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Research Paper

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

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Capsanthin, the main carotenoid of red pepper fruits, is beneficial for human health. To breed pepper (*Capsicum annuum* L.) with high capsanthin content by marker-assisted selection, we constructed a linkage map of doubled-haploid (DH) lines derived from a cross of two pure lines of *C. annuum* ('S3586' × 'Kyoto-Manganji No. 2'). 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%. We grouped DH lines 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.

Key Words: pepper (*Capsicum annuum* L.), capsanthin, QTL, linkage map.

Introduction

Pepper is an important horticultural crop worldwide. Among five cultivated species of pepper, *Capsicum annuum* ($2n = 24$ ($2x$)) is most widely used as a vegetable, a spice, and a food colorant. 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

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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 DH population 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 this study.

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°C. 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°C 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°C 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 5C18-AR II reverse-phase column (Nacalai Tesque, Kyoto, Japan) protected by a guard cartridge (Nacalai Tesque). The oven was operated at 40°C. 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 ith line in the jth replicate, denoted as y_{ij}, is expressed as:

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

where μ is the intercept, g_i is the effect of the ith 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 [$m = 98$ (45 DAF) 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:

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

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

Heritability was estimated as:

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

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, 2004b, 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 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.

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

Table 1. SSR markers newly mapped in this study

Marker name	Forward primer (5′-3′)	Reverse primer (5′-3′)	Motif	Linkage group	Expected product size (bp)[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'.

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 (Experiments 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' at both ripening stages (**Figs. 1**, **2**). This result agreed with that of Konishi and Matsushima (2011).

The capsanthin content in the SM-DH population evaluated in experiments 1 (2013) and 2 (2016) showed a normal distribution (**Fig. 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. 3**). Heritability of capsanthin content was quite low at 45 DAF and it was 0.155 at 90 DAF (**Table 2**).

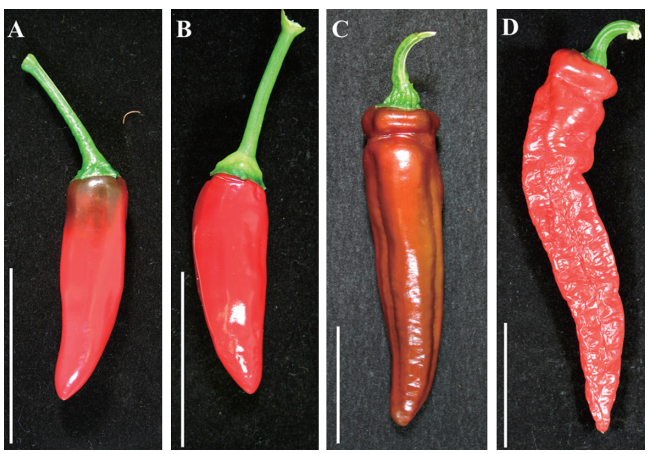

Fig. 1. Fruits of parental lines at two ripening stages. (A) 'S3586', 45 DAF (B) 'S3586', 90 DAF (C) 'Kyoto-Manganji No. 2', 45 DAF (D) 'Kyoto-Manganji No. 2', 90 DAF. Bars indicate 5 cm.

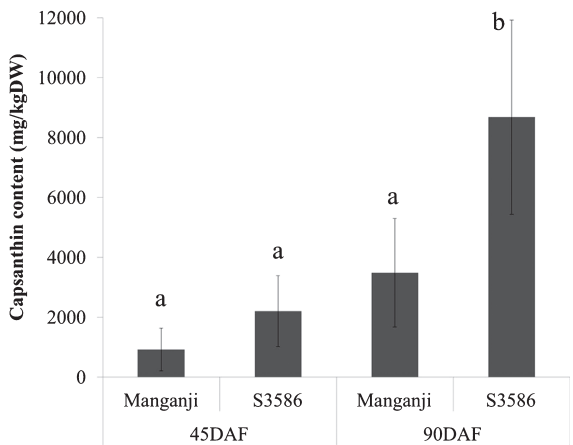

Fig. 2. Capsanthin content of parental lines at two ripening stages. Values are means of 7–9 measurements in two experiments. Error bars represent the standard deviation. Means sharing the same letter are not significantly different according to the Tukey–Kramer multiple-comparison test.

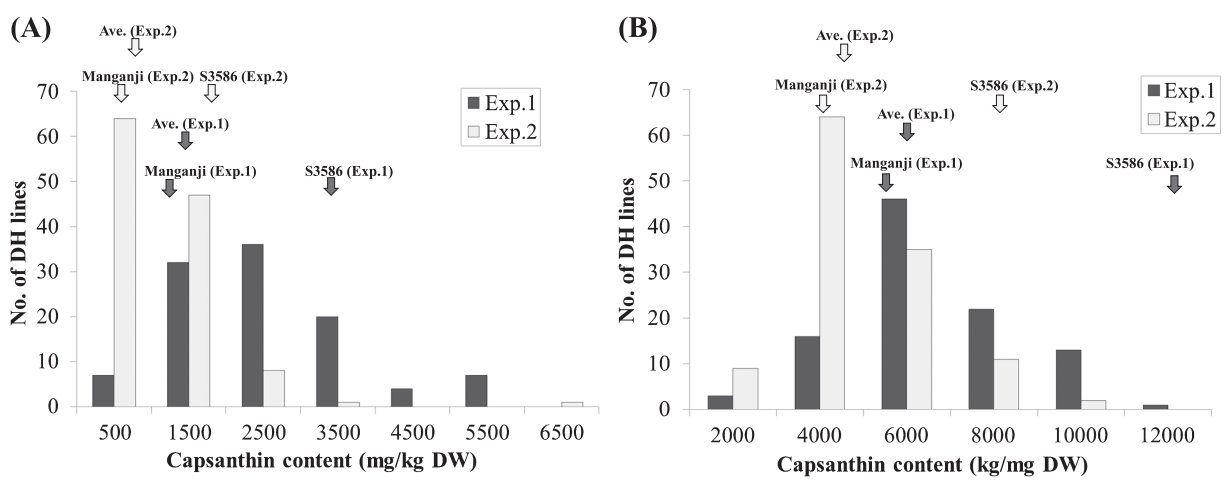

Fig. 3. Frequency distribution of capsanthin content in SM-DH lines. (A) 45 DAF. (B) 90 DAF. Arrowheads indicate the mean values for the parents and average of SM-DH.

Table 2. Heritability of capsanthin content

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. 4**). The average distance between markers was about 9 cM. In this study, 8 new SSR markers were mapped (**Table 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. 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.

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. 6**, **Table 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 3**). An insignificant LOD score peak at 90 DAF was observed close to *Cst15.1* (**Fig. 6**).

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

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. 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 633 MJ/m^2 in experiments 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 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 3**, **4**). Capsanthin is accumulated during the development of pepper fruit (**Fig. 2**), and *Cst15.1* was the only QTL detected when capsanthin content at both 45 DAF and 90 DAF was considered as a

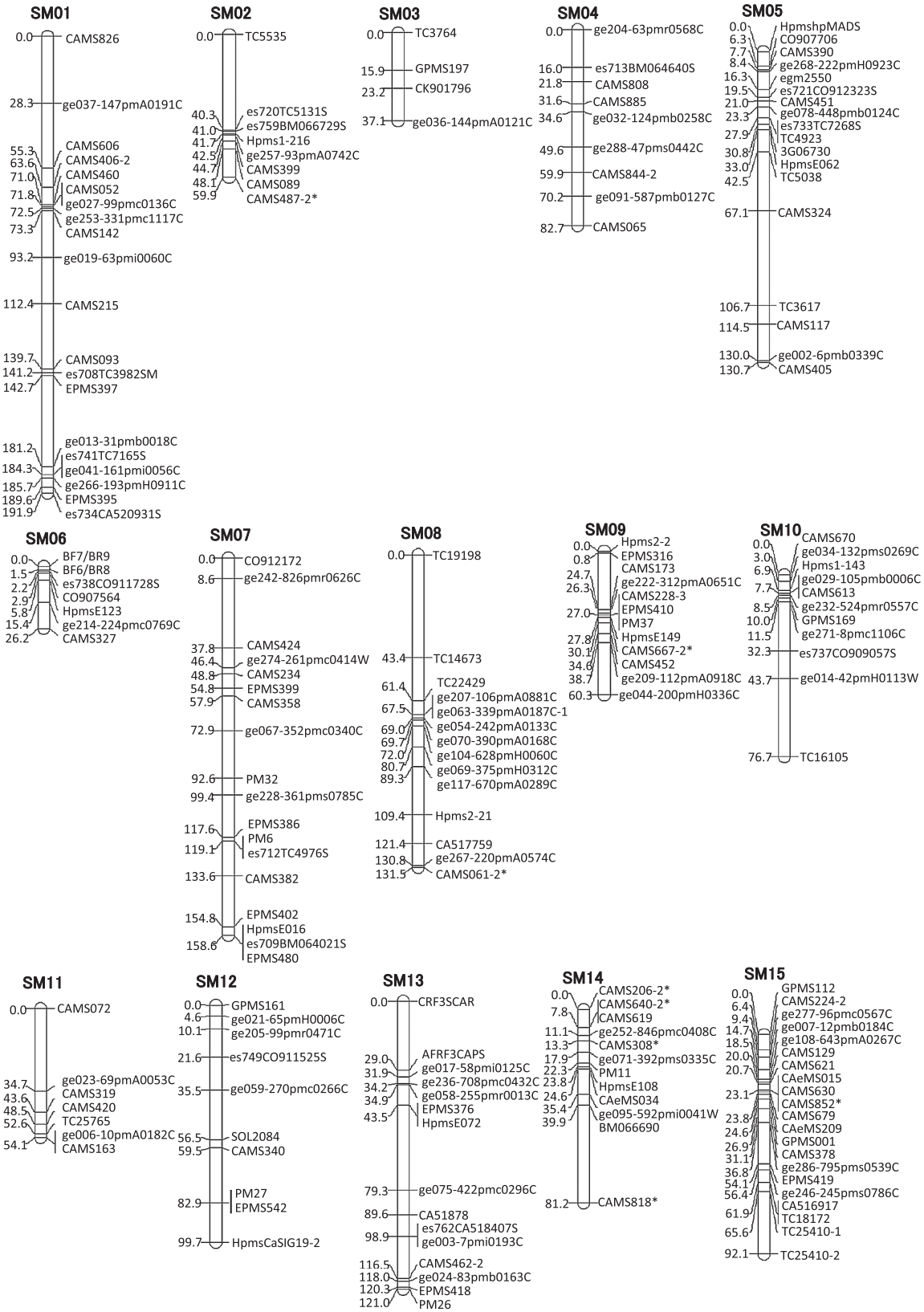

Fig. 4. Linkage map of the SM-DH population ('S3586' × 'Kyoto-Manganji No. 2'). SSR markers "PM", "ge" and "es" were reported by Sugita *et al.* (2006, 2013), "CAMS" and "CAeMS" by Minamiyama *et al.* (2006) and Mimura *et al.* (2010, 2012), "Hpms" by Lee *et al.* (2004a), "HpmsE" by Yi *et al.* (2006), 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).

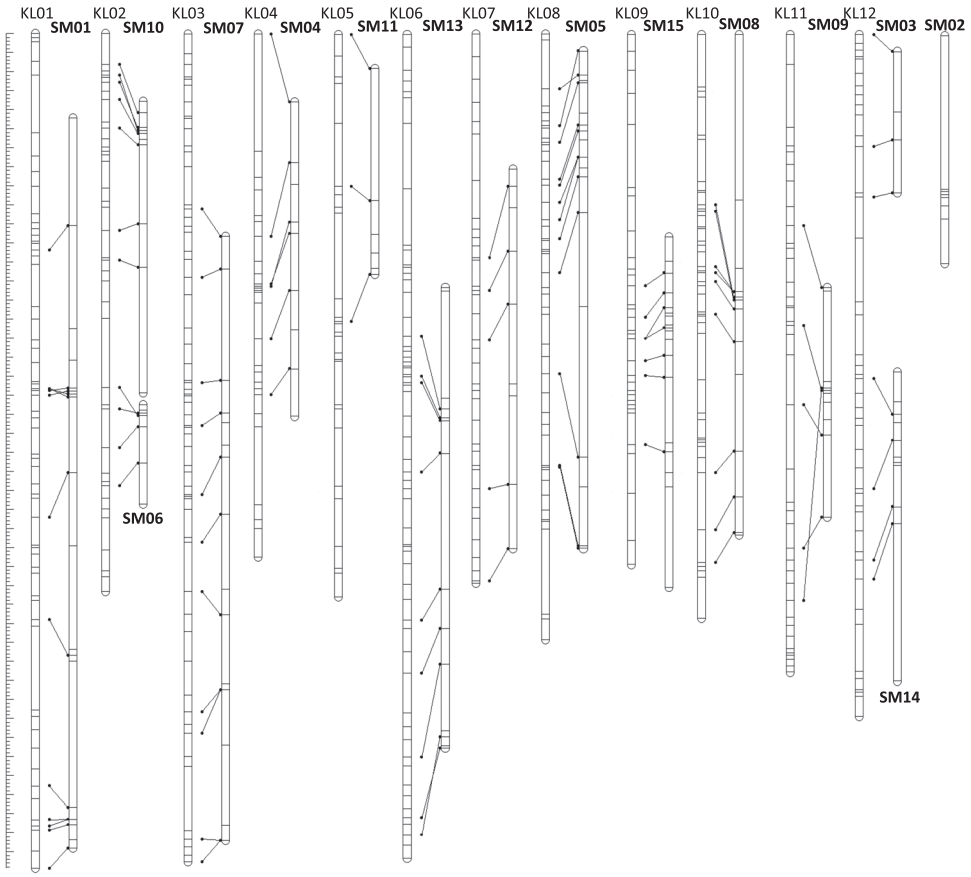

Fig. 5. Comparison between KL-DH and SM-DH maps. KL01–KL12 are 12 linkage groups of the KL-DH map corresponding to the 12 pepper chromosomes (Sugita *et al.* 2013). SM01–SM15 are linkage groups of the SM-DH map constructed in this study. Identical markers on both maps are connected by lines.

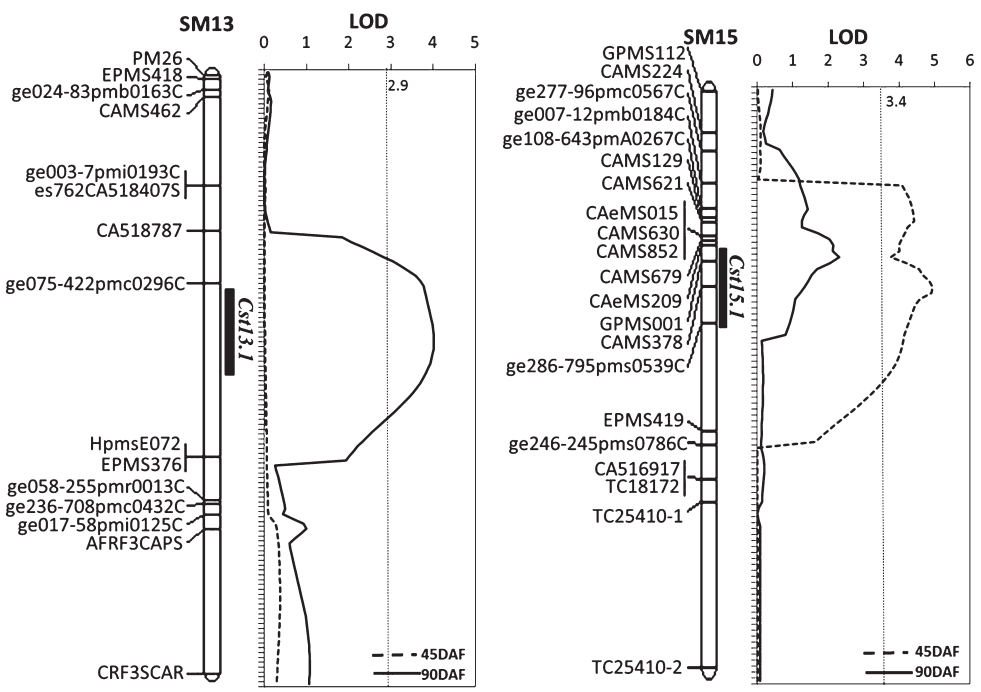

Fig. 6. Positions of two QTLs for capsanthin content on the SM-DH linkage map. Positions of QTLs with 1-LOD support intervals are shown by black boxes. Vertical dotted lines indicate the LOD thresholds.

Table 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 $P < 0.05$.

Table 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.

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 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**), 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.

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