	-0.578	0.327	-0.700	0.262
lutein	-0.497	0.511	0.447	-0.540
β-cryptoxanthin	-0.314	-0.685	-0.283	-0.594

Table 9. Changes in fruit weight during the fruit development.

sample name		I (young)		II (green grown)		III (half-veraison)		IV (veraison)		V (fully ripened)	
Lg1	FW	0.05	± 0.014	0.21	± 0.018	0.27	± 0.005	0.41	± 0.026	0.65	± 0.045
	DW	0.01	± 0.002	0.03	± 0.003	0.04	± 0.001	0.05	± 0.003	0.09	± 0.008
M23-1	FW	0.14	± 0.011	0.37	± 0.022	0.45	± 0.016	0.56	± 0.111	0.87	± 0.056
	DW	0.02	± 0.002	0.05	± 0.004	0.07	± 0.019	0.08	± 0.017	0.15	± 0.012
M27-1	FW	0.14	± 0.022	0.42	± 0.042	0.50	± 0.028	0.52	± 0.065	0.98	± 0.066
	DW	0.02	± 0.002	0.05	± 0.006	0.06	± 0.007	0.06	± 0.009	0.16	± 0.014
M27-4	FW	0.06	± 0.013	0.20	± 0.027	0.29	± 0.034	0.29	± 0.023	0.39	± 0.044
	DW	0.01	± 0.001	0.03	± 0.003	0.04	± 0.005	0.04	± 0.005	0.07	± 0.006
M37-2	FW	0.12	± 0.012	0.37	± 0.012	0.45	± 0.029	0.59	± 0.064	0.98	± 0.063
	DW	0.02	± 0.002	0.05	± 0.001	0.06	± 0.002	0.08	± 0.009	0.17	± 0.012
Lc23	FW	0.14	± 0.031	0.41	± 0.023	0.47	± 0.015	0.48	± 0.061	0.74	± 0.032
	DW	0.02	± 0.003	0.06	± 0.004	0.06	± 0.003	0.06	± 0.009	0.11	± 0.005
Lc27	FW	0.14	± 0.016	0.65	± 0.004	0.83	± 0.016	1.03	± 0.083	1.25	± 0.121
	DW	0.02	± 0.002	0.07	± 0.002	0.09	± 0.003	0.12	± 0.008	0.16	± 0.020
Lc37	FW	0.30	± 0.195	0.44	± 0.178	0.58	± 0.046	0.86	± 0.027	1.27	± 0.083
	DW	0.04	± 0.026	0.06	± 0.024	0.07	± 0.007	0.11	± 0.005	0.19	± 0.016

Data are shown in gram per one fruit.
The values are expressed as mean ± SD (standard deviations).
FW: fresh weight
DW: dry weight

Table 10. Carotenoid contents in fruits during maturing in each strain.

carotenoid	sample name development stage	Lg1				M23-1				M27-1				M27-4			
α-carotene	I (young)	0.041	±	0.0177	bA	0.020	±	0.0019	bA	0.049	±	0.0081	bA	0.011	±	0.0027	bA
	II (green grown)	0.479	±	0.1616	aA	0.119	±	0.0061	abB	0.140	±	0.0529	abB	0.081	±	0.0153	aB
	III (half-veraison)	0.335	±	0.0516	abA	0.157	±	0.0758	abBC	0.174	±	0.0374	abB	0.122	±	0.0386	aBC
	IV (veraison)	0.525	±	0.1374	aA	0.199	±	0.0824	abB	0.234	±	0.0709	aB	0.108	±	0.0148	aB
	V (fully ripened)	0.622	±	0.0116	aA	0.091	±	0.0271	abC	0.277	±	0.0273	aB	0.107	±	0.0047	aC
β-carotene	I (young)	0.427	±	0.1506	cA	0.756	±	0.0619	bA	0.844	±	0.0790	cA	0.326	±	0.0923	cA
	II (green grown)	1.722	±	0.2048	bAB	2.273	±	0.2596	bAB	2.287	±	0.1197	bAB	1.114	±	0.2022	bB
	III (half-veraison)	0.780	±	0.0967	cA	2.704	±	1.7864	bA	2.018	±	0.4860	bA	1.338	±	0.3336	abA
	IV (veraison)	1.393	±	0.1007	bA	2.280	±	0.8768	bA	2.549	±	0.4346	bA	1.380	±	0.1615	abA
	V (fully ripened)	2.447	±	0.2503	aCD	6.618	±	0.2188	aA	5.875	±	0.3118	aAB	1.888	±	0.1137	aD
lutein	I (young)	0.863	±	0.3659	bA	1.200	±	0.2445	abA	1.668	±	0.1804	cA	0.483	±	0.0659	bA
	II (green grown)	2.314	±	0.3350	aBC	2.961	±	0.2721	aAB	4.207	±	0.3110	aA	1.387	±	0.2418	aCD
	III (half-veraison)	0.485	±	0.0818	bB	2.051	±	1.0220	abAB	3.241	±	0.7098	abA	1.531	±	0.2166	aAB
	IV (veraison)	0.380	±	0.0425	bC	1.483	±	0.6046	abABC	2.662	±	0.3756	bcA	1.400	±	0.2543	aBC
	V (fully ripened)	0.210	±	0.0660	bD	0.800	±	0.3627	bCD	2.444	±	0.5538	bcB	0.910	±	0.3422	abCD
β-cryptoxanthin	I (young)	0.006	±	0.0037	dA	0.017	±	0.0238	bA	0.008	±	0.0114	bcA	0.020	±	0.0056	cA
	II (green grown)	0.109	±	0.0367	cdA	0.023	±	0.0181	bB			tr	cB	0.032	±	0.0188	cB
	III (half-veraison)	0.248	±	0.0655	cA	0.062	±	0.0641	bB	0.096	±	0.0561	bcB	0.093	±	0.0220	abB
	IV (veraison)	0.489	±	0.0422	bA	0.031	±	0.0110	bC	0.167	±	0.0750	bB	0.069	±	0.0229	bcBC
	V (fully ripened)	1.366	±	0.0540	aA	0.247	±	0.0198	aC	0.786	±	0.0598	aB	0.132	±	0.0155	aCD
total	I (young)	1.327	±	0.6580	cA	1.993	±	0.3846	bA	2.559	±	0.3102	cA	0.840	±	0.1676	bA
	II (green grown)	4.624	±	0.4368	aABC	5.376	±	0.5936	abAB	6.633	±	0.5221	bA	2.613	±	0.5784	aC
	III (half-veraison)	1.848	±	0.3195	bcA	4.974	±	3.6057	abA	5.529	±	1.3001	bA	3.083	±	0.7386	aA
	IV (veraison)	2.788	±	0.1891	bAB	3.993	±	1.9211	abAB	5.612	±	1.0233	bA	2.958	±	0.3370	aAB
	V (fully ripened)	4.645	±	0.4219	aB	7.756	±	0.6874	aA	9.381	±	0.5280	aA	3.037	±	0.2925	aB

Data are shown as μg/fruit.
The values are shown as mean ± SD (standard deviations).
Different alphabetical letters besides mean they are different among the stages in the same strains (small letters) and among the strains in the same stages (capital letters) at the 5% level by Tukey's HSD test.
tr: trace (less than limit of quantification)

Table 10 (continued). Carotenoid contents in fruits during maturing in each strain.

carotenoid	sample name / development stage	M37-2				Lc23				Lc27				Lc37			
α-carotene	I (young)	0.038	±	0.0173	bA	0.014	±	0.0104	aA	0.027	±	0.0051	aA	0.028	±	0.0190	bA
	II (green grown)	0.077	±	0.0320	abB	0.074	±	0.0487	aB	0.071	±	0.0215	aB	0.028	±	0.0146	bB
	III (half-veraison)	0.103	±	0.0163	abBC	0.029	±	0.0078	aC	0.096	±	0.0183	aBC	0.027	±	0.0078	bC
	IV (veraison)	0.204	±	0.0910	abB	0.035	±	0.0118	aB	0.112	±	0.0500	aB	0.034	±	0.0213	abB
	V (fully ripened)	0.246	±	0.0630	abB	0.021	±	0.0117	aC	0.079	±	0.0258	aC	0.091	±	0.0246	aC
β-carotene	I (young)	0.672	±	0.1885	cA	0.694	±	0.2776	bA	0.746	±	0.1389	cA	1.287	±	0.8057	aA
	II (green grown)	1.556	±	0.0578	bcAB	2.200	±	0.3970	abAB	2.802	±	0.3409	abA	1.871	±	0.9105	aAB
	III (half-veraison)	1.398	±	0.2848	bcA	1.799	±	0.5404	abA	2.464	±	0.4050	bcA	1.223	±	0.4151	aA
	IV (veraison)	2.350	±	0.8844	bA	1.675	±	0.4582	abA	2.544	±	0.5841	bA	2.068	±	0.5384	aA
	V (fully ripened)	6.646	±	0.5296	aA	2.408	±	0.7778	aCD	4.349	±	0.8899	aBC	3.211	±	0.8318	aCD
lutein	I (young)	0.898	±	0.1949	aA	0.950	±	0.2175	bcA	1.239	±	0.0629	bA	1.641	±	1.1744	aA
	II (green grown)	1.871	±	0.3806	aBCD	2.422	±	0.7263	aBC	2.948	±	0.4806	abAB	0.713	±	0.3728	aD
	III (half-veraison)	1.424	±	0.0913	aAB	1.467	±	0.1299	abcAB	1.995	±	0.7597	abAB	0.656	±	0.0824	aB
	IV (veraison)	1.305	±	0.3577	aBC	0.712	±	0.1840	cdBC	1.869	±	0.4035	bAB	1.039	±	0.2552	aBC
	V (fully ripened)	0.981	±	0.3492	aCD	1.996	±	0.0266	abBC	4.541	±	0.7975	aA	1.847	±	0.0862	aBC
β-cryptoxanthin	I (young)			tr	aA			tr	aA			tr	aA			tr	aA
	II (green grown)			tr	aB			tr	aB			tr	aB			tr	aB
	III (half-veraison)	0.034	±	0.0212	aB			tr	aB			tr	aB			tr	aB
	IV (veraison)	0.086	±	0.0557	aBC			tr	aC			tr	aC			tr	aC
	V (fully ripened)	0.086	±	0.0542	aDE			tr	aE			tr	aE			tr	aE
total	I (young)	1.608	±	0.4332	bA	1.658	±	0.6190	bA	2.012	±	0.1234	cA	2.957	±	2.4371	aA
	II (green grown)	3.505	±	0.4656	bBC	4.696	±	1.3645	aABC	6.821	±	0.7788	bAB	2.612	±	1.5801	aC
	III (half-veraison)	2.960	±	0.4628	bA	3.296	±	0.6874	abA	4.555	±	0.8319	bA	1.906	±	0.4623	aA
	IV (veraison)	3.944	±	1.6913	bAB	2.422	±	0.4684	abA	4.524	±	1.0888	bAB	3.141	±	0.8395	aAB
	V (fully ripened)	7.960	±	1.2052	aA	4.424	±	0.9703	aB	8.968	±	1.1031	aA	5.148	±	0.9806	aB

Data are shown as μg/fruit.
The values are shown as mean ± SD (standard deviations).
Different alphabetical letters besides mean they are different among the stages in the same strain (small letters) and among the strains in the same stages (capital letters) at the 5% level by Tukey's HSD test.
tr: trace (less than limit of quantification)

Table 11. Correlation coefficient among the concentration of each carotenoid and each stage.

	α-carotene I	α-carotene II	α-carotene III	α-carotene IV	α-carotene V
α-carotene V	0.97	0.97	0.97	0.98	1.00
β-carotene V	0.03	-0.13	0.05	0.10	-0.03
lutein V	-0.51	-0.58	-0.59	-0.61	-0.59
β-cryptoxanthin V	0.95	0.96	0.96	0.97	0.96

	β-carotene I	β-carotene II	β-carotene III	β-carotene IV	β-carotene V
α-carotene V	0.95	0.83	-0.19	0.16	-0.03
β-carotene V	0.12	0.06	0.69	0.62	1.00
lutein V	-0.43	-0.53	-0.02	-0.04	-0.31
β-cryptoxanthin V	0.95	0.90	-0.08	0.19	-0.03

	lutein I	lutein II	lutein III	lutein IV	lutein V
α-carotene V	0.87	0.71	-0.12	-0.11	-0.59
β-carotene V	0.07	0.38	0.65	0.48	-0.31
lutein V	-0.27	-0.30	0.23	0.30	1.00
β-cryptoxanthin V	0.94	0.81	-0.03	-0.05	-0.53

	β-cryptoxanthin I	β-cryptoxanthin II	β-cryptoxanthin III	β-cryptoxanthin IV	β-cryptoxanthin V
α-carotene V	0.23	0.94	0.97	0.99	0.96
β-carotene V	0.20	-0.16	-0.04	-0.08	-0.03
lutein V	-0.06	-0.57	-0.56	-0.55	-0.53
β-cryptoxanthin V	0.20	0.91	0.96	0.98	1.00

Values represent the correlation coefficient between carotenoid concentration (mg/100 g FW) at each growth stage and carotenoid concentration in fully-ripened fruits.

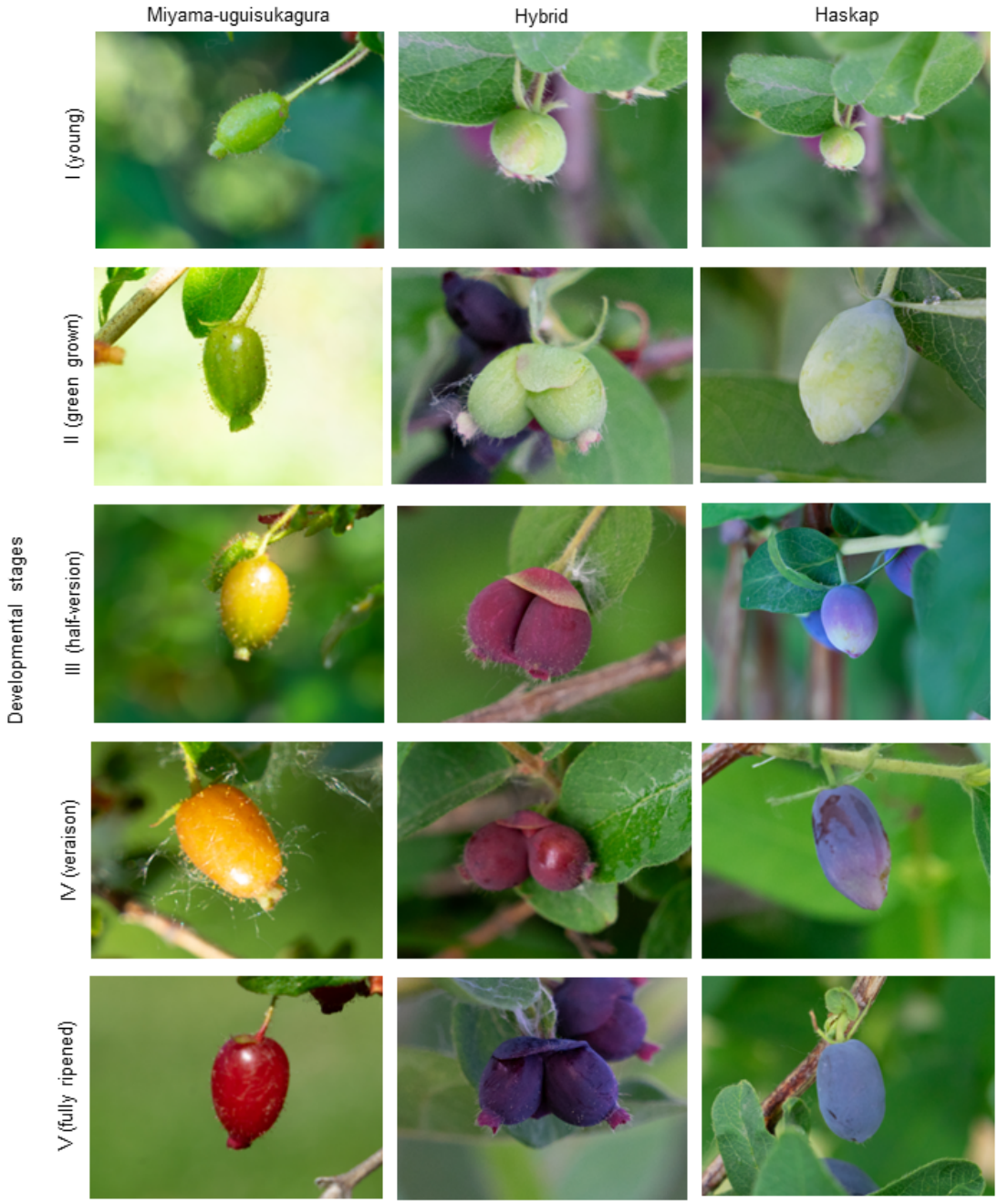

Fig. 7. Fruit images of miyama-uguisukagura, interspecific hybrid, and haskap in each developmental stages.

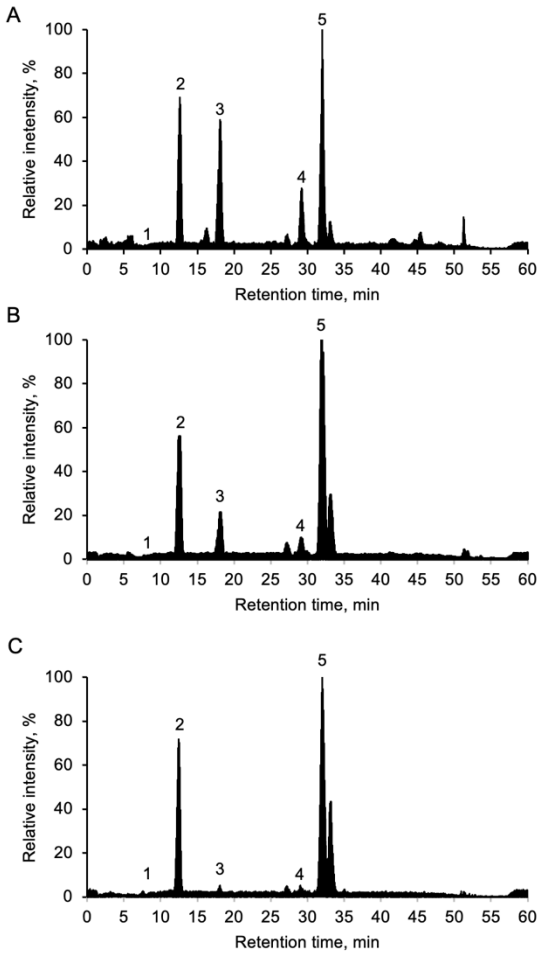

Fig. 8. Comparison of UHPLC/MS/MS chromatograms of fully ripened (stage V) fruit of interspecific hybrid between miyama-usuisukagura and haskap and it parents.

(A) miyama-uguisukagura (Lg1) (B) interspecific hybrid (M27-1) (C) haskap (Lc27).

Total ion chromatograms of MRM (multi reaction monitoring) scans are shown. Peak numbers correspond to 1, lutein; 2, trans-β-apo-8'-carotenal (internal standard); 3, β-cryptoxanthin; 4, α-carotene; 5, β-carotene.

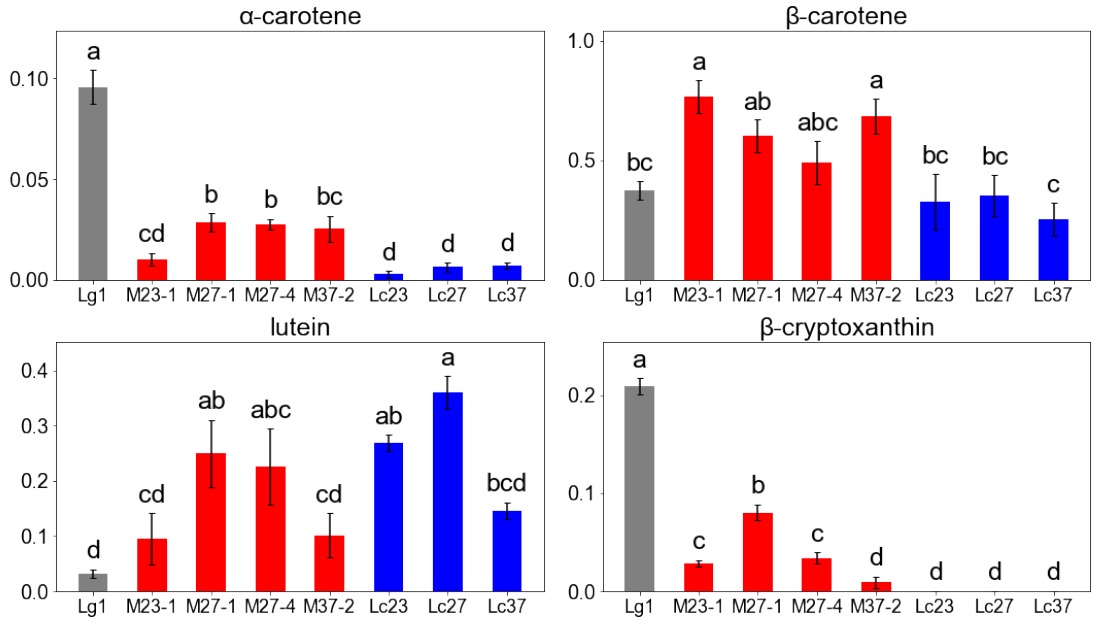

Fig. 9. Carotenoid concentration in the fruits of each strain at stage V (fully ripened fruit).

Data are represented as mg /100 g FW.

Data are shown as mean and SD (standard deviation).

Different alphabetical letters above bars imply significant differences among strains.

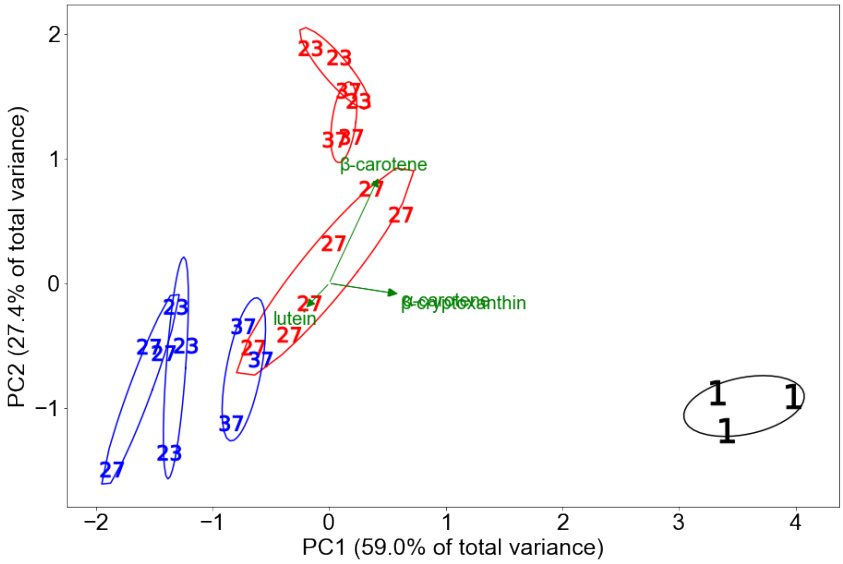

Fig. 10. Principal component analysis (PC1 vs PC2) of each strain using four carotenoid concentration data on stage V (fully ripened fruit).

The plot strings represent the strains, and the colors represent the species as follows: black, Miyama-uguisukagura; red, interspecific hybrid; blue, haskap. Green colored vectors represent each carotenoid eigenvectors of covariance matrix. 95% confidence ellipses were drawn for each strain.

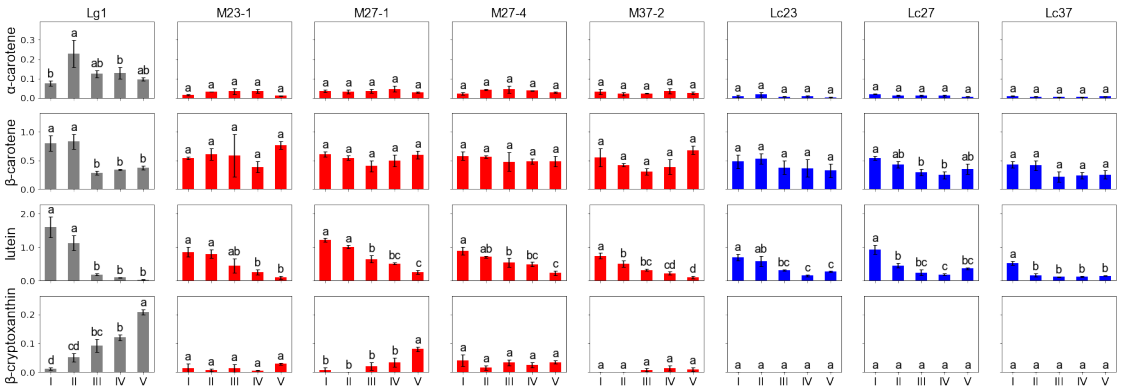

Fig. 11. Carotenoid concentration in the fruits of each strain at all stages.

Data are represented as mg/100 g FW.

Data are shown as mean and SD (standard deviation).

Different alphabetical letters above bars imply the significant differences among stages in each strain.

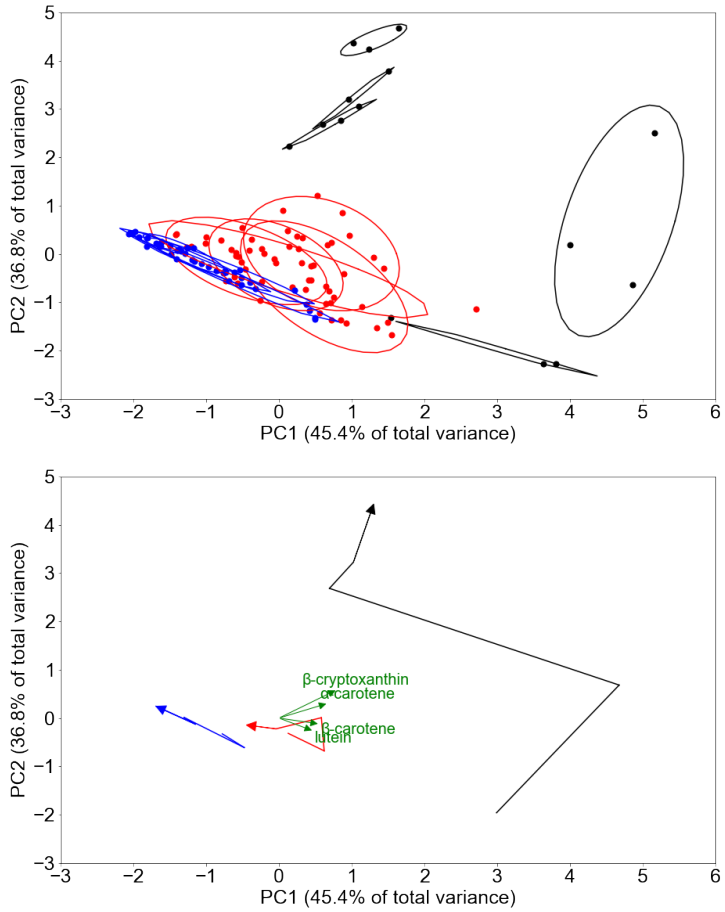

Fig. 12. Principal component analysis (PC1 vs PC2) of each strain using four carotenoid concentration data on stage V (fully ripened fruit).

The colored plots represent the species as follows: black, Miyama-uguisukagura; red, interspecific hybrid; blue, haskap. Green vectors represent each carotenoid eigenvectors of covariance matrix. 95% confidence ellipses were drawn for each species and each stage. Black, red, blue arrows are connected among ellipse centers from stage I (young fruit) to stage V (fully ripened fruit). Arrowhead represents the stage V. Arrow colors correspond with the plot colors.

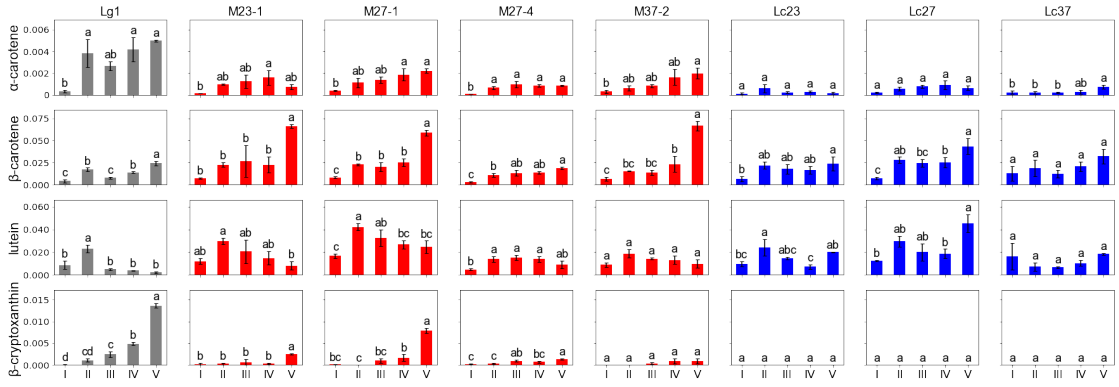

Fig. 13. Carotenoid concentration per fruit in each strain in all stages.

Data are represented as mg per one fruit.

Data are shown as mean and SD (standard deviation).

Different alphabetical letters above bars imply the significant differences among stages in each strain.

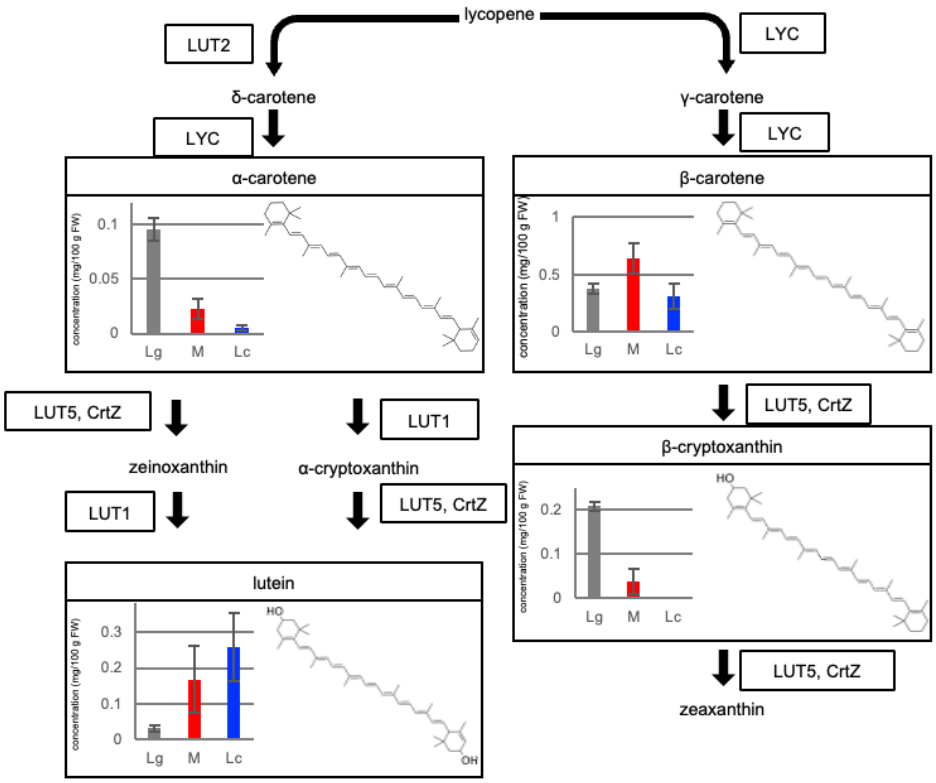

Fig. 14. Speculative carotenoid biosynthesis and concentration in this study.

Boxed characters beside arrows indicate biosynthetic enzymes.

The bar graph indicates the average and standard deviation of carotenoid concentration in mature fruits of Miyama-uguisukagura (gray), interspecific hybrid (red), and haskap (blue).

CrtZ, beta-carotene 3-hydroxylase; LUT1, carotenoid epsilon hydroxylase; LUT2, lycopene beta/epsilon cyclase; LUT5, beta-ring hydroxylase; LYC, lycopene cyclase.

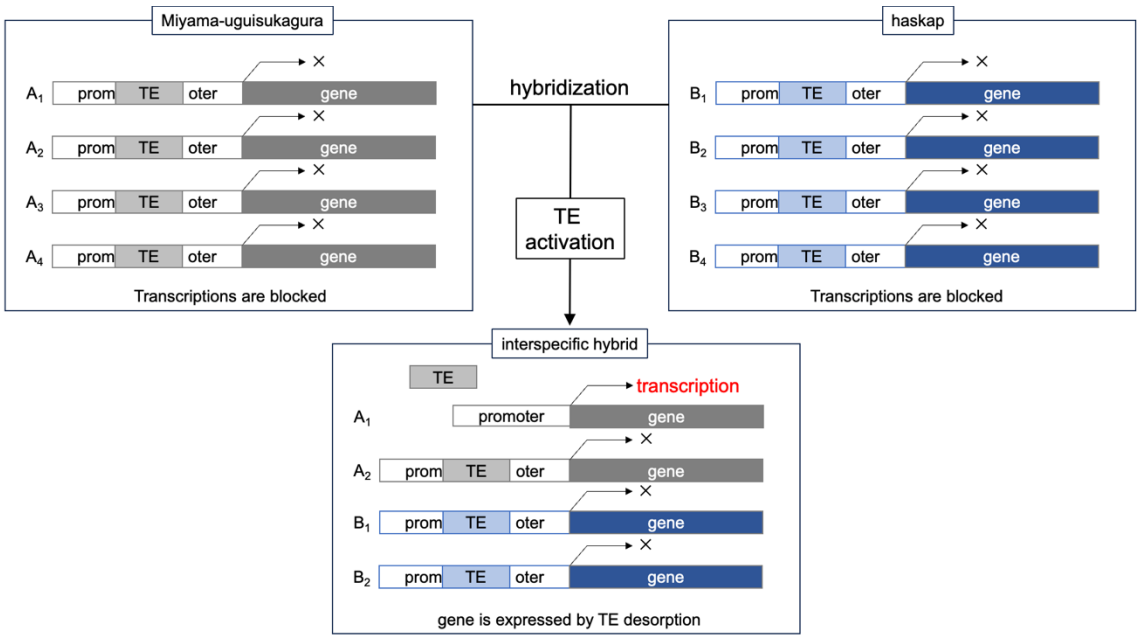

Fig. 15. Conceptual image of gene expression pattern changes by transposon desorption in promoter region caused by interspecific hybridization.

Chapter 4 General discussion

This thesis describes the carotenoid changes during fruit maturation by interspecific hybridization between miyama-uguisukagura and haskap. The levels of some carotenoids increased in fully ripened fruits by interspecific hybridization. Carotenoid levels have been described to increase by interspecific hybridization (Hamada et al., 2016; Toshima et al., 2021; Nakkanong et al., 2012). However, in these studies, herbs, not trees, were used as plant materials. This thesis may be the first report showing that carotenoid levels increased by interspecific hybridization of tree plants.

This study revealed the carotenoid composition and previous studies revealed the anthocyanin composition in interspecific hybrids between miyama-uguisukagura and haskap (Fujita et al. 2020a; Fujita et al. 2020b). Fruit color is mainly affected by the color pigments, chlorophyl, carotenoid, and anthocyanin (Lancaster et al., 1997). The main color pigment in haskap is cyanidin 3-glucoside, a type of anthocyanin (Terahara et al., 1993; Chaovanalikit et al., 2004; Jordheim et al., 2007; Svarcova et al., 2007; Wojdyło et al., 2013; Celli et al., 2015; Zhao et al., 2015; Wang et al., 2016). Fujita et al. (2020b) revealed that the skin colors of miyama-uguisukagura, interspecific hybrid, and haskap are red, red-purple, and violet-blue, respectively, and the pulp colors are orange, grayed-orange, and yellow-green, respectively. The concentration of anthocyanin was commonly high in haskap, intermediate in interspecific hybrid, and low in miyama-uguisukagura. The concentration of the four carotenoids observed in interspecific hybrids (0.78-0.96 mg/100 g FW) was higher than that of miyama-uguisukagura (0.71 mg/100 g FW) or that of haskap (0.41-0.72 mg/100 g FW). Interspecific hybrids showed similar color independence in strains and intermediate color between miyama-uguisukagura and haskap. These results suggest that other factors such as pH and co-pigmentation contribute to fruit skin colors (Harborne, 1958; Yabuya et al., 1997; Heredia et al., 1998; Takeda et al., 2010). Further simulations with various additional factors are needed to improve the accuracy of fruit color prediction.

In this thesis, it was revealed that the accumulation pattern was different among strains. It may be caused by spatio-temporal changes in gene expression related to carotenoid biosynthesis and decomposition during ripening. Tsaniklidis et al. (2016) showed that some genes are expressed at specific stages and in specific tissues during tomato fruit development. Such a spatio-temporal

regulation differences among strains may leads to the changes in final metabolite levels. Moreover, these changes were supposed to be caused by genome structure differences and environmental stress. As described in Chapter 3, genome structure is changed by transposon activation caused by interspecific hybridization (Fukai et al. 2022), which leads to gene expression differences. In allotetraploid (interspecific hybrid) *Arabidopsis kamtchatica*, most of gene derived from parents show a similar expression level whereas in several gene groups, expression levels originated from one parent were higher than those from another parents (Paape et al. 2016). Combining genomic and spatio-temporal transcriptomic data with those of the present study, the traits of interspecific hybrid progeny are expected to be predicted. One of the advantages of interspecific hybrids (allopolyploid) in genomic studies is that it is easier to identify the origin of the expressed gene than for intraspecific hybrids. Hybrid genomes are composed of half of the maternal genome and half of the paternal genome, which generates different transcript isoforms. In intraspecific hybrids, the structural difference between isoforms is small, which makes it difficult to identify from which parents the gene expression pattern originated. Interspecific hybridization solves this problem and a tool to identify the gene origin was also developed (Kuo et al., 2020). This describes the changes in molecular accumulation patterns during ripening by interspecific hybridization. Combined with molecular biological analysis data, this study improves our understanding of the mechanism of hybrid vigor and provides a basis for the prediction of progeny traits.

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Summary

Interspecific hybridization in plant breeding has been used to introduce novel traits that are not present in the original breeding target species. However, it is difficult to predict the traits of the hybrid and unexpected phenotypes may emerge after it is grown because genes that are not present in the original species, which is the breeding target species are introduced from another species by interspecific hybridization. Prediction of traits in hybrids should accelerate plant breeding; more fundamental phenotype information of interspecific hybrids and their parents is needed. Color is one of important traits in fruits that is suitable information for predicting hybrid traits because it is visually recognized and color pigments can be quantified. However, few studies have reported color pigment comparisons between interspecific hybrids and their parents, especially how the color pigments are accumulated during developmental stages. Because color pigments are synthesized and degraded by biosynthesis, the fundamental information for high-accuracy prediction is expected to be obtained by analysis during developmental stages. In this study, the use of interspecific hybrids between miyama-uguisukagura and haskap was used as a model to predict the hybrid traits. Haskap belongs to *Lonicera*, Caprifoliaceae, and has blue-purple oval fruits. Miyama-uguisukagura belongs to the same genus and has red oval fruits. Interspecific hybrids produced from these species have red-purple, twin-shaped fruits. In a previous study during my master course, anthocyanin levels in fruits were measured, and six anthocyanins were detected. Four anthocyanins, including cyanidin 3-glucoside, the main color pigment of haskap fruit, were found at high levels in haskap, low levels in the miyama-uguisukagura, and intermediate levels in the interspecific hybrids. However, in cyanidin 3,5-glucoside and peonidin 3,5-diglucoside, the interspecific hybrids showed a higher level than their parents, highlighting the "hybrid vigor" of the interspecific hybrids. The fruit color is mainly composed of anthocyanins, carotenoids, and chlorophyll. The color change by interspecific hybridization could not be explained only by anthocyanin composition changes. A previous study showed the presence of carotenoids in uguisukagura, a variety related to miyama-uguisukagura, implying that the miyama-uguisukagura also contains carotenoids. It suggests the possibility of carotenoid improvement in haskap by interspecific hybridization with miyama-uguisukagura. The present study focused on carotenoids and investigated how carotenoid levels varied between interspecific hybrids and their parents varied depending on the

stage of fruit maturity.

Compared to that for many natural products, quantification of carotenoid is difficult to perform using LC/MS/MS because carotenoids are not efficiently ionized and are easily degraded. In this study, extraction conditions, separation conditions, and ionization and mass spectrometry conditions were optimized. Using a C30 column for separation and an ionization method known as DUIS, which combines ESI and APCI, was suitable for carotenoid detection. Four carotenoids were detected: α-carotene, β-carotene, lutein, and β-cryptoxanthin. The accumulation patterns of the four carotenoids were also analyzed at different stages of fruit maturation.

One strain of miyama-uguisukagura, three strains of haskap, and four strains of interspecific hybrids were used as plant material. Fruits were harvested at five different stages of maturity and analyzed.

First, the carotenoid content in fully ripened fruits was investigated. β-Carotene was the major carotenoid and showed higher levels in the interspecific hybrids than in the parental strains of miyama-uguisukagura and haskap. β-Cryptoxanthin was not quantified in all haskap lines, whereas β-cryptoxanthin was below the limit of quantification in the interspecific hybrids. However, β-cryptoxanthin was quantifiable in the interspecific hybrids, suggesting that its biosynthesis level was significantly altered by interspecific hybridization.

Next, the carotenoid accumulation pattern at different stages of ripening was investigated. Carotenoid concentrations did not increase or decrease linearly in most carotenoids and strains. Lutein concentrations tended to decrease in the early stages. These results are consistent with previous studies that claim lutein decreases during ripening in many fruit trees. In addition, β-cryptoxanthin levels increased from the early stages to the last stage in miyama-uguisukagura and from the veraison stage to the fully-ripened stage in the interspecific hybrid strains containing high levels of β-cryptoxanthin. These results indicate a change in the accumulation pattern by interspecific hybridization.

Because carotenoids share a common metabolic pathway in plants, carotenoid biosynthesis among strains was discussed. β-Cryptoxanthin is biosynthesized by the LUT5 or CrtZ gene from β-carotene as a precursor. The amounts of β-cryptoxanthin and β-carotene at maturity suggest that these genes are strongly expressed in the presence of β-carotene, especially in miyama-uguisukagura,

weakly expressed in interspecific hybrids, and almost not expressed in the haskap. The accumulation pattern of β-cryptoxanthin was also different between miyama-uguisukagura and interspecific hybrids, suggesting that there may be a difference in a temporal expression. The carotenoids detected in this study were divided into the δ-carotene pathway (α-carotene and lutein) and the γ-carotene pathway (β-carotene and β-cryptoxanthin.) Carotenoids derived from δ-carotene tended to be more abundant in haskap, whereas carotenoids derived from γ-carotene tended to be more abundant in the miyama-uguisukagura and interspecific hybrids. These pathways are related to the LUT2 and LYC genes, suggesting that LUT2 is highly expressed in haskap, whereas LYC is highly expressed in miyama-uguisukagura and interspecific hybrids.

Combined with previous studies, the overall trend in color pigment was as follows; low anthocyanin levels and intermediate carotenoid levels in the miyama-uguisukagura; intermediate anthocyanin levels and high carotenoid levels in interspecific hybrids; high anthocyanin levels and low carotenoid levels in haskap. These results are consistent with the corresponding fruit colors, suggesting that the red-purple color of the interspecific hybrid fruits is results from the presence of both anthocyanins and carotenoids.

In this study, a method for quantifying carotenoids in fruits was optimized and the carotenoid accumulation patterns in the fruits of miyama-uguisukagura, haskap, and their interspecific hybrids were analyzed. These results were used to investigate the factors that cause changes in fruit color and were discussed from the perspective of biosynthetic pathways. Combined with gene expression analysis and genomic data, this study helps solving the mechanism underlying hybrid vigor and provides a basis for the prediction of progeny traits, which is expected to accelerate breeding in the future.

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要約（Summary in Japanese）

種間交雑は植物の育種の手段の 1 つとして，改良する種にない形質を取り入れるために用いられてきた．種間交雑では元の種にない新たな遺伝子が導入されるため，実際に栽培されるまで，形質によっては期待されるものが得られるか予測が難しく，また，予想外の表現型が現れることがある．雑種の形質の予測によって，育種の加速化が期待されるが，そのためには種間雑種とその両親の形質に関する基本的な情報が必要である．果実色は果樹において重要な形質の一つで，視覚的に認識でき，色素を定量化できるため，形質を予測するための情報の一つとして適していると考えられる．しかし，種間雑種と両親の色素成分を比較した報告，特に，成熟期間の中でどのように蓄積されるかを調査した報告は限られている．色素成分は生合成によって生成・分解されることから，成熟段階ごとに解析を行うことによって，より精度の高い予測モデル構築のための基盤的情報を取得できると考えた．種間雑種の形質を予測するためのモデルとして，ハスカップとミヤマウグイスカグラの種間雑種の利用を着想した．ハスカップはスイカズラ科スイカズラ属に属し，青紫色の楕円状の果実を持つ．近縁種である同属のミヤマウグイスカグラは赤色の楕円状の果実を持つ．これらから作出された種間雑種は赤紫色で双子状の果実を持っていた．博士前期課程における研究で，果実のアントシアニン量を測定したところ，6 つのアントシアニンが検出された．これらのうち，ハスカップ果実の主な色素である cyanidin 3-glucoside を含む 4 つのアントシアニン量は，ハスカップで多く，ミヤマウグイスカグラで少なく，種間雑種で中間的であった．しかし，cyanidin 3,5-diglucoside および peonidin 3,5-diglucoside においては，種間雑種において両親より量が増える雑種強勢が起こっていた．植物の果実色は主にアントシアニン，カロテノイド，クロロフィルによって構成されている．ミヤマウグイスカグラとハスカップを種間交雑したことによる果実色の変化はアントシアニンだけ

では説明できなかった．ミヤマウグイスカグラの近縁種であるウグイスカグラでカロテノイドを検出した先行研究からミヤマウグイスカグラでもカロテノイドが多く含まれていることが考えられた．種間交雑による果実色の変化にはカロテノイドも関与している可能性があることから，本研究ではカロテノイドに着目し，種間雑種と両親との間のカロテノイド量が果実の成熟段階によってどのように変化するかを解析した．

カロテノイドは，イオン化が起こりにくく，分解が起こりやすいため，多くの天然物と比べて LC/MS/MS での定量が難しい．本研究では，抽出条件，分離条件，イオン化・質量分析の条件の最適化を行った．C30 カラムを使用した分離を行い，ESI と APCI を組み合わせた DUIS として知られるイオン化法によって，4 つのカロテノイド（α-carotene, β-carotene, lutein, β-cryptoxanthin）の検出，定量が可能となったため，果実の成熟段階ごとの蓄積パターンを解析した．植物材料として，ミヤマウグイスカグラ 1 系統，ハスカップ 3 系統，種間雑種 4 系統を用いた．それぞれの果実を 5 つの成熟段階に分けて収穫し，分析に供試した．

完熟期のカロテノイド含有量について調査したところ，β-carotene は主要なカロテノイドで，完熟期果実において種間雑種は両親のミヤマウグイスカグラおよびハスカップより高い値を示した．β-cryptoxanthin はハスカップのすべての系統で定量限界以下だったが，種間雑種では定量が可能であり，種間交雑によって生合成のレベルが大きく変化したことが示唆された．

次に，成熟段階ごとのカロテノイド含有量について調査した．カロテノイド濃度はほとんどの種類，系統において直線的には増減しなかった．多くの果樹で lutein 濃度は成熟期間中に減少することが知られており，本研究においても，成熟期間の初期段階で減少する傾向があった．また，β-cryptoxanthin はミヤマウグイスカグラでは成熟段階の初期から完

熟まで増加していたものの，種間雑種で多く β-cryptoxanthin を多く含む系統においては成熟段階の後半で増加しており，蓄積パターンが変化していることが明らかとなった．

カロテノイドは植物に共通する代謝経路を持つため，これらの結果を踏まえ，系統間のカロテノイド生合成について考察した．β-cryptoxanthin は β-carotene を前駆体として LUT5 もしくは CrtZ によって生合成される．β-carotene と β-cryptoxanthin の完熟期の成分量の結果から，これらの遺伝子は β-carotene の存在下において，特にミヤマウグイスカグラで強く発現し，種間雑種では弱く発現，ハスカップでの発現量はほとんどないことが示唆された．また，β-cryptoxanthin の蓄積パターンがミヤマウグイスカグラと種間雑種で異なっていたことから，発現タイミングの違いもあることが考察された．また，本研究で検出したカロテノイドは δ-carotene を由来とする α-carotene および lutein と，γ-carotene を由来とする β-carotene および β-cryptoxanthin に分けられる．ハスカップでは δ-carotene に由来するカロテノイドが，ミヤマウグイスカグラ，種間雑種では γ-carotene に由来するカロテノイドが多い傾向にあった．これらの経路は LUT2 と LYC が関わっており，ハスカップでは LUT2 が，ミヤマウグイスカグラや種間雑種では LYC が多く発現していることが示唆された．

色素全体の傾向として，ミヤマウグイスカグラではアントシアニン量が少なく，カロテノイド量が中間的，種間雑種ではアントシアニン量が中間的でカロテノイド量は多く，ハスカップではアントシアニン量が多く，カロテノイド量が少なかった．これらの結果は果実色と概ね一致しており，種間雑種果実の赤紫色はアントシアニンとカロテノイドの両方が含まれることによって呈されたものであると考えられた．

本研究では，果実に含まれるカロテノイドの定量手法を確立し，ミヤマウグイスカグラ，ハスカップおよびそれらの種間雑種の果実におけるカロテノイドの蓄積パターンの解析を行った．これらの結果から果実色の変化の要因を探るとともに，生合成経路からの考察を

行った．今後，本研究で測定された成分分析の結果と生合成に関連する遺伝子発現解析やゲノム解析を組み合わせることによって，種間交雑における形質の予測に活用され，育種の加速化が期待される．