The analysis of fruit characteristics for pepper breeding

（トウガラシ育種のための果実形質の解析）

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2021

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Chapter 1. General Introduction

Pepper (*Capsicum* spp.) is an important horticultural crop worldwide. Among the five cultivated pepper species, *Capsicum annuum* ($2n = 24$ ($2x$)) is the most widely cultivated and is used as a vegetable, spice, and food colorant. We believe that pepper was introduced into Japan in the 16th century (Iwai and Watanabe 2008). Many local pepper cultivars have been grown throughout Japan since the plant's introduction; however, many of these cultivars have been lost. The consumption of local vegetables is attracting increasing attention, as conservation and the effective use of resources become highly prioritized. To meet local needs, local pepper producers seek to increase pepper fruit value and quality. In this study, we performed genetic research and breeding to promote the production of capsanthin, a functional ingredient with the potential to add value to local pepper cultivars. Additionally, we researched postharvest water loss and fruit texture as anatomical traits that could be targeted for improving fruit quality.

Pepper fruits, especially of mature red pepper, are an excellent source of natural pigments. The carotenoid pigments of pepper include capsanthin, capsanthin-5,6-epoxide, capsorubin, zeaxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, β-carotene and cucurbitaxanthin A, and are synthesized and accumulated during fruit ripening. There are a great number of variations on the color and carotenoid content in pepper germplasm. And the most highly valued characteristic is high content of carotenoid. To breed and select carotenoid rich varieties, red-to-yellow isochromic fractions ratio and the capsanthin-to-zeaxanthin ratio are most useful and appropriate index together with the total carotenoid content (Hornero-Mendez et al. 2000, 2002, Wall et al. 2001). Because capsanthin is the main carotenoid, the relative content for capsanthin reaches 60% in carotenoid of red pepper fruits (Hornero-Mendez et al. 2002).

Capsanthin acts as a potential antioxidant, which has been shown to be effective as a free-radical scavenger. Also, capsanthin is usually esterified partially and/or totally with fatty acid in nature (Mínguez-Mosquera and Hornero-Mendez 1994a,1994b), and it is shown that the radical scavenging ability of capsanthin is not influenced by esterification (Bae et al. 2012, Howard et al. 2000, Matsufuji et al. 1998). Furthermore, capsanthin and

its esters reduces the risk of cancer due to exhibiting potent anti-tumor-promoting activity (Maoka et al. 2001). Additionally, capsanthin reduces the risk of cardiovascular diseases because capsanthin had an HDL-cholesterol-raising effect on plasma without detectable differences in total cholesterol (Aizawa and Inakuma 2009).

Genetic control of carotenoid content in pepper fruits has been reported mainly for carotenoid biosynthesis pathways and color variation of fruits (Hurtado-Hernandez and Smith 1985). Genes for carotenoid biosynthesis pathway, for instance, *PSY* for phytoene synthase, *Lcyb* for lycopene-β-cyclase, *Crtz* for β-carotene hydroxylase and *CCS* for capsanthin–capsorubin synthase were identified so far (Bouvier et al. 1994, 1998, Hugueney et al. 1995, Romer et al. 1993). These are key genes for control yellow, orange and red colors of fruits (Huh et al. 2001, Lefebvre et al. 1998). Especially, *CCS* controls the red color (Tian et al. 2015). Further, orange and yellow colors of fruits are due to deletion or silencing of *CCS* gene (Ha et al. 2007, Lang et al. 2004, Rodriguez-Uribe et al. 2012).

Although there are many variations in pigment content in pepper germplasm, few studies about genetic controls of quantitative variations in carotenoid content have been reported. In one study, 4 QTLs were identified for fruit color of red pepper fruits, by quantifying lightness, chroma and hue parameters (Ben Chaim et al. 2001). In another study, 2 QTLs, *pc8.1* and *pc10.1*, were identified that control chlorophyll content. The QTL *pc8.1* also affected carotenoid content in ripe fruit. However, in subsequent generations there was not consistent effect of this QTL on carotenoid content (Brand et al. 2012).

Therefore, to access the genetic mechanisms for controlling content of red color pigment, capsanthin, in pepper, we mapped QTLs for the content in Doubled Haploid population (DH) derived from a cross between genetic resource and local cultivar. The genetic resource ‘S3586’ has high capsanthin content and local cultivar ‘Kyoto-Manganji No. 2’ has low capsanthin content. In QTL mapping using population with fixed genotypes such as DH and recombinant inbred lines, utilization of all biological replication in one analysis is very important factor for reduction of nongenetic residual variance and increase in accuracy of the mapping (Broman and Sen, 2009). Because our segregating population is DH, we can create multiple individuals with the same genotype for two

experiments. Hence, we performed QTL mapping mainly using phenotypes from two datasets (Experiments 1 and 2) at one time. Further, we discuss how to increase capsanthin content by marker-assisted selection in practical breeding of pepper using QTLs detected in chapter 2.

Manganji pepper, include some cultivars, used in Chapter 2 as the parent of the DH population and one of the local cultivars grown in Kyoto Prefecture. Manganji pepper is known for its large, tasty fruits that are typically harvested at the green mature stage, about 30 to 35 days after flowering (DAF). Mature red fruits of Manganji pepper are infrequently shipped over long distances because they have low capsanthin content, which corresponds with poor coloring. Additionally, shipping Manganji pepper makes it difficult to control the outflow of genetic resources, as the mature fruits contain mature and viable seeds. Therefore, we bred a new F_1 red pepper cultivar with high capsanthin content (Chapter 3).

Generally, peppers suffer from high postharvest water loss at every stage of ripening because the fruit is hollow. This water loss reduces the economic value of the fruit because of the subsequent reductions in freshness, firmness, and glossiness. High postharvest water loss is, therefore, an important defect in peppers for consumption as a vegetable. To control the postharvest water loss, Lownds et al. (1994) reported the effect of storage temperature and packaging. However, methods for reducing postharvest water loss by storage condition will not contribute to a fundamental solution. Therefore, it is necessary to clear the cause of high water loss and process of breeding.

The cuticular membrane covering the fruit surface is thought to play a role in fruit water loss (Saladié et al. 2007) and resistance to pathogens and insects (Isaacson et al. 2009; Saladié et al. 2007). In several studies, associations between the amounts of cuticle, cuticular wax, and cutin, and postharvest water loss have been investigated. A negative correlation between the rate of water loss and total amount of cuticular wax in pepper fruit was found in some studies (Lownds et al. 1993; Maalekuu et al. 2004) but not others (Parsons et al. 2012). Additionally, Maalekuu et al. (2005) found no correlation between fruit water loss and whole cuticular weight, whereas Parsons et al. (2012) found a positive correlation between fruit water loss and total cutin monomer amounts in pepper fruit.

Moreover, Parsons et al. (2012) reported that fruits containing higher amounts of terpenoids relative to aliphatic wax in the cuticle tended to have a higher rate of water loss. Thus, the role of the cuticular membrane in postharvest water loss remains unclear.

Several studies of the relationships between the physiological properties of pepper fruit and postharvest water loss have been conducted (Maalekuu et al. 2005; Smith et al. 2006). Maalekuu et al. (2005) found negative correlations between the rate of water loss and fruit fresh weight, fruit pericarp weight, pericarp surface area, and initial water content, but there was a positive correlation between the rate of water loss and dry matter content. There was no relationship between the rate of water loss and pericarp thickness.

Chaïb et al. (2007) found correlations between the cellular structure of the pericarp and texture of tomato fruit. Fruit texture is one of the most important components of fruit quality for consumers. There are several studies on the influence of storage and blanching on fruit texture in bell pepper (Hernández-Carrión et al. 2014; Papageorge et al. 2003), but few studies regarding breeding to improve fruit texture. The objective of chapter 4 was to clarify the effects of cuticle development and anatomical traits on postharvest water loss and texture of pepper fruit.

Chapter 2. Detection of quantitative trait loci for capsanthin content in pepper (*Capsicum annuum* L.) at different fruit ripening stages

Introduction

Pepper fruits, especially of mature red pepper, are an excellent source of natural pigments. There are a great number of variations on the color and carotenoid content in pepper germplasm. Capsanthin is the main carotenoid and the relative content for capsanthin reaches 60% in carotenoid of red pepper fruits (Hornero-Mendez et al. 2002). Capsanthin acts as a potential antioxidant (Bae et al. 2012, Howard et al. 2000, Matsufuji et al. 1998), reduces the risk of cancer (Maoka et al. 2001). Additionally, capsanthin reduces the risk of cardiovascular diseases because capsanthin had an HDL-cholesterol-raising effect on plasma without detectable differences in total cholesterol (Aizawa and Inokuma 2009).

Genetic control of carotenoid content in pepper fruits has been reported mainly for carotenoid biosynthesis pathways and color variation of fruits (Hurtado-Hernandez and Smith 1985). Genes for carotenoid biosynthesis pathway were identified so far (Romer et al. 1993, Bouvier et al. 1994, Hugueney et al. 1995, Bouvier et al. 1998). Although there are many variations in pigment content in pepper germplasm, few studies about genetic controls of quantitative variations in carotenoid content have been reported.

Therefore, to access the genetic mechanisms for controlling content of red color pigment, capsanthin, in pepper, we mapped QTLs for the content in DH population derived from a cross between genetic resource ‘S3586’ and local cultivar ‘Kyoto-Manganji No. 2’. In QTL mapping using population with fixed genotypes such as DH and recombinant inbred lines, utilization of all biological replication in one analysis is very important factor for reduction of nongenetic residual variance and increase in accuracy of the mapping (Broman and Sen, 2009). Because our segregating population is DH, we can create multiple individuals with the same genotype for two experiments. Hence, we performed QTL mapping mainly using phenotypes from two datasets (Experiments 1 and 2) at one time.

Materials and Methods

Plant materials

The pepper genetic resource ‘S3586’ (*C. annuum*, Laboratory of Plant Genetics and Breeding, Shinshu University, Matsushima et al. 2009) was crossed with cultivar ‘Kyoto-Manganji No. 2’ (*C. annuum*, Biotechnology Research Department, Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Research Center, Seika, Kyoto, Japan, Minamiyama et al. 2012). A segregating doubled-haploid (SM-DH) population (n = 141) was developed by anther culture of an F_1 plant as described by Dumas de Vaulx et al. (1981).

SM-DH lines were grown in a greenhouse of the Biotechnology Research Department (Seika, Kyoto, Japan) during the summers of 2013 (Experiment 1) and 2016 (Experiment 2). One plant of each SM-DH line was used for analysis. Seeds were sown in trays filled with vermiculite on 11 March 2013 and 4 February 2016. After 4 weeks, seedlings were transplanted into rockwool cubes; the cubes were placed on rockwool slabs on 17 May 2013 and 22 April 2016. The temperature in the greenhouse was maintained above 16 ºC. Plants were grown in hydroponic solution (M nutrient prescription, M Hydroponic Research Co., Ltd., Aichi, Japan) with an electrical conductivity of 1.0 to 1.2 dS/m. Five fruits were harvested from each plant at each of the two ripening stages, 45 and 90 days after flowering (DAF). 45 DAF was turning color stage and 90 DAF was full maturity stage. Usually, we harvest ‘Kyoto-Manganji No. 2’ at green mature stage, about 30 to 35 DAF, but at this stage capsanthin mostly is not detected. The peduncles and the seeds were removed, and the fruits were cut into small pieces and kept at −30 ºC until analysis.

Pigment extraction and saponification

Because a large proportion of capsanthin in pepper fruits is esterified with many kinds of fatty acids, qualitative and quantitative analysis of all capsanthin esters by high-

performance liquid chromatography (HPLC) is very difficult. Generally, prior to HPLC analysis, capsanthin esters are hydrolyzed by saponification and then the capsanthin monomer is quantified (Hornero-Mendez et al. 2000, Howard et al. 2000).

Frozen sample (1 g fresh weight) was powdered using a mortar and pestle with sea sand and extracted with ethanolic pyrogallol (3% w/v). Extraction was repeated until the complete loss of color. All extracts were pooled and made up to 50 mL with ethanolic pyrogallol in a volumetric flask.

Each aliquot (10 mL) of the extract was mixed with 1 mL of potassium hydroxide (60% w/v). The tubes were placed in a 70 ºC water bath for 30 min with shaking continuously during saponification. The tubes were then cooled in water to room temperature, and 22.5 mL of sodium chloride (10 g/L) was added into each tube. Then the suspension was extracted three times with 15 mL of n-hexane/ethyl acetate (9:1 v/v). The upper layer was collected, and evaporated to dryness, and the residue was dissolved in 5 mL of ethanolic pyrogallol (3% w/v). All samples were filtered through 0.45-μm nylon membrane filters (Minisart-RC, Sartorius, Göttingen, Germany).

HPLC analysis

Qualitative and quantitative HPLC analysis was performed according to the modified method of Goda et al. (1995) using a Shimadzu LC-10A quaternary pump equipped with a diode array detector (Shimadzu, Kyoto, Japan) and a Cosmosil 5C-AR II reverse-phase column (Nacalai Tesque, Kyoto, Japan) protected by a guard cartridge (Nacalai Tesque). The oven was operated at 40 ºC. The sample injection volume was 10 μL. Samples were eluted with acetone in water as follows: 70% acetone for 5 min; 70%–90% linear gradient for 5 min; 90% acetone for 3 min; 90%–100% linear gradient for 20 min; and 100% acetone for 5 min. The flow rate was 1.0 mL/min. For quantification, a capsanthin standard was obtained from Extrasynthese S.A. (Lyon, France) and a β-carotene standard was obtained from Wako Pure Chemical Industries (Osaka, Japan). All samples were analyzed before and after saponification. Loss of capsanthin during saponification was calculated from the loss of β-carotene. All analysis was carried out in 3 to 5 replications.

Heritability of traits

We used one-way ANOVA tables for phenotypes of the SM-DH lines in each experiment, using line (genotype) as a factor, to estimate heritability (h^2) of capsanthin content at 45 DAF and 90 DAF. The phenotypic value of the *i*th line in the *j*th replicate, denoted as y_{ij}, is expressed as:

$$y_{ij} = \mu + g_i + e_{ij} \ (i = 1, 2, \ldots, m; j = 1, 2),$$

where μ is the intercept, g_i is the effect of the *i*th line, e_{ij} is the residual error with $g_i \sim N(0,\sigma_g^2)$ and $e_{ij} \sim N(0,\sigma_e^2)$, and m is the number of lines [m = 98 (45DAF) or 94 (90 DAF)]. The expectations of sums of squares between and within lines, S_B and S_W, are expressed as:

$$E(S_B) = 2(m-1)\sigma_g^2 + (m-1)\sigma_e^2$$

and

$$E(S_W) = m\sigma_e^2$$

From these formulae, estimates of the genetic variance σ_g^2 and residual variance σ_e^2, $\hat{\sigma}_g^2$ and $\hat{\sigma}_e^2$, are obtained as:

$$\hat{\sigma}_e^2 = S_W / m \quad \text{and}$$

$$\hat{\sigma}_g^2 = \{S_B - (m-1)\hat{\sigma}_e^2\} / \{2(m-1)\} \quad .$$

Heritability was estimated as:

$$\hat{h}^2 = \hat{\sigma}_g^2 / (\hat{\sigma}_g^2 + \hat{\sigma}_e^2) \quad .$$

Isolation of genomic DNA and genotyping

Genomic DNA from the leaves of parental lines and the SM-DH population was isolated using a Nucleon PhytoPure DNA extraction kit (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, England). Simple sequence repeat (SSR), single nucleotide polymorphism (SNP), sequence characterized amplified repeat (SCAR) and cleaved amplified polymorphic sequence (CAPS) primer pairs used in this study were selected on the basis of the published marker locus data (Gulyas et al. 2006, Kim and Kim 2006, Lee et al. 2004a,b, Mimura et al. 2010, 2012, Minamiyama et al. 2006, 2007, Nagy et al. 2007,

Sugita et al. 2006, 2013, Yi et al. 2006). Some SSR markers designed by Minamiyama et al. (2006) were newly mapped in this study (Table 2-1). PCR with SSR primers (Sugita et al. 2006, 2013) was performed by a post-labeling method with a bar-coded split tag as described in Konishi et al. (2015). PCR products were sequenced on a 3730x1 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Fragment length was determined by GeneMapper v3.7 software (Applied Biosystems). Labeling and analysis of SSR markers developed by Minamiyama et al. (2006) were performed as in that report. The 5′ ends of the forward primers were labeled with D2-, D3- or D4-fluorescent dye. PCR products were sequenced on a Beckman CEQ 200xL sequencer (Beckman Coulter, Fullerton, CA, USA). Fragment length was determined on a CEQ 8000 genetic analysis system (Beckman Coulter). SNPs were genotyped by the Tm-shift method as in Fukuoka et al. (2008). PCR using SCAR and CAPs markers was performed as in Lee et al. (2004b), Gulyas et al. (2006) and Kim and Kim (2006). PCR products were separated on 2% agarose gels and stained with ethidium bromide.

Table 2-1. SSR markers newly mapped in this study

Marker name	Forward primer (5'–3')	Reverse primer (5'–3')	Motif	Linkage	Expected product[a]
CAMS_061-2	cgatttcagtgggtgcctat	cgcactgaaaaaggagatgg	(gt)3...(tg)5...(tg)4...(ga)3	8	231
CAMS_206-2	tggagcatgcgtaaactcac	gtgactaacccccctgctgtc	(ac)22cc(ac)13	14	286
CAMS_308	gtgcttcgcatgcttgtatc	tctgaaagatgacagataattgtgg	(ta)3(tg)3(ta)7tgt(ag)11	14	215
CAMS_487-2	ggatgaggtcagtatgggact	tttgcatgcctgcagaataa	(ag)13	2	260
CAMS_640-2	atgggctaatgatcacgaca	cgtttacatgcgtcgttattg	(taa)8	14	158
CAMS_667-2	cgatccgtgaaagttactcaa	ggcaccccaaacttttttagtc	(ag)12...(ga)4...(ga)3	9	222
CAMS_818	gctgcgacctcttcttcttc	ccccactaggtgggaataca	(ctt)5...(caa)5	14	268
CAMS_852	gctgaggttctagccaccag	tgtcgaaccgggacatagat	(tct)9	15	256

All markers in this table were designed by Minamiyama et al. (2006).
[a]Expected product size is indicated for 'Kyoto-Manganji No. 2'.

Construction of a linkage map and QTL analysis

AntMap software (Iwata and Ninomiya 2006) was used to construct LGs; the order of markers was determined using the Kosambi mapping function. The map was compared to the KL-DH map (Sugita et al. 2013; downloaded from VegMarks, http://vegmarks.nivot.affrc.go.jp/) and to the pepper genome (chromosomes) sequence data (CM334 ver. 1.55, Kim et al. 2014; available from the Pepper Genome website, http://peppergenome.snu.ac.kr). Composite interval mapping was performed in QTL Cartographer ver. 2.5 software (https://brcwebportal.cos.ncsu.edu/qtlcart/WQTLCart.htm, Wang et al. 2005). Forward and backward stepwise regression was performed with a threshold of $P < 0.05$. To establish empirical LOD thresholds at the 5% level, 1000 permutation tests were performed. Seven datasets were prepared (Supplemental Table 1) using phenotypes of two cultivations (experiment 1 and 2) at two ripening stages (45 and 90 DAF). These datasets were used to perform QTL mapping. In dataset No .7, all of the phenotypes at two ripening stages (45 DAF and 90 DAF) in two cultivations were entered as a single phenotype.

Analysis of the effect of QTLs on capsanthin content

The SM-DH lines were grouped according to the genotypes of the markers linked to the QTLs, and the phenotypes of the groups were compared to each other.

Results

Phenotypic characterization of capsanthin content in parents and SM-DH population

Capsanthin content increased during ripening (from 45 DAF to 90 DAF) in both parental lines and was higher in ‘S3586’ than in ‘Kyoto-Manganji No.2’ at both ripening stages (Fig. 2-1,2-2). This result agreed with that of Konishi and Matsushima (2011).

Fig. 2-1. Fruits of parental lines at two ripening stages. (A) 'S3586', 45 DAF (B) 'S3586', 90 DAF (C) 'Kyoto-Manganji No. 2', 45 DAF (D) 'Kyoto-Manganji No. 2', 90 DAF. Bars indicate 5cm.

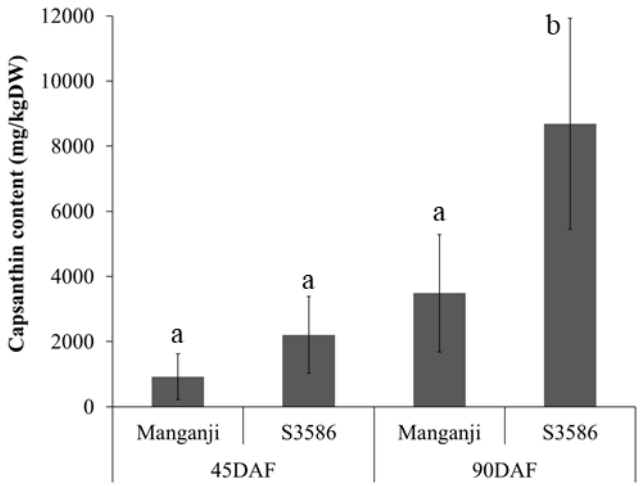


Fig. 2-2. Capsanthin content of parental lines at two ripening stages. Values are means of 7-9 measurements in two experiments. Error bars represent the standard deviation. Means sharing the same letter are not significantly different according to the Tukey–Kramer multiple-comparison test.

The capsanthin content in the SM-DH population evaluated in Experiments 1 (2013) and 2 (2016) showed a normal distribution (Fig. 2-3). Capsanthin content of parental lines and average capsanthin content of SM-DH was higher in Experiment 1 than in Experiment 2 at both ripening stages (45 and 90 DAF) and a histogram of capsanthin content in Experiment 2 shifted lower than in Experiment 1. In 45 DAF, the distribution pattern in Experiment 1 was different from that in Experiment 2 (Fig. 2-3). Heritability of capsanthin content was quite low at 45 DAF and it was 0.155 at 90 DAF (Table 2-2).

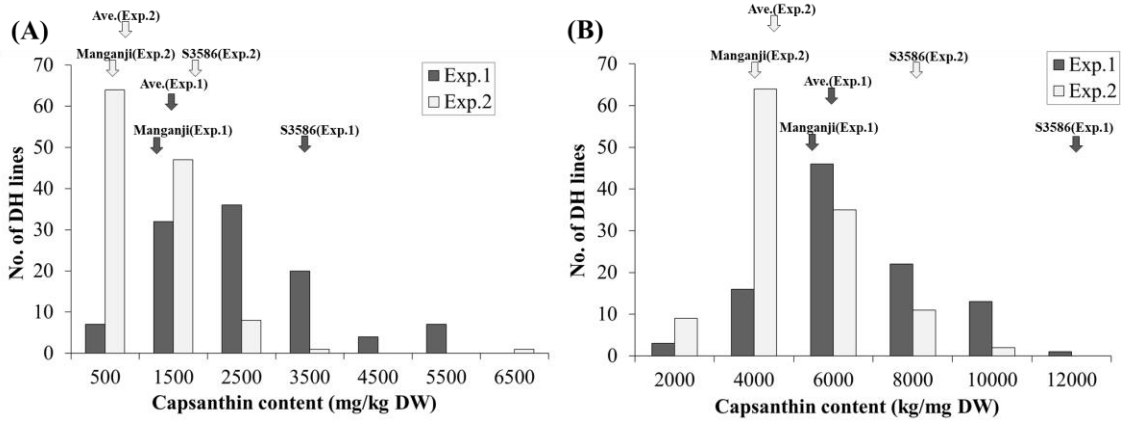


Fig. 2-3. Frequency distribution of capsanthin content in SM-DH lines. (A)45 DAF. (B)90 DAF. Arrowheads indicate the mean values for the parents and average of SM-DH.

Table 2-2. Heritability of capsanthin

Trait	h^2
Content at 45 DAF	−0.068
Content at 90 DAF	0.155

Linkage map construction

To construct a genetic map (designated as the SM-DH map), 160 SSR, 24 SNP, 3 SCAR and 1 CAPS markers were used. The map consisted of 15 LGs covering a total distance of 1403.8 cM (Fig. 2-4). The average distance between markers was about 9 cM. In this study, 8 new SSR markers were mapped (Table 2-1). We were able to assign 14 of the 15 LGs of the SM-DH map to LGs of the KL-DH map (Sugita et al. 2013), which covers nearly the entire genome of *C. annuum* (Fig. 2-5). Comparison with the pepper genome (CM334 ver. 1.55) using the BLAST program showed that the SM-DH map covered 75% of the genome.

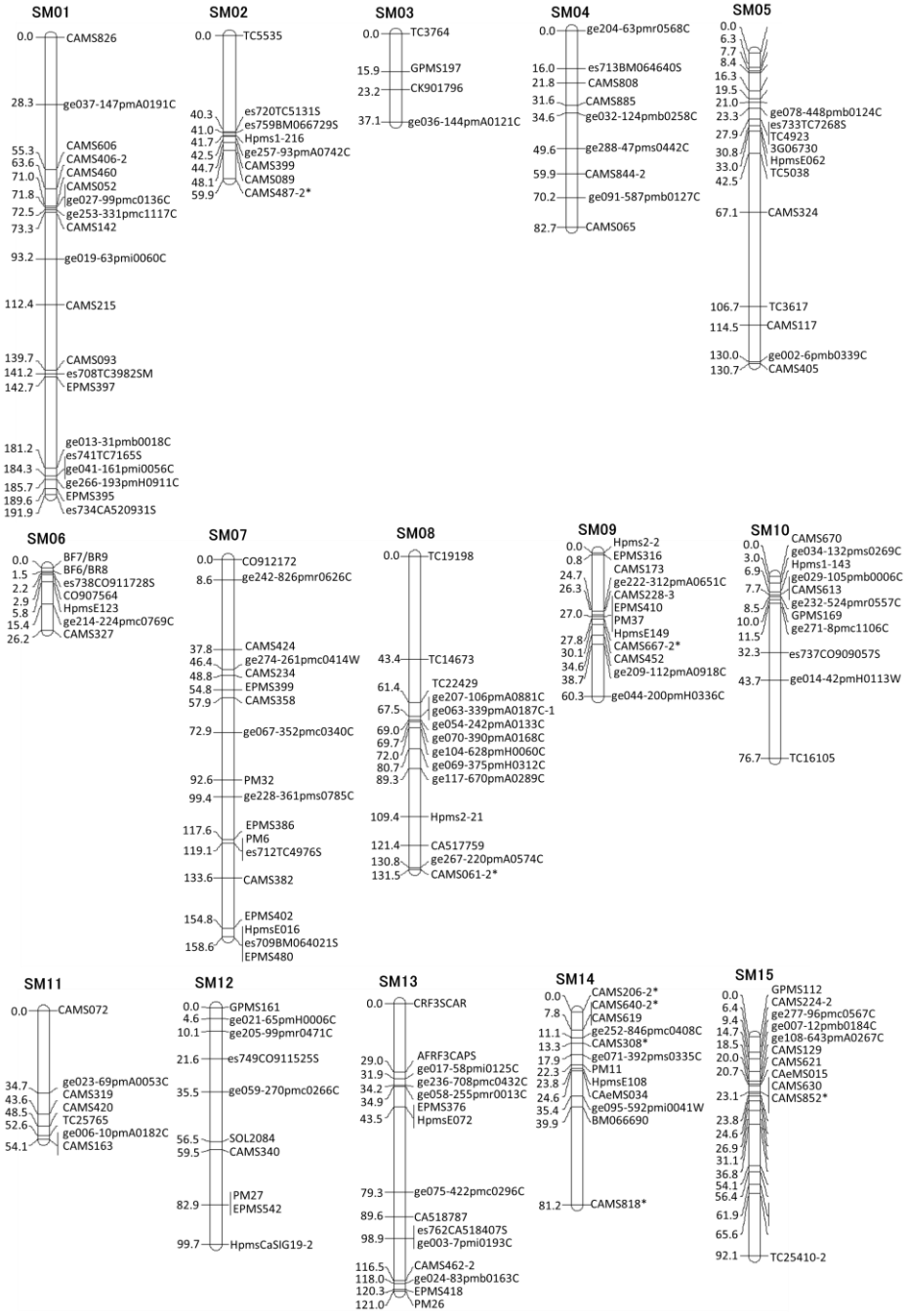


Fig. 2-4. Linkage map of the SM-DH population ('S3586' × 'Kyoto-Manganji No. 2'). SSR markers "PM", "ge" and "es" were reported by Sugita et al. (2006,1013), "CAMS" and "CAeMS" by Minamiyama et al. (2006) and Mimura et al. (2010, 2012), "Hpms" by Lee et al. (2004a), "HpmsE" by Yi et al. (2006), and "GPMS" and "EPMS" by Nagy et al. (2007). Newly mapped SSR markers are indicated with asterisks (*). SNP markers "TC", "CA", "CO" and "CK" were reported by Sugita et al. (2013). SCAR markers BF7/BR9 and BF6/BR8 were reported by Lee et al. (2004b) and CRF3SCAR by Gulyas et al. (2006). The CAPS marker AFRF3CAPS was reported by Kim and Kim (2006)

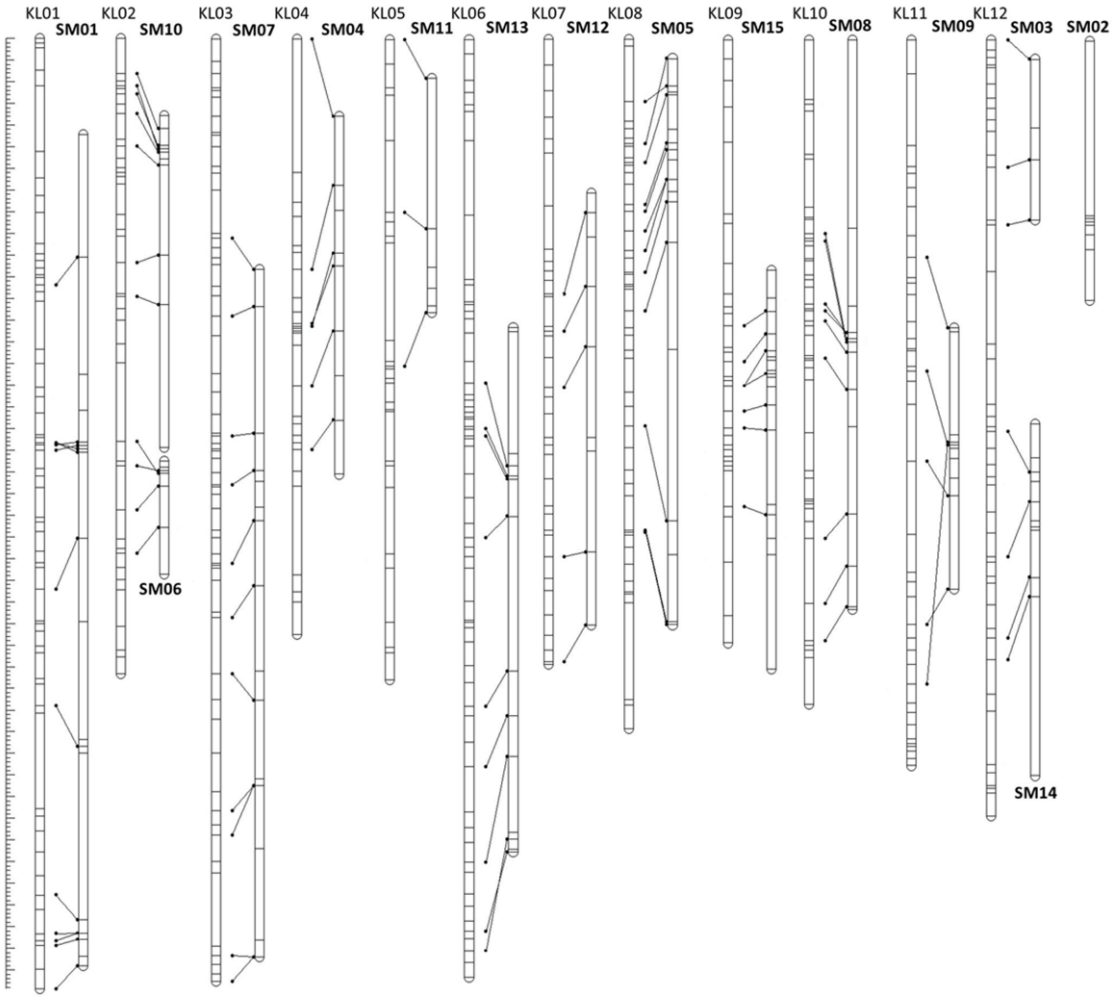


Fig. 2-5. Comparison between KL-DH and SM-DH maps. KL01–KL12 are 12 linkage groups of the KL-DH map corresponding to the 12 pepper chromosomes (Sugita et al. 2013). SM01–SM15 are linkage groups of the SM-DH map constructed in this study. Identical markers on both maps are connected by lines.

QTL analysis

We carried out QTL analysis using 4 datasets on capsanthin content at 45 DAF and 90 DAF obtained from two experiments (Experiments 1 and 2). Further, we also performed QTL analysis using the data on the content at 45 DAF and 90 DAF from each experiment (Dataset 5 and 6, Supplemental Table 1).

Analysis of capsanthin content at 45 DAF from two experiments detected a significant QTL on LG15 (LOD score, 4.95; Fig. 2-6, Table 2-3), which was designated *Cst15.1*. The

LOD score peak was positioned between the SSR markers GPMS001 and CAMS378. The additive effect of this QTL was 501.0, and the allele that increased capsanthin content was derived from 'S3586' (Table 2-3). An insignificant LOD score peak at 90 DAF was observed close to *Cst15.1* (Fig. 2-6).

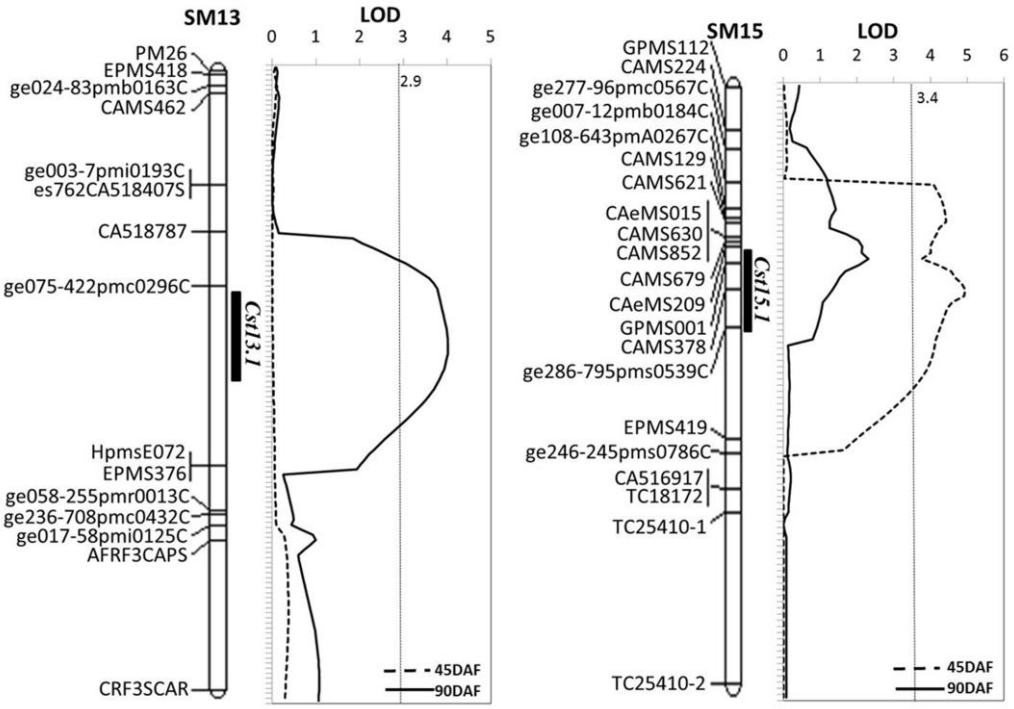


Fig. 2-6. Positions of two QTLs for capsanthin content on the SM-DH linkage map. Positions of QTLs with 1-LOD support intervals are shown by black boxes. Vertical dotted lines indicate the LOD thresholds.

Table 2-3. QTLs for capsanthin content at each ripening stage detected in this study

Experiment	Trait	Dataset[a]	LG	QTL ID	Marker[b]	Position	LOD	R^{2c}	Additive effect[d]	Threshold[e]
1-2	Content at 45 DAF	5	15	*Cst15.1*	GPMS001 CAMS378	28.9	5.0	16.1	501.0	3.3
	Content at 90 DAF	6	13	*Cst13.1*	EPMS376, HpmsE072 ge075-422pmc0296C	51.8	4.0	17.0	778.0	2.7
1	Content at 45 DAF	1	15	*Cst15.1*	GPMS001 CAMS378	28.9	5.0	16.1	501.0	3.4
	Content at 90 DAF	2	13	*Cst13.1*	EPMS376, HpmsE072 ge075-422pmc0296C	51.8	4.0	17.0	778.0	2.8
2	Content at 90 DAF	4	7	*Cst7.1*	CAMS424 ge274-261pmc0414W	45.8	6.0	15.1	-675.9	2.9
			15	*Cst15.1*	CAeMS015 GPMS001	24.6	4.6	10.8	529.5	2.9

[a]Details of datasets are shown in Supplemental Table 1.
[b]Nearest markers on both sides of QTL.
[c]Percentage of phenotypic variation explained.
[d]Positive values indicate alleles from 'S3586'.
[e]The significance threshold for QTL detection by 1000 permutations at $P < 0.05$.

Analysis of capsanthin content at 90 DAF from two experiments detected a significant LOD peak on LG13 (LOD score, 4.02; Fig. 2-6, Table 2-3), which was designated as *Cst13.1*. The LOD peak was positioned between the SSR markers EPMS376/HpmsE072 and ge075-422pmc0296C (Fig. 2-6). The additive effect of this QTL was 778.0 and its allele that increased capsanthin content was derived from 'S3586' (Table 2-3).

In analysis of the content from each single experiment (Dataset1-4), *Cst15.1* was detected at 45 DAF in experiment 1, and at 90 DAF in experiment 2. *Cst 13.1* was also detected at 90 DAF in experiment 1 (Table 2-3). A new QTL, *Cst7.1* was detected only at 90DAF in experiment 2. At 45 DAF of experiment 2, we could not detect any significant QTL.

We also carried out QTL analysis using combined 45 DAF and 90 DAF data (Dataset 7), which we considered as variations of a single phenotype during ripening. In this analysis, we detected *Cst15.1* but not *Cst13.1* (Supplemental Fig. 1).

We grouped SM-DH lines according to the genotypes of markers adjacent to *Cst15.1* and *Cst13.1* on both sides and calculated the mean capsanthin content of each group at each ripening stage. Lines with the homozygous genotypes of *Cst15.1* or *Cst13.1* derived from 'S3586' had higher capsanthin content than the other lines at both 45 DAF and 90 DAF (Table 4). At 45 DAF, the 'S3586' allele of *Cst15.1* seemed to be more effective in increasing capsanthin content than that of *Cst13.1*.

Table 2-4. Capsanthin content in fruits of SM-DH lines grouped according to the genotypes of markers adjacent to the QTLs *Cst15.1* and *Cst13.1*

Traits	Genotypes of QTLs		*N*	Capsanthin content (mg/kg DW)[a]
	Cst15.1	*Cst13.1*		
Content at 45 DAF	M	M	48	956.9 a
	M	S	47	1219.0 ab
	S	M	29	1723.0 b
	S	S	28	1793.3 b
Content at 90 DAF	M	M	45	3586.5 a
	M	S	45	4966.7 b
	S	M	29	4974.8 b
	S	S	28	5667.8 b

[a] Data are means of two experiments.
Means sharing the same letter are not significantly different between line groups according to the Tukey–Kramer multiple-comparison test.
M, homozygous for the 'Kyoto-Manganji No. 2' allele; S, homozygous for the 'S3586' allele.

Discussion

In this study, to access the mechanisms for the genetic control in variation of capsanthin content of pepper (*C. annuum*), QTL mapping using SM-DH lines derived from a cross of high content genetic resource line, 'S3586' and cultivar 'Kyoto-Manganji No. 2' was performed. Capsanthin content of SM-DH lines at two ripening stages (45 DAF and 90 DAF) differed between the two experiments, and its difference ascribes to the variation of the environmental (cultivation) conditions (Fig. 2-3). It is known that carotenoid accumulation is regulated by light signaling (Nisar et al. 2015). In *Capsicum* fruit, light irradiation at immaturity stage of fruit increases the expression of *Psy* gene for phytoene synthase (Nagata et al. 2015). Phytoene synthase is an enzyme upstream in capsanthin biosynthesis (Supplemental Fig. 3) and expression level of *Psy* and content of total carotenoid positively correlate (Rodriguez-Uribe et al. 2012). In this study, total global solar radiation at the nearest observation point from fruit setting (DAF 0) to immaturity stage (DAF 40) was 785 and 633MJ/m^2 in Experiment 1 and 2, respectively (Supplemental Fig. 2). This difference of light condition may account for the difference of capsanthin content in two experiments. In order to verify this hypothesis, it is necessary to investigate into gene expression for carotenoid biosynthesis in the fields under different light condition.

To improve accuracy of QTL detection, we took into account variation among year in the analysis according to Broman and Sen (2009) and found *Cst15.1* at 45 DAF and *Cst13.1* at 90 DAF (Table 3). *Cst 15.1* was detected at 45 DAF of experiment 1 and 90 DAF of experiment 2, but *Cst 13.1* was detected at only experiment 1. Hence, it is possible that *Cst 15.1* has more stable and large effect than *Cst 13.1*. We could select SM-DH lines with higher capsanthin content at both ripening stages in both experiments by using markers adjacent to the two QTLs (Table 2-4), suggesting that the QTLs have stable effects on capsanthin content under environmental conditions tested here.

It is important to breed high-capsanthin-content peppers to be used as health beneficial vegetables. Because the pepper fruits to be used as vegetables are usually harvested before they are fully matured (ex. 90 DAF), it is necessary to accumulate QTLs that increase capsanthin content at early stage (ex. 45 DAF). In particular, *Cst15.1* seems to be efficient for this purpose (Tables 2-3, 2-4). Capsanthin is accumulated during the development of pepper fruit (Fig. 2-2), and *Cst15.1* was the only QTL detected when capsanthin content

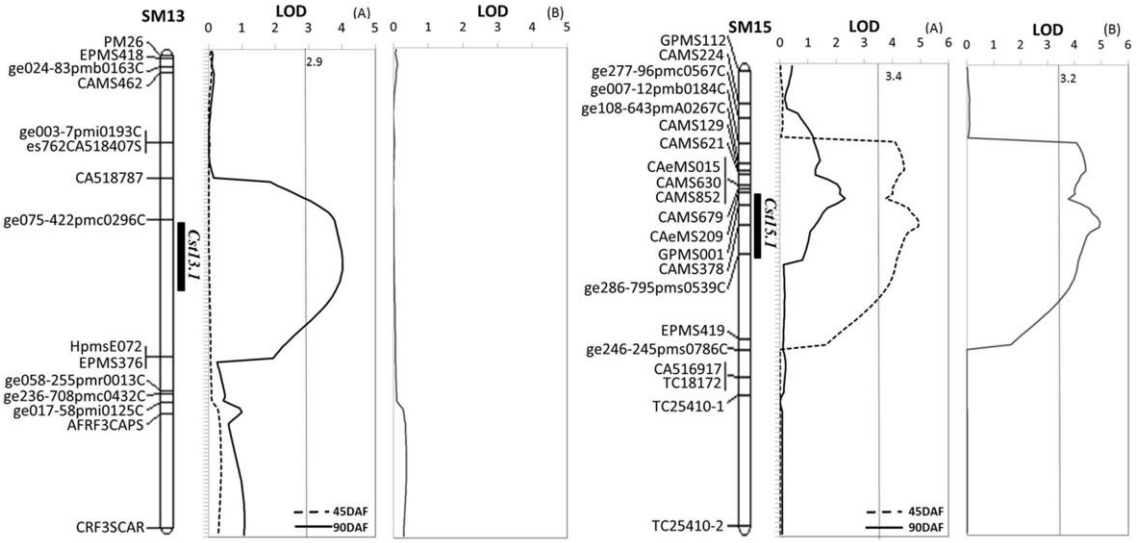
at both 45 DAF and 90 DAF was considered as a variation of a single phenotype (Supplemental Fig. 1), suggesting that *Cst15.1* affects capsanthin content at more than one stage.

The color of pepper fruits starts to change at 45 DAF (turning-color stage), and this change is completed at 90 DAF (full-maturity stage); one QTL was detected at each of the two stages when the analyses were conducted with the two cultivations at each ripening stage as phenotypes (Table 2-3). Hence, the two QTLs may have distinct effects on fruit ripening. However, it is very difficult to identify the candidate genes for the QTLs because the existing regions of the QTLs on the pepper genome (http://peppergenome.snu.ac.kr) are too large to narrow down. On the other hand, *CCS* gene for capsanthin-capsorubin synthase, a key enzyme for capsanthin biosynthesis (Supplemental Fig. 3), begins to be expressed when the fruits starting to ripe (Lefebvre et al. 1998). Also, *CCS* gene was mapped to chromosome 6 (Thorup et al. 2000), and *Cst 13.1* was also mapped to the same chromosome. Additionally, lycopene ε-cyclase gene (*LCY-E*, Supplemental Fig. 3), that probably act in the lutein synthesis pathway not in the capsanthin synthesis pathway, was mapped to chromosome 9 (Thorup et al. 2000) as with *Cst 15.1*. However, to detect the candidate gene of *Cst13.1* and *Cst15.1* and to clarify the relationship of these QTLs with *CCS* and *CLY-E*, it is necessary to use the high-resolution QTL mapping and transcript quantification.

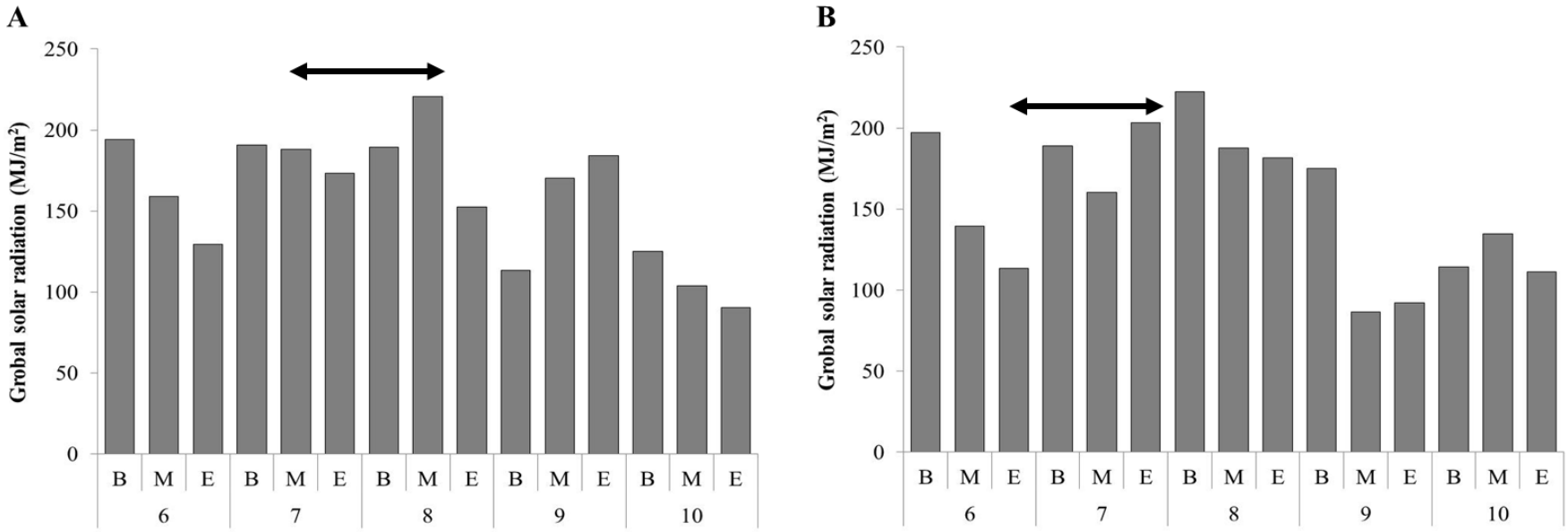
In spite of small h^2 values for capsanthin content at 45 and 90 DAF (Table 2-2), the QTLs detected in this study explained more than 15% of the total phenotypic variation, suggesting that the marker sets flanking these QTLs derived from 'S3586' would be efficient tools to breed peppers with high capsanthin content by marker-assisted selection. Because the SM-DH map covers only 75% of the entire genome, it may be necessary to check whether other QTLs exist in the remaining 25% if additional markers are available.

In pepper, strategies to breed carotenoid-enriched cultivars have been so far limited, probably because of complicated phenotypic evaluation. Selection for capsanthin content using DNA markers linked to QTLs is highly effective. Many QTLs may affect carotenoid biosynthesis and make the plants fit under various environmental conditions. To identify more QTLs for carotenoid content, it is necessary to screen high-carotenoid-content materials from many landraces and genetic resources and to develop many DNA markers for detecting QTLs from those materials in the future.

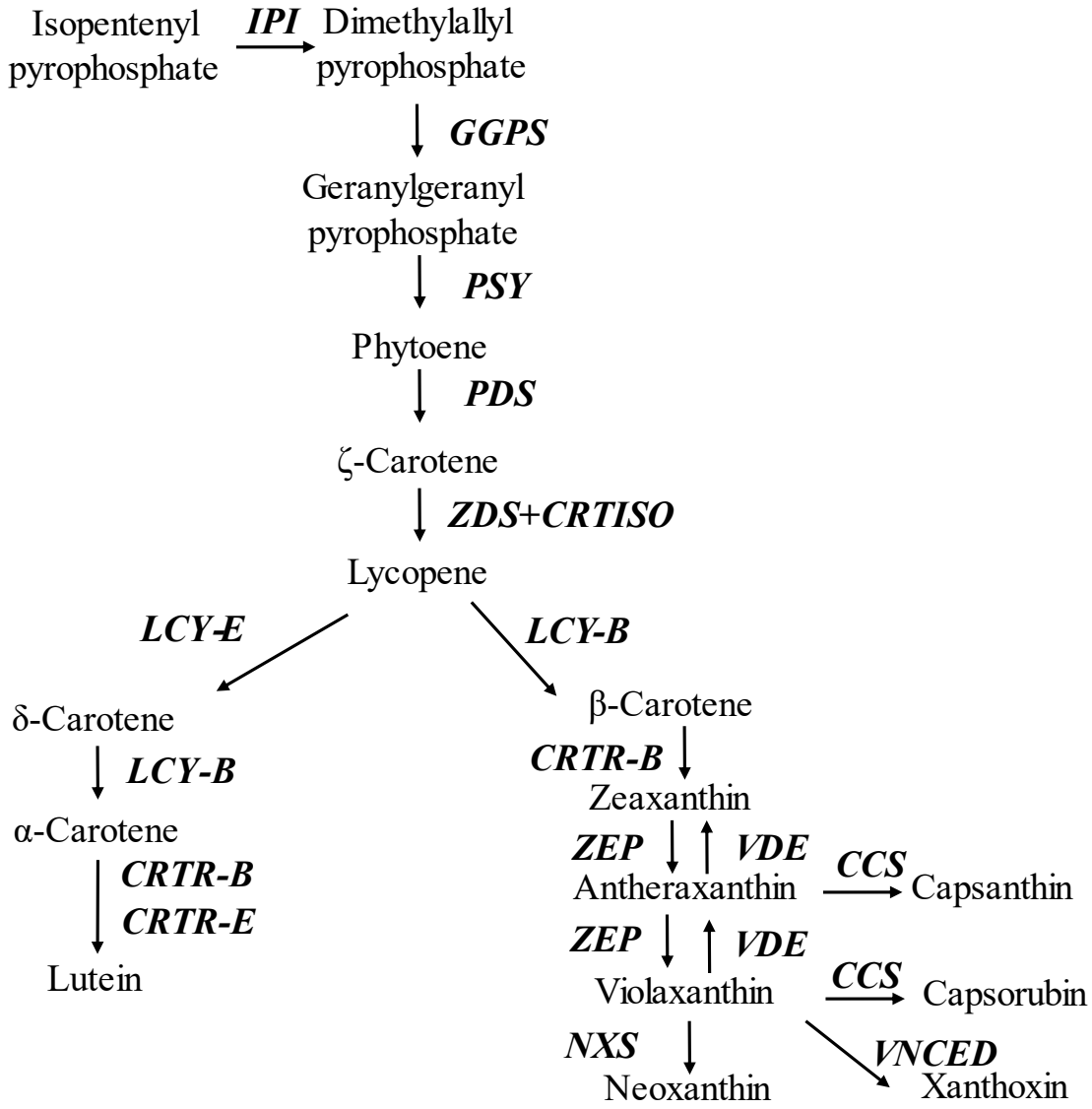
Supplemental Fig. and Table



Supplemental Fig. 1. Position of QTLs for capsanthin content on linkage map. Positions of QTLs with 1-LOD support intervals are shown by black boxes. (A)QTL analysis using the capsanthin content at 45 DAF and 90 DAF as two phenotypes(B)QTL analysis using 45 DAF and 90 DAF data at once as a single phenotype. The vertical dotted line indicates the LOD threshold.



Supplemental Fig. 2. Global solar radiation of Nara-city, the nearest observation point of Seika, during the period of fruiting. (A) Experiment 1 (2013). (B) Experiment 2 (2016). B is the beginning ten days of the month, M is the middle ten days, and E is the last ten days. Allows indicate the immaturity stage period of fruits for analysis.



Supplemental Fig. 3. The carotenoid biosynthesis pathway in *Capsicum*. Abbreviations of enzymes: CCS, capsanthin-capsorubin synthase; CRTISO, carotenoid isomerase; CRTR-B, β-ring hydroxylase; CRTR-E, ε-ring hydroxylase; GGPS, geranylgeranyl diphosphase synthase; IPI, isopentenyl diphosphate isomerase; LCY-B, lycopene β-cyclase; LCY-E, lycopene ε-cyclase, NXS, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin de-epoxidase; VNCED, 9-*cis*-epoxycarotenoid dioxygenase; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase. Figure was modified Rodriguez-Uribe et al. (2012).

Supplemental Table 1. Datasets using in QTL analysis

Dataset	Experiment	DAF	No. of phenotype	No. of genotype
1	1	45	106	141
2	1	90	101	141
3	2	45	121	141
4	2	90	121	141
5	1 and 2	45	227	141
6	1 and 2	90	222	141
7	1 and 2	45 and 90	449	141

Chapter 3. Breeding of high capsanthin- containing red pepper ‘DMSM188’

Introduction

Manganji pepper, a local cultivar cultivated in Kyoto Prefecture, is known for its large, tasty fruit that is usually harvested at the green mature stage, about 30 to 35 days after flowering (DAF). The mature red fruits of Manganji pepper are hardly used because they have low capsanthin content, which limits their coloring. Mature Manganji pepper fruits pose an additional problem for the protection of genetic resources, since the mature fruits also contain mature seeds. To remedy this issue, we bred a new high-capsanthin F_1 red pepper cultivar by a combination breeding cross of cytoplasmic male sterility (CMS) ‘Kyoto-Manganji No. 2’ and selected SM-DH lines derived from crossing the high-capsanthin genetic resource line ‘S3586’ with ‘Kyoto-Manganji No. 2’.

Materials and Methods

Breeding of ‘DMSM188’

The breeding process for ‘DMSM188’ is shown in Fig. 3-1. As a seed parent, CMS Kyoto-Manganji No. 2 was raised through a continuous backcross of ‘Kyoto-Manganji No. 2’ to CMS line DDH9, which was created from the cultivar ‘Daimyo’. ‘Kyoto-Manganji No. 2’ was used to maintain CMS Kyoto-Manganji No. 2. To select the pollen parent, we used the SM-DH line that was developed as described in Chapter 2, using marker-assisted selection of the capsaicinoid synthase gene and fertility restoring gene. We harvested inbred seeds from 53 SM-DH individuals containing the non-pungent and fertility restoring genes. DHS_1 plants, developed from the inbred seed of SM-DH, were grown in a greenhouse during the summer of 2012, and 34 plants were selected for their large fruit size and high capsanthin content. F_1 seeds were then developed by performing a combination breeding cross of CMS Kyoto-Manganji No. 2 and the selected DHS_1 plants. The F_1 plants were grown in a greenhouse during the summers of 2013 and 2014, and line selection was completed. Finally, ‘DMSM188’, with the desired fruit properties

of high capsanthin content, non-pungent flavor, and similar shape to ‘Kyoto-Manganji No. 2’, was selected.

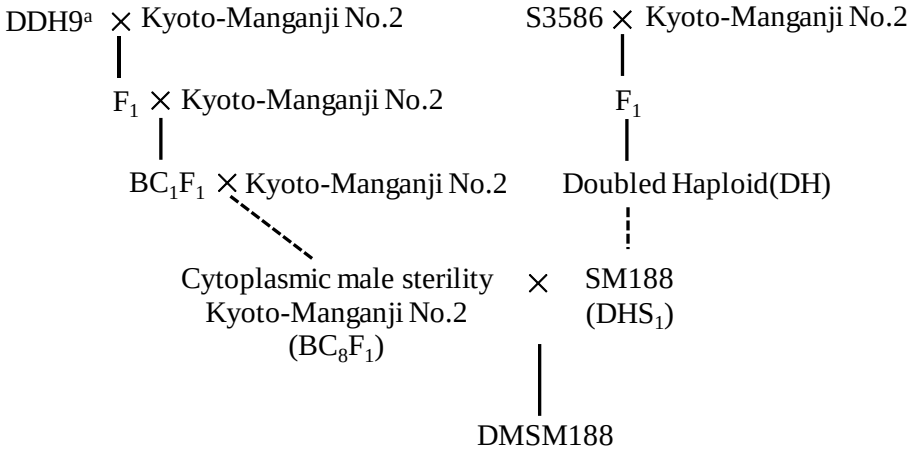


Fig. 3-1. Breeding process for ‘DMSM188’

a: DDH9 was created from another cultivar, ‘Daimyo’.

‘DMSM188’ fruit characteristics and yield trial

‘DMSM188’ and ‘Kyoto-Manganji No. 2’ were grown in a greenhouse at the Biotechnology Research Department (Seika, Kyoto, Japan) in 2015 and 2016. The cultural outline is presented in Table 3-1. The harvest period was defined as the time at which over three quarters of the fruit’s surface had turned red. The yield trial was performed according to ‘Manganji’ shipping standards, in which marketable fruits are graded to three classes: streight(S), a little bit curve(A), and curve(B). Sixty Class S fruits were harvested from July 14 through August 17 of 2015 and their characteristics were surveyed.

Table 3-1. Cultural outline of greenhouse-grown ‘DMSM188’ peppers in Kyoto, Japan in 2015 and 2016

Year	Seeding date (month/day)	Transplanting date (month/day)	Ridge width (m)	Intra-row (cm)	Training style	Pre-planting fertilizer(kg/a)			Replication (plants × reps)
						N	P_2O_5	K_2O	
2015	2/16	4/27	1.5	70	V-style with 4 main stems	3.2	2.3	2.9	6×2
2016	2/4	5/6	1.5	70		2.9	2.2	2.7	5×3

Fruit component analysis

1 Plant material

‘DMSM188’ and ‘Kyoto-Manganji No. 2’ were grown in a greenhouse at the Biotechnology Research Department (Seika, Kyoto, Japan) from 2014 through 2017. Seeds were sown in trays filled with vermiculite. Seedlings were transplanted into rockwool cubes, then placed onto rockwool slabs. The greenhouse temperature was maintained above 16°C. Plants were grown in hydroponic solution (M nutrient prescription, M Hydroponic Research Co., Ltd., Aichi, Japan) having an electrical conductivity of 1.0 to 1.2 dS/m. Five fruits were harvested from each plant at the fixed ripening stage of 60 DAF. The peduncles and seeds were removed, and the fruits were cut into small pieces and kept at –30°C until the time of analysis. All analyses were carried out with six replications. In 2015, fruits were harvested at 40, 50, 60, and 70 DAF to analyze the change in capsanthin content over time.

2 Pigment extraction, saponification, and HPLC analysis of capsanthin

These analyses used the same methods as were described in Chapter 2.

3 Reduced ascorbic acid content

Frozen samples with twice the amount of metaphospholic acid (5% w/v) were homogenized and processed in a centrifugal separator. The transparent supernatant was

diluted five times with metaphospholic acid (5% w/v). Each solution's reduced ascorbic acid content was measured using an RQflex plus 10 (Merck KGaA, Darmstadt, Germany) and Reflectoquant ascorbic acid test (Merck KGaA, Darmstadt, Germany).

4 Free sugar content

Powdered freeze-dried samples (1 g dry weight) were extracted with 50 mL ethanol (80% v/v) and processed in a centrifugal separator, then filtered through a 0.45-μm nylon membrane filter (Minisart-RC, Sartorius, Tokyo, Japan). Qualitative and quantitative high-performance liquid chromatography (HPLC) analysis was performed using a 1260 Infinity refractive index detector (Agilent Technologies, Santa Clara, CA, United States) and an Asahipak NH2P-50 4E column (Showa Denko, Tokyo, Japan). The analysis was performed at an oven temperature of 40°C with a sample injection volume of 10 μL. Samples were eluted with acetonitrile (70% v/v) at a flow rate of 1.0 mL/min. Sucrose, glucose, and fructose standards were prepared for quantification.

5 Free amino acid content

Powdered freeze-dried samples (1 g dry weight) were extracted with 50 mL ethanol (80% v/v) and processed in a centrifugal separator, then filtered through a 0.45-μm nylon membrane filter (Minisart-RC, Sartorius, Tokyo, Japan). Qualitative and quantitative HPLC analysis was performed using a 1260 Infinity quaternary pump equipped with a diode array detector (Agilent Technologies, Santa Clara, CA, United States) and a ZORBAX Eclipse Plus C18 column (Agilent Technologies, Santa Clara, CA, United States). The analysis was performed at an oven temperature of 40°C with a sample injection volume of 10 μL. Samples were injected after derivatization with o-phthalaldehyde and 9-fluorenylmethyl chloroformate. Samples were eluted with solution A (10 mM phosphate-boric buffer, pH 8.2) and solution B (acetonitrile:methanol:water = 45:45:10) as follows: 2% solution B for 0.5 min; 2%–57% linear gradient for 19.5 min; 57%–100% linear gradient for 3.5 min; and 100%–2% linear gradient for 1.5 min. The flow rate was 1.5 mL/min. Amino acid standards were obtained from Agilent Technologies (Santa Clara, CA, United States) for quantification.

Results and Discussion

‘DMSM188’ fruit characteristics and yield

The fruit characteristics of ‘DMSM188’ are shown in Table 3-2. Fruit weight, length, width, and pericarp thickness were lower in ‘DMSM188’ than in ‘Kyoto-Manganji No. 2’ (Fig. 3-2).

‘DMSM188’ yields in 2015 and 2016 are shown in Table 3-3. Yield was evaluated by the weight and number of upper class (classes S and A) or marketable (classes S, A, and B) fruits. In 2015, ‘DMSM188’ had a significantly higher marketable fruit number than did ‘Kyoto-Manganji No. 2’ ($P < 0.01$), but the average marketable fruit weight did not differ significantly between the two cultivars. Contrastingly, in 2016, the marketable fruit number did not differ significantly between cultivars, but ‘DMSM188’ had a significantly lower average marketable fruit weight than did ‘Kyoto-Manganji No. 2’ ($P < 0.01$). In both 2015 and 2016, the average upper class fruit yield did not differ significantly between cultivars, but ‘DMSM188’ had a significantly lower average marketable fruit weight than did ‘Kyoto-Manganji No. 2’ ($P < 0.01$).

On average, ‘DMSM188’ fruit weighed less than ‘Kyoto-Manganji No. 2’ fruit; this was attributed to the fruit’s characteristics. Therefore, to increase the fruit yield of ‘DMSM188’ and approach an equivalent fruit yield to ‘Kyoto-Manganji No. 2’, we examined the appropriate planting density, fertilization, and training practice for ‘DMSM188’.

Table 3-2. Characteristics of ‘DMSM188’ and ‘Kyoto-Manganji No. 2’ peppers grown in a greenhouse in Kyoto, Japan in 2015 and 2016

	Fruit weight (g)	Fruit length (cm)	Fruit diameter (cm)	Fruit length／Fruit diameter	Pericarp thickness (mm)
DMSM188	31.9	19.5	2.6	7.50	2.1
Kyoto-Manganji No. 2	50.5	21.0	3.3	6.45	2.4
	**	**	**	ns	**

** significant at $P < 0.01$; ns: not significant; $N = 60$

Table 3-3. Fruit yield of ‘DMSM188’ and ‘Kyoto-Manganji No. 2’ peppers grown in a greenhouse in Kyoto, Japan in 2015 and 2016

Year	Cultivar	Upper class yield[a]		Marketable yield[b]	
		(g/plant)	(number/plant)	(g/plant)	(number/plant)
2015	DMSM188	2816.7	102.7	5479.0	221.9
	Kyoto-Manganji N0.2	3748.5	91.7	5896.6	158.3
2016	DMSM188	2738.5	106.4	4140.2	182.2
	Kyoto-Manganji N0.2	3755.3	105.8	5610.9	172.2
2015		**	ns	ns	**
2016		**	ns	**	ns

[a] Upper class : S and A

[b] Marketable class : S, A, and B

** significant at $P < 0.01$; ns: not significant; Replication is indicated in Table 3-1.

Fig. 3-2. ‘DMSM188’ (A) and ‘Kyoto-Manganji No. 2’ (B) fruits at 60 days after flowering. Scale bars: 5 cm.

Capsanthin content of 'DMSM188'

The capsanthin contents of 'DMSM188' and 'Kyoto-Manganji No. 2' are shown in Fig. 3-3. From 2014 to 2016, annual changes occurred in the capsanthin contents of both cultivars measured at 60 DAF. These differences ascribe to the year-to-year variation in environmental conditions, especially the total global solar radiation from the time of fruit set to maturity. In all years, however, 'DMSM188' had significantly higher capsanthin content than did 'Kyoto-Manganji No. 2' ($P < 0.01$). The average capsanthin content in 'DMSM188' over the three years of the experiment was 8,981 mg/kg DW; this was more than twice the three-year average capsanthin content in 'Kyoto-Manganji No. 2' (3,634 mg/kg DW). In both cultivars, capsanthin content increased with increasing fruit maturity. However, 'DMSM188' increased in capsanthin content and turned red earlier than did 'Kyoto-Manganji No. 2', and turned a deeper red color than 'Kyoto-Manganji No. 2' (Fig. 3-2, 3-4).

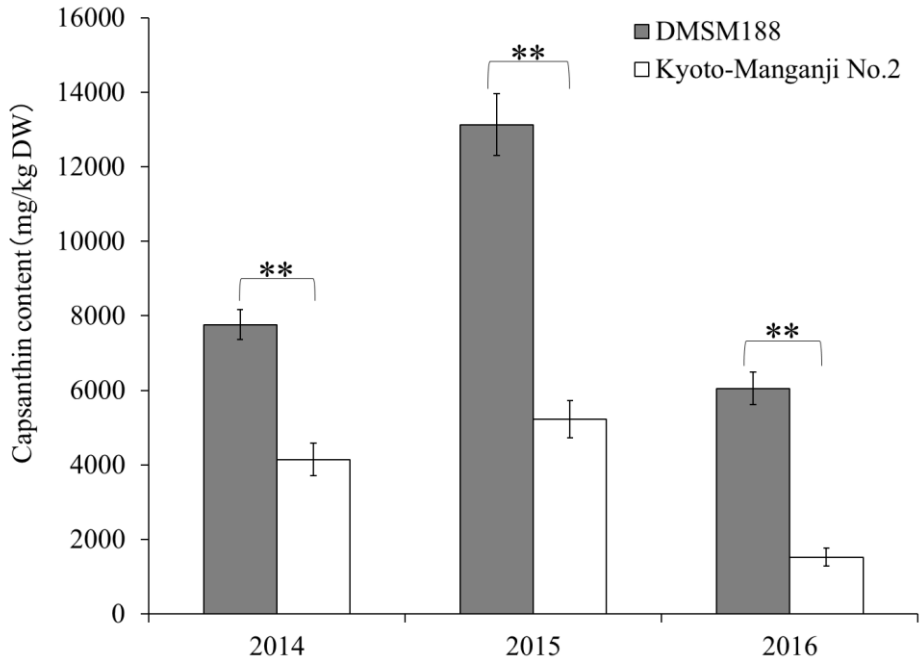


Fig. 3-3. Capsanthin content of 'DMSM188' and 'Kyoto-Manganji No. 2' peppers grown in a greenhouse in Kyoto, Japan from 2014 to 2016.

** significant at $P < 0.01$. Bars = SD.

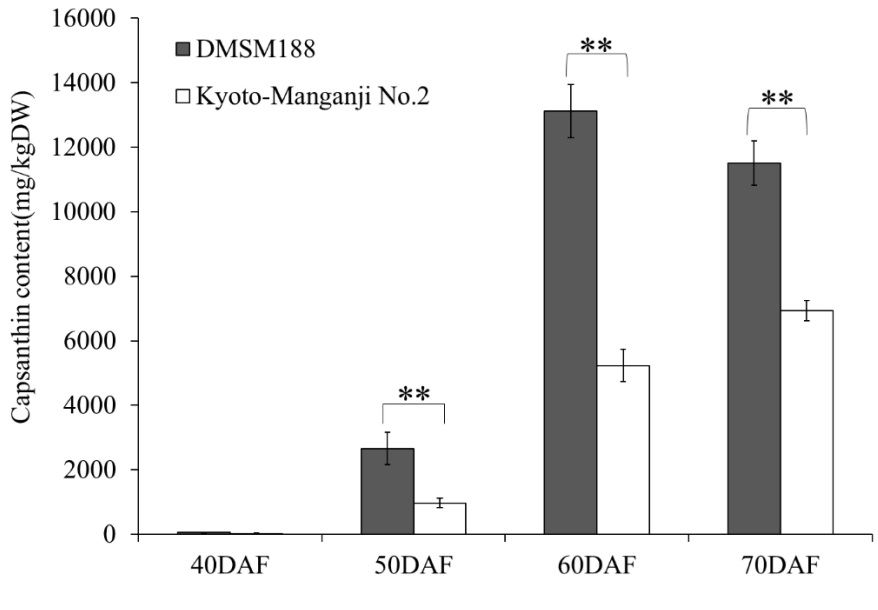


Fig. 3-4. Capsanthin content at different ripening stages of 'DMSM188' and 'Kyoto-Manganji No. 2' peppers grown in a greenhouse in Kyoto, Japan. DAF: days after flowering.

** significant at $P < 0.01$. Bars = SD.

Reduced ascorbic acid content

The ascorbic acid content of 'DMSM188' was higher than that of 'Kyoto-Manganji No. 2' in every year of the experiment (Fig. 3-5). In 'DMSM188', the average ascorbic acid content over three years was 182 mg/100 g FW, 20% higher than the three-year average ascorbic acid content of 'Kyoto-Manganji No. 2' (148 mg/100 g FW).

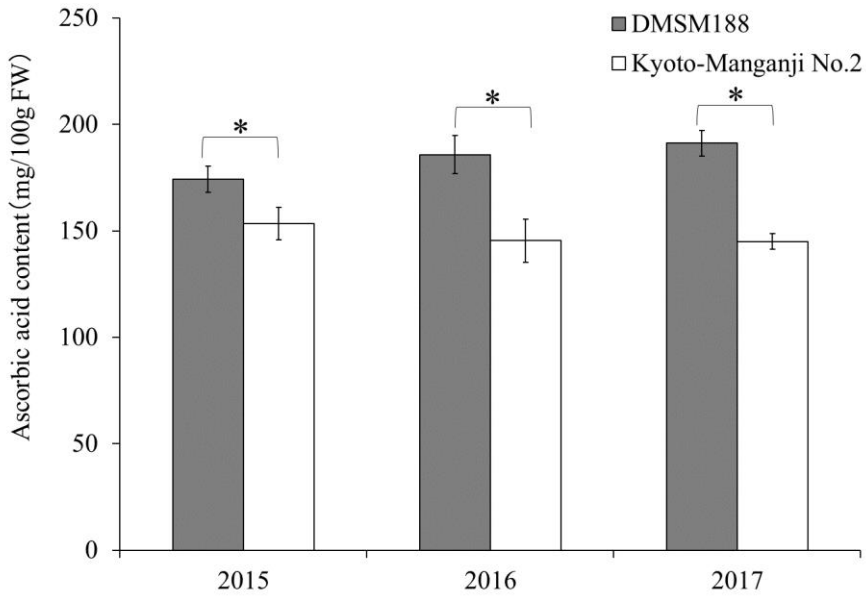


Fig. 3-5. Ascorbic acid content of 'DMSM188' and 'Kyoto-Manganji No. 2' peppers grown in a greenhouse in Kyoto, Japan from 2015 to 2017.

* significant at $P < 0.05$. Bars = SD.

Free sugar content

In 2015 and 2016, the total free sugar content did not differ significantly between ‘DMSM188’ and ‘Kyoto-Manganji No. 2’. However, in 2017, ‘DMSM188’ had a significantly higher total free sugar content than did ‘Kyoto-Manganji No. 2’ ($P < 0.05$; Fig 3-6). In a two-factor factorial ANOVA test covering all three years of the experiment, significant differences were detected among both cultivar and experiment year.

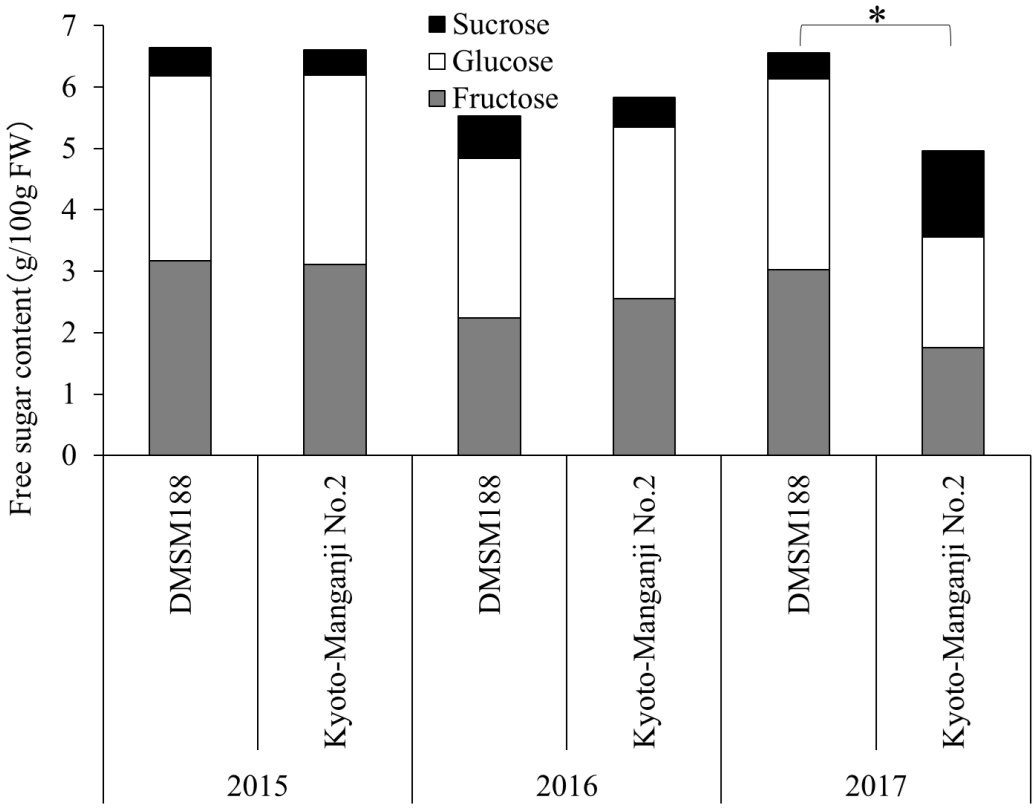


Fig. 3-6. Free sugar content of ‘DMSM188’ and ‘Kyoto-Manganji No. 2’ peppers grown in a greenhouse in Kyoto, Japan from 2015 to 2017.

* significant at $P < 0.05$.

Free amino acid content

In the quantitative HPLC analysis, total amounts of aspartic acid (Asp), glutamic acid (Glu), serine (Ser), glycine (Gly), threonine (Thr), and alanine (Ala) were compared; these amino acids were selected because they are related to sweetness and flavor. 'DMSM188' had higher levels of all six amino acids than did 'Kyoto-Manganji No. 2' in both years of the experiment ($P < 0.05$, 0.01; Fig. 3-7). In 'DMSM188', the average combined value of all six amino acids over three years was 268 mg/100 g FW, 30% higher than the three-year average combined amino acid content of 'Kyoto-Manganji No. 2' (199 mg/100 g FW).

As mentioned above, 'DMSM188' contained higher levels of the functional ingredients capsanthin and ascorbic acid than did 'Kyoto-Manganji No. 2'. 'DMSM188' also contained higher levels of free sugars and amino acids than did 'Kyoto-Manganji No. 2'. These components influence taste, indicating that 'DMSM188' has different taste characteristics than 'Kyoto-Manganji No. 2'.

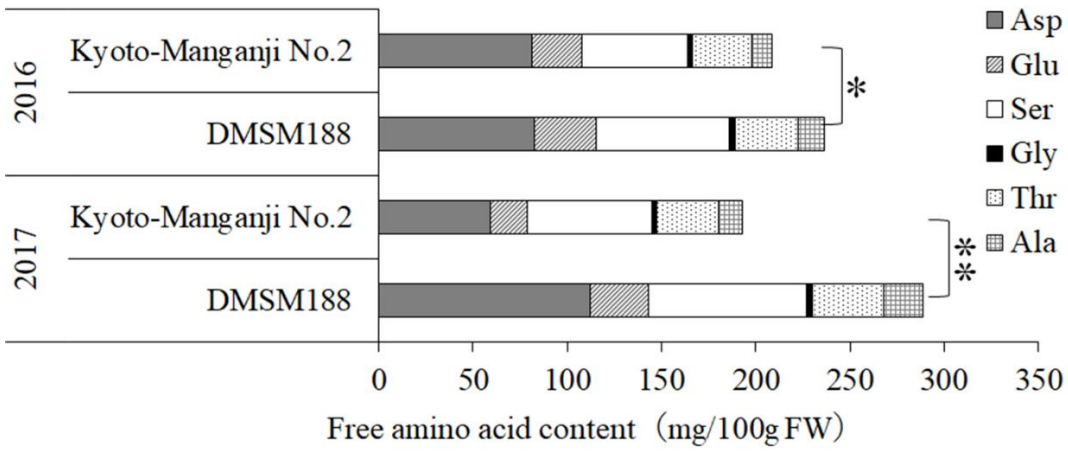


Fig. 3-7. Free amino acid content of 'DMSM188' and 'Kyoto-Manganji No. 2' peppers grown in a greenhouse in Kyoto, Japan in 2016 and 2017.

Asp: aspartic acid; Glu: glutamic acid; Ser: serine; Gly: glycine; Thr: threonine; Ala: alanine

** significant at $P < 0.01$; * significant at $P < 0.05$.

Chapter 4. Relationship of cuticle development with water loss and texture of pepper fruit

Introduction

Pepper is an important horticultural crop globally. Generally, peppers suffer from high postharvest water loss at every stage of ripening because the fruit is hollow. This water loss reduces the economic value of the fruit and high postharvest water loss is an important defect in peppers for consumption as a vegetable.

The cuticular membrane covering the fruit surface is thought to play a role in fruit water loss (Saladié et al. 2007). In several studies, associations between the amounts of cuticle, cuticular wax, and cutin, and postharvest water loss have been investigated. But the role of the cuticular membrane in postharvest water loss remains unclear.

Several studies of the relationships between the physiological properties of pepper fruit and postharvest water loss have been conducted (Maalekuu et al. 2005; Smith et al. 2006) but the relationships remain unclear.

Fruit texture is one of the most important components of fruit quality for consumers. There are several studies on the influence of storage and blanching on fruit texture in bell pepper (Hernández-Carrión et al. 2014; Papageorge et al. 2003), but few studies regarding breeding to improve fruit texture.

The objective of this study was to clarify the effects of cuticle development and anatomical traits on postharvest water loss and texture of pepper fruit.

Materials and methods

Plant materials

The plant materials included 6 commercial cultivars, 21 genetic resources (Plant Genetic and Breeding Laboratory, Shinshu University; Matsushima et al. 2009), and 4 breeding lines (Biotechnology Research Department, Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center) (Table 4-1). Thirty-one cultivars were selected

for character of fruit according to future breeding, elongated shape, and red color in mature fruit, which were grown in the greenhouse of the Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Center during 2017 and 2020. Seeds were sown in vermiculite, and individual seedlings were transplanted into 10 L pots filled with nursery soil (Takii & Co. Ltd., Kyoto, Japan). Seedlings of each cultivar were grown in the greenhouse under a controlled environment of a minimum temperature of 16°C with ambient sunlight and watered either once or twice daily. Mature green fruits of almost maximum size without visible defects were harvested and quickly transported in plastic bags to the laboratory.

Postharvest water loss

Ten mature green fruits of each cultivar were harvested except for S3194, S3197, and S3226; these cultivars did not fruit enough and five fruits were harvested and stored in an incubator at 22°C and 56% relative humidity. Fruit weight was measured just before storage and 3 days after storage. The methods of Leide et al. (2007) for surface area analysis were modified. The pepper fruits were assumed to be cone-shaped. Two photographs of the conical surface of the fruit were obtained and the fruit surface area was calculated using the following formula:

fruit surface area (cm^2) = $2 \times \pi \times X$ (cm^2) / 2

where X represents the average area of the two images calculated using Image J software. To validate this formula, we scanned some fruit with a 3D scanner (FARO Edge Scan Arm ES 9ft, FARO technologies Inc., Lake Mary, USA) and calculated the surface area using Magics software (Materialise, Frankfurt, Germany).

Fruit fresh weight, water content, and pericarp thickness

The initial water content of freshly harvested fruits and dry weight of fruits, the same fruits using measurement of postharvest water loss, were determined by drying in an oven for 24 h at 105°C. Five mature green fruits from every cultivar were cut across the center of the fruit and measured the thickness of the pericarp at five equal intervals across the cut sections.

Microscope assay for cuticular characterization

The sections of the pericarp were separated at approximately 60 μm thickness near the tip of the six pepper fruits using a razor blade and micro slicer (DTK-3000, Dosaka-em Co. Ltd., Kyoto, Japan). In a preliminary study, the cuticular membrane of the fruit tip was the most developed among the various regions of the pepper fruit and the same trend was seen among varieties (data not shown). Ten sections of each cultivars were stained with Sudan IV for 2 min and then rinsed them with distilled water. The sections were mounted on slides in distilled water with a cover slip and then imaged and measured them using a microscope at five intervals in each section (VHX-2000, Keyence Co. Ltd., Osaka, Japan). As shown in Figure 4-1, the aspects of the cuticle that we examined are as follows:
a: the thickness of the cuticular membrane, outside the epidermal cells;
b: the thickness of the cuticular membrane.

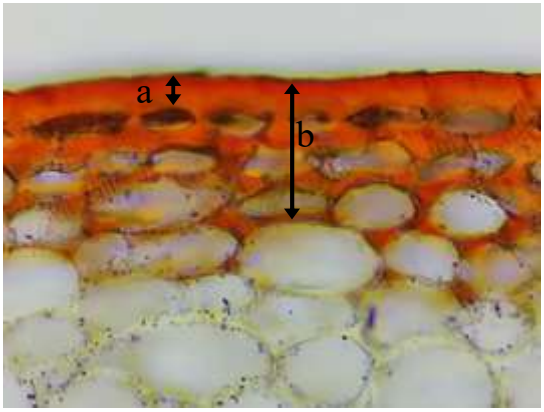


Fig. 4-1. Thickness of the cuticular membrane (a) and (b) of pepper fruit.

The sections of pericarp of approximately 5 mm thickness were separated near the tip of the fruit using a razor blade. The sections were transferred to 2% glutaraldehyde fixative (2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4), which was then replaced with 50% ethanol. Sections were then transferred using a gradient of t-butyl alcohol followed by 50% ethanol, and then replaced with t-butyl alcohol. The sections were dried using freeze dehydration equipment (JFD-310; JEOL Co. Ltd., Tokyo, Japan). The prepared samples were observed using a scanning electron microscope (SEM) (JSM IT-200; JEOL Co. Ltd.) after coating them with Pt in an ion-sputter coater (JEC-3000FC; JEOL Co. Ltd.).

Approximately 5 mm^3 sections of pericarp were separated at the square pole from the middle region of the pepper fruit using a razor blade. The sections were fixed with 2.5% glutaraldehyde solution (2.5% glutaraldehyde in 1/15 M phosphate buffer, pH 7.2, 3 h), post-fixed with OsO_4 solution (1.0% OsO_4 in 1/15 M phosphate buffer, pH 7.2, 3 h), dehydrated using a graded acetone series, replaced with propylene oxide, and embedded in epoxy resin at 38°C overnight and 60°C for 24 h. Ultrathin sections (60 nm) were created using a Leica UC7 ultramicrotome. These sections were mounted on copper grids and stained with 4% uranyl acetate for 16 min and Reynold's lead citrate for 8 min and observed with a transmission electron microscope (TEM) (JEM-1400 Plus; JEOL Co. Ltd.).

Cuticle isolation

The methods of Parsons et al. (2012) for total cuticular extraction were modified. The representative eight cultivars, which varied in rate of water loss, and cut 10 square sections (10 × 10 mm) from the center of the pepper fruit using a razor blade. The cuticular membrane from the sections were isolated using a mix of 0.1% (v/v) pectinase and 0.1% (v/v) cellulase (i.e., 2500 and 670 units L^{-1} of pectinase and cellulase, respectively) and 1 mM NaN_3 to prevent microbial growth in a sodium citrate buffer (0.2 M, pH 3.8). Digestion took place in an incubator shaker set at 37°C and 100 rpm for 3 weeks, and the enzyme solution was changed each week. The cuticular membrane were then separated from the pericarp, rinsed it in distilled water, and dried it at 50° C for 48 h. Five replications were performed for each cultivar.

Total wax and cutin quantitation

The methods of Chefetz (2003) for quantitative analysis of total wax, cutin, and polysaccharide with cutan were modified. The isolated cuticles were dewaxed with chloroform:methanol (1:1) at 50°C for 5 h. The dewaxed cuticles were rinsed in methanol, dried, and weighed. To remove cutin, the dewaxed cuticles were saponified with 1% potassium hydroxide in methanol at 35°C for 48 h. The non-saponifiable fraction was rinsed in methanol, dried, and weighed. The total wax and cutin contents were calculated

by subtraction of the enzymatically isolated cuticular membrane, the dewaxed cuticles, and the non-saponifiable fractions. According to Tsubaki et al. (2013), the non-saponifiable fraction was regarded as polysaccharide with cutan.

Texture analysis

Eight cultivars that were used in cuticle isolation were used for texture analysis. The puncture force was evaluated using a creep meter (RE2-3305; Yamaden Co. Ltd., Tokyo, Japan) with a 20 N force cell, and a cylindrical flat-end plunger with 1.5 mm diameter, with a cross head speed of 0.5 mm s^{-1}. Data were analyzed using BAS-3305-LE ver. 2.5 software (Yamaden Co. Ltd.). Pepper pieces (1 × 3 cm) that were cut from the center of the fruit with a razor blade, were placed skin-side down. Measurements were repeated on 20 pieces of fruit from each cultivar.

Turgor pressure of the pericarp

The turgor pressure of the pericarp was calculated from the difference between the osmotic pressure and diffusion pressure deficit (DPD) of the fruit pericarp. DPD was analyzed using the Chardakov dye method and modified protocol of Yuda and Okamoto (1967). Two sets of five ranges of sucrose solutions were prepared. The pericarp tissue was placed in each solution of one set and left for 1 h. A bit of methylene blue solution was added to the solution of the other set. A drop of the stained solution was layered on the first set of solutions, the tissue was removed, and the DPD was determined from how the stained drop was diluted in the solution. At the same time, the supernatant of the crushed fruits was stained and the osmotic pressure was determined based on how the stained drop was diluted in the sucrose solution sets.

Statistical analysis

Statistical analyses were conducted using Statcel4 (Yanai 2015), an add-in for Microsoft Excel software. Correlation coefficients were calculated using Spearman's rank test.

Results

Surface area analysis

The surface area values calculated from the photographic images were closely correlated with the values from the 3D scanner data (Fig. 4-2). Therefore, we estimated the fruit surface area using the following revised formula:

fruit surface area (cm^2) = $1.0502(\pi \times X) - 10.607$

where X represents the average area of the two images.

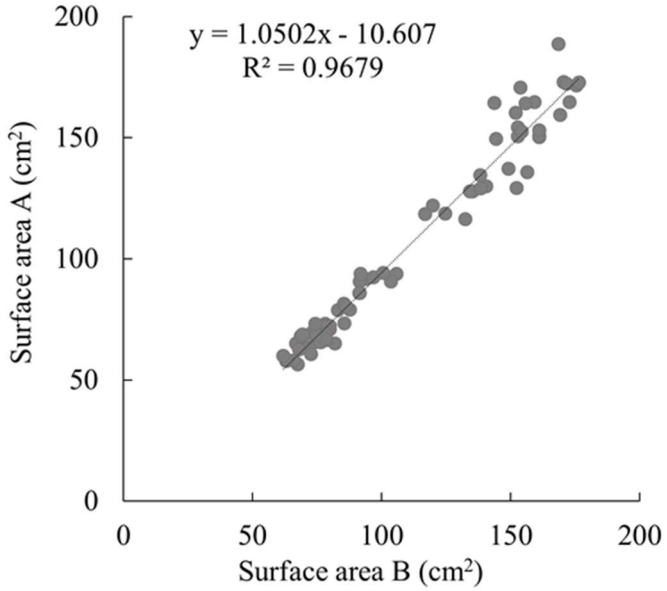


Fig. 4-2. Relationship between the surface area of fruit measured using different methods.

A: Surface area values calculated by 3D scanner data, B: Surface area values calculated from photographic images. $X = 2 \times \pi \times X/2$, where X represents the average area of two images calculated using Image J software.

Postharvest water loss and correlation with physical traits and thickness of the cuticular membrane

The water loss of 31 cultivars ranged from 6.8 to 50.2 mg cm^{-2} per 3 d (Table 4-1). A negative correlation between postharvest water loss and fruit fresh weight, and a weak negative correlation between water loss and pericarp thickness were found (Table 4-1).

Table 4-1. Fruit characterization and cuticular thickness of 31 pepper cultivars

Cultivar[a]	Water Loss (mg cm^{-2} 3 d^{-1})	Fruit fresh weight (g)	Water content (%)	Pericarp thickness (mm)	Cutiular thickness a (μm)	Cutiular thickness b (μm)
Fushimi	14.5 ± 1.5	12.1 ± 0.5	90.5 ± 0.3	1.7 ± 0.0	10.5 ± 0.4	40.2 ± 0.6
Manganji	12.0 ± 0.5	44.5 ± 1.5	92.4 ± 0.1	2.4 ± 0.1	9.8 ± 0.4	20.7 ± 2.9
Takanotsume	40.4 ± 4.8	2.8 ± 0.2	85.1 ± 0.7	1.0 ± 0.0	15.5 ± 0.4	72.5 ± 1.9
Fntonaga	27.4 ± 1.9	11.9 ± 0.9	88.5 ± 0.7	1.7 ± 0.1	12.4 ± 0.4	114.8 ± 2.2
Nikko	23.5 ± 3.2	4.2 ± 0.3	86.4 ± 0.6	1.1 ± 0.1	10.8 ± 0.4	61.0 ± 1.4
Ecuadorian	7.0 ± 1.7	32.3 ± 2.7	90.9 ± 0.3	2.7 ± 0.1	15.1 ± 0.9	29.7 ± 2.6
S3155	7.7 ± 1.1	34.6 ± 2.9	93.4 ± 0.2	3.4 ± 0.1	12.8 ± 0.5	24.2 ± 1.1
S3157	29.4 ± 4.6	3.7 ± 0.4	90.7 ± 0.2	2.0 ± 0.0	7.8 ± 0.3	62.4 ± 2.6
S3159	17.7 ± 2.6	13.2 ± 0.8	89.6 ± 0.5	1.8 ± 0.1	14.2 ± 0.5	54.4 ± 2.4
S3172	35.6 ± 2.4	2.6 ± 0.1	81.8 ± 1.1	1.0 ± 0.0	14.4 ± 0.3	103.2 ± 2.5
S3175	12.7 ± 0.9	2.9 ± 0.2	89.7 ± 0.7	0.9 ± 0.1	16.6 ± 0.6	28.0 ± 3.4
S3187	15.0 ± 2.2	24.1 ± 2.2	92.9 ± 0.1	2.1 ± 0.1	11.9 ± 0.4	65.7 ± 2.4
S3194	12.3 ± 2.4	6.8 ± 0.7	89.4 ± 1.1	2.0 ± 0.1	15.4 ± 0.4	29.5 ± 2.4
S3197	11.1 ± 0.4	10.3 ± 0.5	90.0 ± 1.1	2.0 ± 0.0	14.4 ± 0.6	79.4 ± 2.1
S3202	29.1 ± 3.3	3.3 ± 0.3	89.2 ± 0.4	1.2 ± 0.1	13.1 ± 0.4	56.7 ± 2.9
S3204	31.1 ± 4.2	2.1 ± 0.2	89.6 ± 0.3	1.9 ± 0.0	16.4 ± 0.7	68.5 ± 3.3
S3223	19.8 ± 4.5	4.0 ± 0.3	89.9 ± 0.3	1.4 ± 0.0	10.3 ± 0.7	64.3 ± 2.7
S3225	20.9 ± 2.0	2.7 ± 0.1	87.5 ± 1.1	0.9 ± 0.0	13.7 ± 0.4	78.5 ± 1.4
S3226	15.6 ± 1.7	3.6 ± 0.4	87.9 ± 1.0	1.2 ± 0.0	17.8 ± 0.6	71.2 ± 2.6
S3227	12.7 ± 0.5	2.4 ± 0.1	89.4 ± 0.3	0.8 ± 0.0	8.8 ± 0.3	70.9 ± 1.2
S3229	32.2 ± 2.3	2.5 ± 0.2	88.0 ± 0.3	1.0 ± 0.0	11.0 ± 0.4	55.2 ± 1.7
S3313	33.8 ± 2.3	1.9 ± 0.1	82.8 ± 0.7	0.9 ± 0.0	18.1 ± 0.3	86.3 ± 3.2
S3340	50.2 ± 4.1	2.6 ± 0.2	82.5 ± 1.3	1.0 ± 0.1	16.0 ± 0.6	105.1 ± 2.3
S3344	36.5 ± 4.4	4.3 ± 0.3	81.0 ± 0.7	2.4 ± 0.1	21.0 ± 0.4	116.3 ± 2.2
S3583	7.4 ± 0.7	13.9 ± 1.2	93.7 ± 0.1	2.4 ± 0.2	10.9 ± 0.4	25.2 ± 0.8
S3586	18.0 ± 1.5	8.0 ± 0.6	85.4 ± 0.7	2.6 ± 0.1	13.3 ± 0.4	41.7 ± 1.3
S3592	34.6 ± 4.4	3.2 ± 0.1	87.5 ± 0.6	1.2 ± 0.1	11.0 ± 0.5	110.9 ± 1.4
FE20	6.8 ± 0.7	13.1 ± 1.1	91.7 ± 0.2	1.3 ± 0.1	11.5 ± 0.4	28.6 ± 0.7
FE26	8.8 ± 1.4	14.0 ± 0.8	92.7 ± 0.2	2.2 ± 0.0	10.5 ± 0.4	26.3 ± 0.9
FE89	15.3 ± 2.1	14.9 ± 1.5	91.0 ± 0.3	2.3 ± 0.1	12.7 ± 0.4	23.5 ± 1.6
FE115	13.9 ± 1.2	16.4 ± 1.7	90.7 ± 0.4	1.9 ± 0.1	11.1 ± 0.3	27.9 ± 1.1
Correlation between water loss						
r	-	-0.69**	-0.8**	-0.47**	0.30^{NS}	0.72**

Note: [a]Abbreviations with "S" were genetic resources of the Plant Genetic and Breeding Laboratory, Shinshu University (Matsushima et al. 2009) and "FE" were breeding lines of the Biotechnology Research Department, Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Research Center.

Each value represents the average ± standard errors. ** and NS indicate significance at $P<0.01$ and non-significance using Spearman's rank test.

The thickness of the cuticular membranes varied from 7.8 to 21.0 μm (a, outside the epidermal cells) and 20.7 to 116.3 μm (b) (Table 4-1). There was no correlation between water loss and the thickness of the cuticular membrane (a) (Fig. 4-1) but the thickness of the cuticular membrane along with the epidermal cells (b) was strongly correlated with water loss (Table 4-1).

Micromorphology of the pericarp epidermis

Under the microscope, cuticular membrane that was stained by Sudan IV wedged between epidermal cells were observed (Fig. 4-3A, B). The cultivar Manganji had a thinner cuticular membrane wedged between epidermal cells (Fig. 4-3A) but S3340 had a thicker membrane between the fourth and fifth layers of subepidermal cells (Fig. 4-3B). Under a SEM, development of the cuticular membrane was observed in the same area stained by Sudan IV (Fig. 4-3C). Additionally, under a TEM, an amorphous fibrous structure was found in the cuticular layer vertically wedged between subepidermal cells (Fig. 4-3D, E).

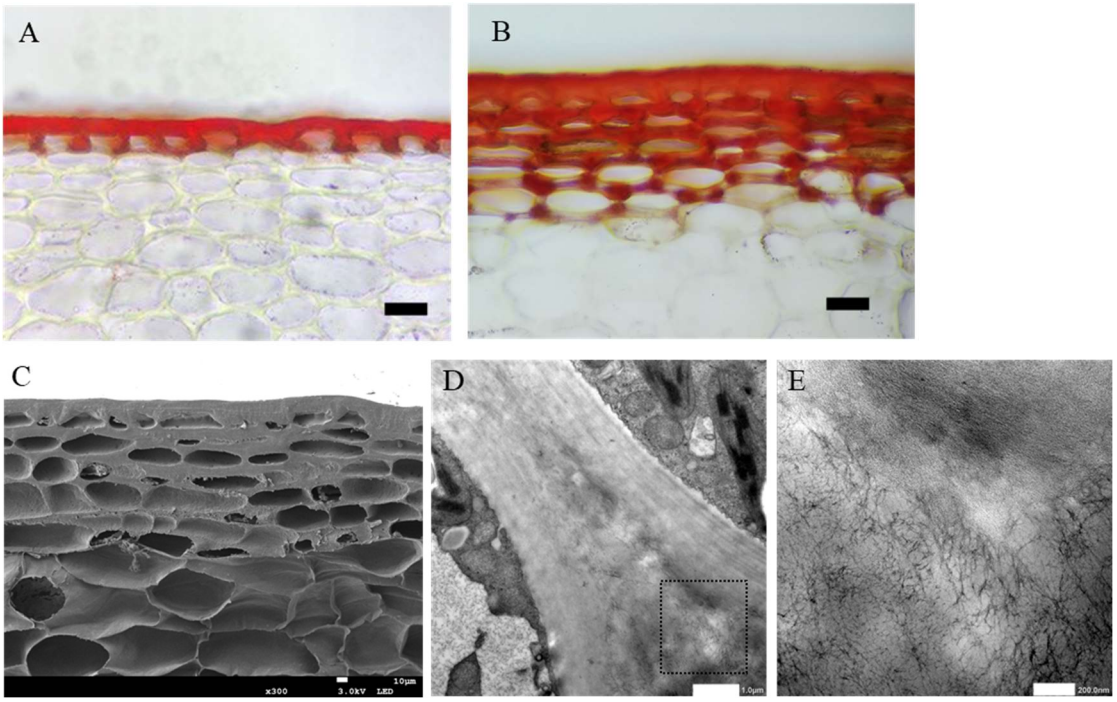


Fig. 4-3. Micromorphology of the cuticular membrane in the pepper fruit cultivars Manganji (A) and S3340 (B, C, D, E). Scale bars indicate 30 μm (A, B), 10 μm (C), 1 μm (D), 200 nm (E). E is an enlarged view inside the square frame of D.

Total wax, cutin, and polysaccharide with cutan content

The cuticular membrane consists of cutin polymer, with waxes that impregnate and cover the cutin, polysaccharides derived from the walls of epidermal cells, and cutan (Domínguez et al. 2011). In addition, in the pepper fruit, the cuticular membrane included the epidermal cells that are buried in cuticle. The weight of the cuticular membrane, and total wax, total cutin, and polysaccharide with cutan content of the eight selected cultivars were quantified under a range of water loss and cuticular membrane thickness conditions. The weight of the cuticular membrane ranged from 0.87 to 7.40 mg cm^{-2}, and there were differences between cultivars (Table 4-2). There were positive correlations between water loss and cuticular membrane weight, total cutin weight, and polysaccharide with cutan weight (Table 4-2).

Table 4-2. Cuticle, wax, and cutin weight of pepper fruit

	Water Loss	Cuticle weight	Total wax weight	Cutin weight	Polysaccharide with cutan weight
Cultivar[a]	(mg cm^{-2} 3 d^{-1})	(mg cm^{-2})	(μg cm^{-2})	(μg cm^{-2})	(μg cm^{-2})
Fushimi	14.5 ± 1.5	1.73 ± 0.02	51.8 ± 2.4	1369.4 ± 20.9	308.2 ± 13.5
Manganji	12.0 ± 0.5	1.61 ± 0.10	30.8 ± 8.8	1228.2 ± 47.8	347.6 ± 46.9
Takanotsume	40.4 ± 4.8	4.73 ± 0.21	33.8 ± 5.5	3849.4 ± 166.8	843.6 ± 43.4
Futonaga	27.4 ± 1.9	7.40 ± 0.32	217.0 ± 21.5	6142.4 ± 276.3	1037.6 ± 42.8
Nikko	23.5 ± 3.2	4.04 ± 0.25	334.8 ± 171.2	3051.4 ± 94.7	654.8 ± 26.2
S3155	7.7 ± 1.1	0.87 ± 0.02	27.8 ± 1.1	606.8 ± 19.5	234.8 ± 27.2
S3340	50.2 ± 4.1	7.28 ± 0.06	107.4 ± 7.6	5739.2 ± 53.5	1428.4 ± 16.0
S3586	18.0 ± 1.5	1.91 ± 0.02	65.6 ± 18.1	1402.4 ± 20.8	437.0 ± 15.0
Correlation between water loss					
r		0.93**	0.62^{NS}	0.93**	0.95**

Note: [a]See Table 1 for abbreviations. Each value represents the average ± standard errors. ** and NS indicate significance at $P<0.01$ and non-significance using Spearman's rank test.

Texture analysis

The force-displacement curve of the pepper fruit is shown in Figure 4-4. In this study, the force-displacement curve was divided into two phases based on the degree of inclination, according to Yoshikawa et al. (1982). Phase I was defined as the start of analysis to the small change in force with displacement, just before a rapid increase in

force. Phase II was defined as the end of phase I to the maximum value of force, at which the plunger broke through the pericarp (Fig. 4-4). Table 4-3 shows that there was no correlation between the maximum force of phase II with the average force of phase I. However, there was a significant positive correlation between the maximum force and displacement of phase II.

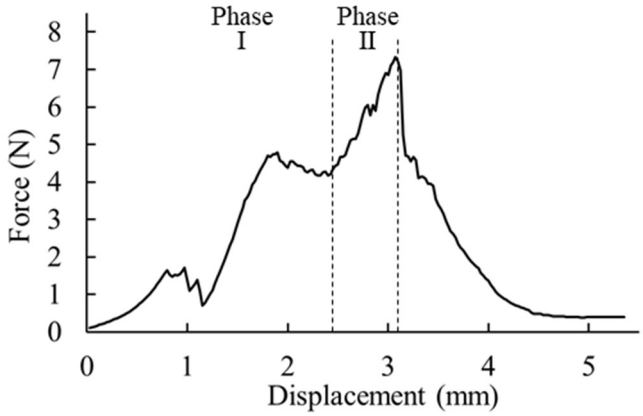


Fig. 4-4. Parameters extracted from the force-distance curves of a texture analysis of pepper fruit. The curve was divided into two phases based on the degree of inclination. Phase I was defined as the start of analysis to the small change in force with displacement, just before the rapid increase in force. Phase II was defined as after phase I to the maximum value of force at which the plunger broke through the pericarp.

Table 4-3. Texture analysis of pepper fruit

Cultivar[a]	Average force of phase I (N)	Maximum force of phase II (N)	Displacement of phase II (mm)
Fushimi	1.97 ± 0.10	4.79 ± 0.48	0.62 ± 0.06
Manganji	2.45 ± 0.09	4.09 ± 0.40	0.24 ± 0.03
Takanotsume	1.49 ± 0.20	7.04 ± 0.90	0.71 ± 0.07
Futonaga	2.28 ± 0.10	7.88 ± 0.72	0.87 ± 0.06
Nikko	1.47 ± 0.14	4.93 ± 0.60	0.54 ± 0.05
S3155	2.85 ± 0.08	4.52 ± 0.70	0.23 ± 0.02
S3340	0.85 ± 0.05	8.14 ± 1.03	1.01 ± 0.04
S3586	1.75 ± 0.14	7.82 ± 0.84	0.92 ± 0.08
Correlation coefficient values			
r		$-0.64^{NS\ b}$	$0.93^{**\ c}$

Note: [a]See Table 1 for abbreviations. Each value represents the average ± standard errors. ** and NS indicate significance at $P<0.01$ and non-significance using Spearman's rank test. [b]Correlation between average force of phase I and maximum force of phase II. [c]Correlation between maximum force of phase II and displacement of phase II.

There was a positive correlation between the average force of phase I and the thickness of the pericarp, but no correlation with turgor pressure of the pericarp (Table 4-4). A positive correlation was found between the maximum force of phase II with the weight of the cuticular membrane, cutin content, which was the main component of the cuticular membrane, and polysaccharide-cutan content (Table 4-4). There was no correlation between the maximum force of phase II with wax content (Table 4-4). In addition, a positive correlation was found between the displacement of phase II with the weight of the cuticular membrane, cutin content, which was the main component of the cuticular membrane, and polysaccharide-cutan content (Table 4-4).

Table 4-4. Correlation coefficient values between the average force of phase I, maximum force of phase II, and displacement of phase II and the weight of cuticle, total wax, cutin, polysaccharide-cutan, thickness of pericarp, and turgor pressure of pericarp

	Cuticle weight	Total wax weight	Cutin weight	Polysaccharide with cutan weight	Pericarp thickness	Turgor pressure of pericarp
Average force of phase I	-	-	-	-	0.83^{*}	-0.67^{NS}
Maximum force of phase II	0.88^{**}	0.67^{NS}	0.88^{**}	0.86^{*}	-0.60^{NS}	0.53^{NS}
Displacement of phase II	0.76^{*}	0.54^{NS}	0.76^{*}	0.76^{*}	-0.52^{NS}	0.34^{NS}

Note: **, * and NS indicate significance at $P<0.01$, $P<0.05$ and non-significance using Spearman's rank test.

Discussion

It is clear from our results that the thickness and weight of the cuticular membrane is correlated with water loss (Tables 4-1, 4-2). Leide et al. (2007) found a positive correlation between cuticle weight and water loss in tomato fruit. Parsons et al. (2012) found a positive correlation between total cutin content and fruit water loss. These results are consistent with our findings (Table 4-2). However, Maalekuu et al. (2005) found no correlation between fruit water loss and whole cuticle weight. Rate of water loss was expressed per unit surface area of the pericarp. Maalekuu et al. (2005) calculated the surface area based on the total pericarp weight and weight of a fixed area of the pericarp. However, this method is not precise because the thickness of the fixed area pericarp is not

constant. Therefore, their results may have differed because the measurement method used was not appropriate.

Postharvest water loss was negatively correlated with fruit fresh weight (Table 4-1). This result may be because lower weight fruits tended to have a thicker cuticle than higher weight fruits in this study. Additionally, a positive correlation was found between the thickness of the cuticular membrane with water loss in similar-sized fruits (data not shown).

The cuticular membrane wedged between subepidermal cells (Fig. 4-3A, C), and an amorphous fibrous structure in the cuticular layer wedged between subepidermal cells were observed (Fig. 4-3D, E). Generally, the structure of the cuticular membrane, as viewed from the outer side, comprises a thin layer of epicuticular wax, lamellate cuticle proper, and cuticle layer with inhomogeneous structure. The cuticle layer has a bi-layered structure with external and internal layers, adjacent to the epidermal cell wall (Bargel et al. 2006; Jeffree 2006; Mérida et al. 1981). The cuticle proper, which contains wax and cutin, is the main barrier of the cuticle membrane (Bargel et al. 2006; Riederer and Schreiber 2001). The lamellate structure of the cuticle proper is presumed to be the path for molecules diffusing across the cuticle and there is no correlation between the thickness of the cuticle proper and its permeability (Baur et al. 1999). On the other hand, the cuticle layer, which contains cutin, wax, and polysaccharides, has an inhomogeneous reticular structure (Jeffree 2006), which adds little to the barrier properties (Baur et al. 1999).

In the present study, cultivars with thick cuticle development in the pericarp exhibited higher water loss (Table 4-1). In addition, the cuticle that wedged between subepidermal cells have amorphous and fibrous structures (Fig. 4-3). According to a report by Domínguez et al. (2011), the observed fibrous structure is polysaccharides derived from epidermal cells. Therefore, these results suggest that development of a cuticle layer with limited barrier properties between the subepidermal cells, forms a path for diffusion and increases water loss.

It is known that pathways of water loss in fruit are the cuticular membrane, the calyx, and the stomata. In eggplant, there is a large amount of water loss from the calyx and correlations have been observed between the calyx area and the rate of water loss (Díaz-

Pérez 1998). However, judging from our preliminary tests (data not shown), the contribution of the calyx to water loss was limited. Moreover, pepper fruit have few stomata (Weryszko-Chmielewska and Michałojć 2011), and no stomata were observed on pepper fruit used in this study. Therefore, most of the water loss from pepper fruit was through the cuticular membrane of the pericarp.

With a few exceptions, it is believed that pepper fruits with thin pericarp have thick cuticles because thick cuticles provide mechanical support to maintain the integrity of fruit organs. Pepper fruits with thick cuticles tend to lose water at a higher rate than that with thin cuticles, but fruits on trees do not shrivel during the growing period.

Even though it is assumed that cuticular thickness and weight are correlated to some extent, the most important factor for water loss across the pericarp may be the depth to which the cuticle extends into the anticlinal wall, permeating through the walls of the additional underlying epidermal cell layers. The contribution of the permeability of cutin, the main component of the cuticle, was much larger than that of wax (Schönherr 1976). Additionally, the solubility parameters of the ingredients comprising the cuticle increased in the order inner side, epicuticular wax, cuticle proper, cuticle layer (Khayet and Fernandez 2012). Although wax from pepper fruit surface functions as a main barrier for water loss, shown by drastic increase of water loss from fruit dewaxed by chloroform (data was not shown), higher permeability of cuticle wedged deeply between subepidermal cells accelerate water loss. Therefore, rate of water loss depends on the thickness of cuticular membrane. This is the first report of a correlation between the visual thickness of the cuticular membrane and water loss in pepper fruit. In addition, cultivars with a thick cuticular membrane containing a lot of cutin and polysaccharide showed greater water loss.

In addition, a positive correlation was found between the average force of phase I, which indicates hardness of the mesocarp (Yoshikawa et al. 1982), and thickness of the pericarp. The turgor pressure of the pericarp was measured because higher turgor pressure has a higher compaction force in pericarp tissues, however, we found no correlation.

A positive correlation was found between the maximum force and displacement of phase II—indicating hardness and toughness of the exocarp, respectively (Yoshikawa et al.

1982)—and weight of the cuticular membrane (Table 4-3). In the many pepper cultivars, subepidermal cells of the exocarp buried in the cuticular membrane were observed. Therefore, fruits with a thick cuticle had a hard, tough texture (Tables 4-3, 4-4). Moreover, 70 to 83% of the cuticular membrane weight was cutin and 14 to 23% was polysaccharides with cutan (Table 4-2). López-Casado et al. (2007) found that the cuticular membrane is a complex of cutin with polysaccharides and this complex possesses both elastic and viscoelastic properties, while stiffness is primarily provided by the polysaccharides; the cutin matrix imparts plasticity. These conclusions are in agreement with our findings that fruit with a high content of cutin and polysaccharides were tough and hard (Tables 4-3, 4-4).

In this study, the puncture force was evaluated from inside to outside of the fruit because at the measurement from outside to inside the value of force is affected by pericarp thickness. When we eat fruits of pepper, teeth bites on the fruit are observed on both sides of the pericarp. In this case, soft flesh is crushed and the hard parts of the pericarp are bit off. Therefore, hardness and toughness valued in this study coincide with the actual texture.

These results lead to the conclusion that the cuticular membrane has a close relationship with water loss and texture. Therefore, quantification of the cuticular membrane is important for breeding of pepper fruit for improvements in water loss and texture. In addition, for breeding of pepper, S3155 is a promising material with less water loss and tender fruit texture. Enzymes and these candidate genes of cuticle biosynthesis are already reported (Yeats and Rose, 2013), but reports of QTLs for development of cuticle in pepper is limited. QTLs for the development of cuticles are necessary to make molecular marker for selection of water loss and texture. In this study, the composition of cutin and polysaccharide were not analyzed. Therefore, further research on the correlation between water loss with each component of cutin and polysaccharide is required.

Chapter 5. General Discussion and Conclusions

To enhance the value of local pepper cultivars, it is necessary to create new means of consuming them. In Kyoto Prefecture, all of the locally grown pepper cultivars are harvested when they are immature. However, peppers undergo drastic changes to their carotenoid content and taste components as they mature. Therefore, making the best possible use of mature fruits is an important tool in growing the local pepper market.

Capsanthin is the most characteristic component of mature red peppers. However, Manganji pepper, a local cultivar from Kyoto, has a low capsanthin content. To determine the mechanisms for the genetic control of capsanthin content in peppers, we used quantitative trait locus (QTL) mapping with SM-DH lines that were derived from crossing a high-content genetic resource line, 'S3586', and the cultivar 'Kyoto-Manganji No. 2' (as described in Chapter 2). To increase the accuracy of QTL detection, we analyzed year-to-year variation according to Broman and Sen (2009). From our analysis, we found *Cst15.1* at 45 days after flowering (DAF) and *Cst13.1* at 90 DAF. We detected *Cst 15.1* at 45 DAF in Experiment 1 and at 90 DAF in Experiment 2; however, *Cst 13.1* was detected only in Experiment 1. Hence, it is possible that *Cst 15.1* has a stabler and larger effect than does *Cst 13.1*.

In both experiments, we selected SM-DH lines with higher capsanthin contents in both immature and mature fruits by using markers adjacent to the two QTLs; this suggests that the QTLs have stable effects on capsanthin content under environmental conditions. Because peppers are usually harvested before they are fully ripe (approximately 90 DAF), it is necessary to accumulate QTLs that increase the capsanthin content at an early stage (e.g., 45 DAF). *Cst15.1* is particularly suited for this purpose. Capsanthin accumulates during pepper development. When we considered the capsanthin content at both 45 and 90 DAF to be a variation of a single phenotype, *Cst15.1* was the only QTL detected. This suggests that *Cst15.1* affects capsanthin content at more than one stage of fruit development.

The existing QTL regions of the pepper genome (http://peppergenome.snu.ac.kr) are too large to narrow down, making it difficult to identify candidate genes. To identify these

genes, we must use high-resolution QTL mapping and transcript quantification. Additionally, because the SM-DH map covers only 75% of the entire genome, it is necessary to check whether other QTLs exist in the remaining 25%, if additional markers are available.

In Chapter 3, SM-DH lines with higher capsanthin content were selected by using markers adjacent to the capsaicinoid synthase gene and fertility restoring gene. We performed combination breeding crosses of the cytoplasmic male sterility 'Kyoto-Manganji No. 2' seed parent, and a selected SM-DH line pollen parent. From the resulting F_1, we selected 'DMSM188' fruit with high capsanthin content, non-pungent flavor, and similar shape to 'Kyoto-Manganji No. 2'.

The pepper cultivar 'DMSM188' was found to have two heterozygous QTLs (as described in Chapter 2) and a capsanthin content of twice or more than that of 'Kyoto-Manganji No. 2'. These findings support the validity of using marker-assisted selection adjacent to QTLs, and the potential for using first-filial hybrid breeding to produce high-capsanthin peppers.

Because peppers are hollow, they suffer from high postharvest water loss at every stage of ripening; this water loss reduces the fruit's economic value. Postharvest water loss is an even more serious problem in mature fruits, which have a lower water content than do unripe fruits. In Chapter 4, we assessed cuticle development and anatomical traits related to postharvest water loss in peppers.

Our results clearly demonstrated that the thickness and weight of the cuticular membrane were positively correlated with water loss, and that postharvest water loss was negatively correlated with fruit fresh weight. This may be because in our study, lower-weight fruits tended to have thicker cuticles than higher-weight fruits. We additionally found that the cuticular membrane thickness was positively correlated with water loss in similar-sized fruits.

As viewed from the outside, the cuticular membrane structure comprises a thin layer of epicuticular wax, the lamellate cuticle proper, and a cuticle layer with an inhomogeneous structure. The cuticle layer has a bi-layered structure with external and internal layers adjacent to the epidermal cell wall. The cuticle proper, containing wax and cutin, is the

cuticle membrane's main barrier, and its lamellate structure is presumed to be the path for molecular diffusion across the cuticle. On the other hand, the cuticle layer, which contains cutin, wax, and polysaccharides, has an inhomogeneous reticular structure, which adds little to its barrier properties.

In the present study, cultivars with thicker cuticle development in the pericarp exhibited higher water loss. In addition, we observed that the cuticular membrane and an amorphous fibrous structure in the cuticular layer were wedged between the subepidermal cells. According to a report by Domínguez et al. (2011), the observed fibrous structure was composed of polysaccharides derived from epidermal cells. Therefore, these results suggest that the development of a cuticle layer with limited barrier properties between the subepidermal cells forms a path for diffusion and increases water loss.

Fruit texture is one of the most important quality components for consumers. Nonetheless, few breeding studies have focused on improving fruit texture. In Chapter 4, we assessed pepper cuticle development and the anatomical traits related to fruit texture.

We found a positive correlation between the average force of phase I, which indicates mesocarp hardness, and pericarp thickness. We also found a positive correlation between the maximum force and displacement of phase II—indicating hardness and toughness of the exocarp, respectively —and the weight of the cuticular membrane. Moreover, we observed many pepper cultivars in which the exocarp's subepidermal cells were buried in the cuticular membrane. Therefore, fruits with thick cuticles had a hard, tough texture. López-Casado et al. (2007) found that the cuticular membrane was a complex of cutin and polysaccharides, and that this complex possessed both elastic and viscoelastic properties. Contrastingly, stiffness was found to be primarily a function of the polysaccharides, whereas the cutin matrix imparted greater plasticity. These conclusions are in agreement with our findings that fruits with high cutin and polysaccharide contents are tough and hard in texture.

Our results lead us to conclude that the properties of the cuticular membrane are closely related with water loss and texture. Therefore, quantifying these properties is important for breeding peppers with reduced water loss and improved texture. Evaluating cuticle thickness is a particularly effective strategy for wide, rapid selection during early-stage

breeding because it can be measured using simple methods. Conversely, cuticle weight is useful in detailed selection, because individual differences in cuticle weight within a cultivar are indicative of water loss. Enzymes and candidate genes for cuticle biosynthesis have already been reported, but there are limited reports of QTLs for cuticle development in peppers; such QTLs are necessary to create molecular markers to select for the qualities of water loss and texture.

In Chapter 3, we discovered that although ‘DMSM188’ had a high capsanthin content, its water loss was only marginally higher than that of ‘Kyoto-Manganji No. 2.’ This result was attributed to ‘S3586’, the pollen parent material used in breeding, which had a thicker cuticle than ‘Kyoto-Manganji No. 2.’ In future breeding efforts, cultivars with high capsanthin content, reduced water loss, and tender fruit texture should be prioritized.

Summary

Pepper is an important horticultural crop worldwide. Among five cultivated species of pepper, *Capsicum annuum* is most widely used and many kinds of pepper local cultivars were cultivated in Japan. To enhance the value of pepper local cultivar, it is necessary to create the new direction of consumption. Every pepper local cultivar in Kyoto prefecture is harvested at unmatured stage. But pepper is changed drastically in carotenoid and taste components content by maturity. Therefore, to make the best possible use of mature fruit is important tool for market growth of pepper local cultivars.

Capsanthin, the main carotenoid of red pepper fruits, is beneficial for human health. In Chapter 2, to breed pepper with high capsanthin content by marker-assisted selection, a linkage map of doubled-haploid (DH) lines derived from a cross of two pure lines of *C. annuum* ('S3586' × 'Kyoto-Manganji No. 2') was constructed. The map, designated as the SM-DH map, consisted of 15 linkage groups and the total map distance was 1403.8 cM. Mapping of quantitative trait loci (QTLs) for capsanthin content detected one QTL on linkage group (LG) 13 at 90 days after flowering (DAF) and one on LG 15 at 45 DAF; they were designated *Cst13.1* and *Cst15.1*, respectively. *Cst13.1* explained 17.0% of phenotypic variance and *Cst15.1* explained 16.1%. The DH lines were grouped according to the genotypes of markers adjacent to *Cst13.1* and *Cst15.1* on both sides. The DH lines with the alleles of both QTLs derived from 'S3586' showed higher capsanthin content at 45 and 90 DAF than the other lines. This is the first identification of QTLs for capsanthin content in any plant species. The data obtained here will be useful in marker-assisted selection for pepper breeding for high capsanthin content.

In Chapter 3, SM-DH lines with higher capsanthin content were selected using markers adjacent to the capsaicinoid synthase gene and fertility restoring gene. Combination breeding cross of cytoplasmic male sterility Kyoto-Manganji No.2, seed parent, and selected SM-DH lines, pollen parent, were done and 'DMSM188' that fruit have high capsanthin content, non-pungent and similar shape with 'Kyoto-Manganji' was selected from F_1. 'DMSM188' contain higher capsanthin and ascorbic acid, these are functional ingredients, than 'Kyoto-Manganji No.2'. Additionally, 'DMSM188' contain higher free

sugar and amino acids which influence taste, therefore ‘DMSM188’ have different feature in taste to ‘Kyoto-Manganji No.2’.

Postharvest water loss in pepper fruit reduces its shelf life. In mature fruit, postharvest water loss is more serious problem because water content of mature fruit is lower than that of unmatured fruit. Fruit texture is one of the most important components of fruit quality for consumers. In Chapter 4, the anatomical traits of pepper fruit related to postharvest water loss and texture were assessed. There was a strong positive relationship between postharvest water loss and the thickness of the cuticular membrane, cuticular weight, total cutin weight, and polysaccharide-cutan weight. An amorphous fibrous structure that forms a path for diffusion and increases water loss was observed in the thick cuticle of the pericarp. In addition, positive correlations between the hardness of the exocarp and the weight of cuticular membrane, cutin content, and polysaccharide-cutan content were found. These results indicate that the thickness of the cuticular membrane wedged between subepidermal cells may influence water loss through the pericarp of pepper fruit and fruit with a high cutin and polysaccharide content have a hard tough texture.

Acknowledgements

I wish to express my sincere gratitude to Dr. Satoshi Terabayashi, Emeritus Professor of Kyoto Prefectural University and Professor of Setsunan University, for his continuous encouragement and guidance, and to Dr. Akihro Itai, Professor of Kyoto Prefectural University, for his invaluable comments during this study.

I extend my gratitude to Dr. Akio Ohyama, National Agriculture and Food Research Organization, for his technical advice and assistance in QTL analysis and Dr. Syuichi Date, Lecturer of Kyoto Prefectural University, for his direction on specimen preparation of TEM and Dr. Noriyuki Furutani, Kyoto Prefectural Agriculture, Forestry and Fisheries Technology Research Center, for technical advises in texture analysis.

I deeply thank to Dr. Ken'ichi Matsushima and Dr. Mineo Minami, Associate Professor and Emeritus Professor of Shinshu University, and Dr. Toru Sugita, Professor of Minami Kyushu University, and Dr. Takahiro Minamiyama, Professor of Kyoto University of Education, for providing the pepper genetic resource and primers used in this study.

I am also thankful to Kyoto Prefectural Biotechnology Research Department staffs for their assistance of my research.

Finally, I am deeply grateful to my parents and my brother for their understanding, support and sincere encouragement for my study.

List of publications

Original articles

1. Konishi A., Furutani N., Minamiyama Y., Ohyama A.
   Detection of quantitative trait loci for capsanthin content in pepper (*Capsicum annuum* L.) at different fruit ripening stages. Breeding Science 69: 30-39 (2019).

2. Konishi A., Ozaki K., Suetome N.
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3. Konishi A., Terabayashi S., Itai A.
   Relationship of Cuticle Development with Water Loss and Texture of Pepper Fruit. Canadian Journal of Plant Science 102: 103-111(2022).

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