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Identification of Large Deletion of *Ccs* Responsible for Non-Red Fruit Color in Pepper (*Capsicum annuum*) and Development of DNA Marker to Distinguish the Deletion

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Abstract

Chili pepper (*Capsicum* spp.) fruit color is an important agronomical trait. It has been known that a large deletion in the 5' upstream region of the *Ccs* gene generates non-red fruit color in pepper, but the accurate size and position of the deletion and whether all the non-red cultivars had the same large deletion or not were unclarified. In this study, to identify the *Ccs* upstream large deletion, we carried out diagnostic PCR using six forward primers at 300 - 900 bp intervals in the 5' untranslated region of *Ccs* with a fixed reverse primer for a yellow fruit pepper "Sonia Gold". Then it was revealed that 4430 bp from −3234 bp position in upstream region to 1196 bp position in exon was deleted in *Ccs* of "Sonia Gold". The allele having this deletion was named *ccs-del*. Probably this allele is substantially the same as *ccs-p1* having 4879 bp deletion reported previously. Based on the sequence determined, we developed a PCR marker to distinguish *ccs-del*. Genotyping of 16 cultivars of *C. annuum* showed that 14 had *ccs-del* and the remaining two had another mutant allele *ccs-3*. This result indicates that *ccs-del* is the most common allele and widely shared in non-red fruit cultivars in *C. annuum*. Genotyping of 16 cultivars of *C. chinense* clarified that one cultivar each possessed *ccs-del* and *ccs-3*. These results indicate that major alleles responsible for non-red fruit color in *C. annuum* were shared across species throughout interspecific introgression.

Keywords

Non-Red Fruit Pepper, *Ccs*, Identification of Large Deletion, *ccs-3*, Development of DNA Marker

1. Introduction

Chili pepper (*Capsicum* spp.) is a major crop cultivated all over the world used as vegetable and spice. The various and vibrant fruit color of pepper such as red, orange and yellow is an important agronomical trait that arises consumer willingness to buy and makes our tables colorful. Furthermore, some pigments have positive effect to our health such as providing antioxidant activity and acting as dietary precursor of vitamin A [1] [2].

The fruit color of pepper is determined by types and amounts of carotenoids. Carotenoids are natural pigment classified into tetraterpene, existing in almost all organisms living in an environment under sunlight and oxygen conditions. Carotenoids are synthesized in the biosynthesis pathway that starts from isopentenyl pyrophosphate (IPP) and undergoes via various enzymatic reactions [3] [4] [5] [6] [7]. The final products of the pathway are capsanthin and capsorubin that are red pigments making color of pepper mature fruit red [7] [8] [9]. Previous studies reported that non-red pepper fruit color such as orange and yellow are generated by mutations in *Psy* or *Ccs* genes involved in the carotenoid synthesis pathway. The *Psy* gene encodes phytoene synthase that catalyzes geranylgeranyl pyrophosphate into phytoene [7]. The *Ccs* gene encodes capsanthin-capsorubin synthase that acts in the final step of the pathway, catalyzing antheraxanthin and violaxanthin into capsanthin and capsorubin, respectively [7]. As for the major cultivated pepper species *C. annuum* L., mutation in the *Ccs* gene mainly caused non-red fruit color. The *Ccs* gene is 1497 bp in length of coding region consisting of single exon [10]. Several studies reported that a large deletion located in the upstream region of the *Ccs* gene was responsible for the non-red fruit color [11] [12] [13]. Popovsky and Paran (2000) performed PCR to amplify entire region of the *Ccs* gene and reported that no amplification was detected in yellow fruit cultivar [12]. Lang *et al.* (2004) carried out Southern hybridization for the *Ccs* gene using red and orange fruit cultivars and indicated conservation of downstream of the coding region and lack of upstream of the coding and 5' flanking regions of the *Ccs* gene in orange fruit cultivar [13]. These results suggested that at least 1 kb large deletion involving both the promoter and upstream of coding region has occurred in the *Ccs* gene of non-red fruit color pepper. Although it was roughly understood that the deletion was more than 1 kb and located on the upstream region of the *Ccs* gene, the accurate length and position of the deletion, namely, how long and from where to where at single nucleotide level, have not been clarified because of its largeness. Furthermore, it has been unknown whether all non-red fruit color in the different cultivars is caused by the identical deletion. In fact, two *Ccs* alleles, *ccs-p1* and *ccs-p2*, having different size large deletion in promoter region have been reported in *Capsicum* [14]. Regarding the *Psy* gene, several mutant alleles causing non-red fruit color have been found in the second major pepper species *C. chinense* Jacq [14] [15] [16].

Along with the progress of genomics, the entire genomic sequence of *Capsicum annuum* L. has been published [17] [18]. Genomics has innovatively pro-

gressed investigation of crop important genes, therefore genes controlling useful biological characters have been identified in many crops, for example, major domestication gene *tb1* in maize, semidwarf gene *sd1* in rice and spike morphology determinative gene *Q* in wheat [19] [20] [21]. Also as for pepper, QTLs responsible for fruit size were identified using the entire genome sequence [22]. The information of entire genome sequence enables us to approach the detail of the large deletion. In this study, we tried to identify the accurate size and position of the large deletion of the *Ccs* gene in a non-red fruit pepper and develop a marker to distinguish the deletion. Then using the developed marker, the *Ccs* gene of non-red pepper genetic resources were genotyped and the identity of the cause of non-red fruit color in pepper was discussed.

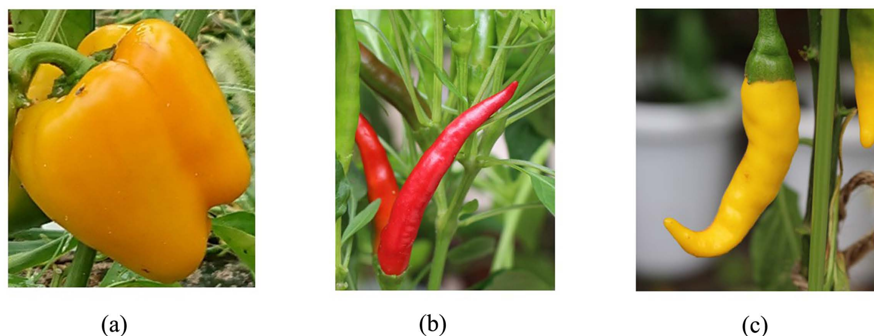
2. Materials and Methods

2.1. Plant Materials

A commercial cultivar *C. annuum* cv. "Sonia Gold" was used as non-red fruit color pepper cultivar. "Sonia Gold" is a bell (blocky) type sweet pepper with yellow fruit color that is commonly cultivated as vegetable (**Figure 1(a)**). As a comparison, a corn type red fruit hot pepper *C. annuum* cv. "Takanotsume" that is a traditional cultivar used as spice in Japan was used (**Figure 1(b)**). As for genotyping, each of the 16 cultivars of *C. annuum* and *C. chinense* were used (**Table 1**). The total DNA was extracted from leaves of each of the accessions with CTAB method [23].

2.2. Diagnostic PCR

Based on the genome sequence of *C. annuum*, six forward primers (Ccs5up_F-762, F-1671, F-2393, F-2745, F-3080, F-3784) were designed at 300 - 900 bp intervals in the 5' untranslated region of the *Ccs* gene. The numbers following F- in the primer names indicates the upstream position when the first nucleotide of the exon is defined as 1. Using the six forward primers with a fixed reverse primer Ccs_R+1494, diagnostic PCR was carried out using Tks Gflex DNA polymerase (Takara Bio) in 20 μL reaction volume with 40 ng genomic DNA as template. The condition of PCR amplification was 1 cycle at 94˚C for 1 min, 30 cycles at



(a) (b) (c)

Figure 1. Mature fruit of (a) "Sonia Gold", (b) "Takanotsume" and (c) "Kiiro Togarashi".

Table 1. Non-red fruit color pepper used for genotyping.

No.	Cultivar name	Source	Mature fruit color
C. annuum			
1	Sweet Palermo Golden	Market	Yellow
2	Mild na Aji no Paprika Yellow	Market	Yellow
3	California Wonder Golden 2019	Market	Yellow
4	Sonia Gold	Market	Yellow
5	Cayenne Pepper Golden	Market	Yellow
6	Señorita Golden	Market	Yellow
7	Señorita Orange	Market	Orange
8	14C23-1	Our laboratory[a]	Yellow
9	14C23-2	Our laboratory	Yellow
10	msGTY-1	TOKITA Seed Co.	Orange
11	Wonder Bell (Yellow)	Market	Yellow
12	California Wonder Golden	Market	Yellow
13	California Wonder Orange	Market	Orange
14	Color Piment Yellow	Market	Yellow
15	Color Piment Orange	Market	Orange
16	Kiiro Togarashi	Market	Yellow
C. chinense			
1	Habanero Orange	Market	Orange
2	Habanero Swarp Orange	Market	Orange
3	Grif 9325	USDA	Orange
4	PI 281444	USDA	Orange
5	PI 438614	USDA	Orange
6	PI 438645	USDA	Orange
7	Habanero Lemon	Market	Yellow
8	Yellow Buht Jolokia	Market	Yellow
9	Scotch Bonnet Yellow	Market	Yellow
10	PI 224448	USDA	Yellow
11	PI 315019	USDA	Yellow
12	PI 390611	USDA	Yellow
13	PI 485593	USDA	Yellow
14	PI 670898	USDA	Yellow
15	CGN 17023	CGR the Netherland	Yellow
16	CGN 22854	CGR the Netherland	Yellow

[a]The materials segregated from commercial variety and maintained in our laboratory.

94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 5 min, followed by a final extension at 72˚C for 3 min. Amplification was checked by electrophoresis in 1% agarose gel with 1 × TAE buffer. The primers used were listed in Table 2.

2.3. Genotyping of the *Ccs* Gene Using Developed PCR Marker

Using the normal allele specific and the large deletion specific forward primers (Ccs5del F1 and Ccs5del F2, respectively) with a fixed reverse primer (Ccs5del R) developed in this study, multiplex PCR was conducted for each of the 16 cultivars of *C. annuum* and *C. chinense*. PCR condition was the same as diagnostic PCR described above, except that the extension time was changed to 30 sec.

Table 2. Primer list used in this study.

Name	Sequence (5' → 3')	Purpose	Reference
Ccs5up_F-762	AGGAATACTACAAGGCCTCCA	diagnostic PCR	
Ccs5up_F-1671	GATGAAAATTTGGGCTCCGC	diagnostic PCR	
Ccs5up_F-2393	TGAAACTGAATAAGCTGCGACT	diagnostic PCR	
Ccs5up_F-2745	ACTAATACAGCTGCCGTCCA	diagnostic PCR	
Ccs5up_F-3080	GCCGCCCATTCTTTCTAGTC	diagnostic PCR	
Ccs5up_F-3784	ACGGCAACTCAACACAAGTG	diagnostic PCR	
Ccs R+1494 (CCS-d[a])	AAGGCTCTCTATTGCTAGATTGCCCAG	diagnostic PCR and RT-PCR	Lang *et al.* (2004) [13]
Ccs5del F1	TGAGAAGTGTCCTTGAGGAAGA	multiplex PCR to distinguish the large deletion	
Ccs5del F2	TCTGGTGCTTTTAGAGTTTGGA	multiplex PCR to distinguish the large deletion	
Ccs5del R	TTTAACCAAGGGGACAGTGC	multiplex PCR to distinguish the large deletion	
Ccs F+174	ACCCACATCAAAGCCAGAGT	RT-PCR	
Ccs R+352 (CCS-534[a])	ACATGGAAAGTAGTAAAGG	RT-PCR	Lang *et al.* (2004) [13]
Ccs F+1284 (CCS-1484[a])	GGAAGGTACTAGGAGATTGTT	RT-PCR	Lang *et al.* (2004) [13]
ccs3dCAPS F	GTACTGGCTGAGGCCATCGT	dCAPS for *ccs-3*	
ccs3dCAPS R[b]	AAACAATCTCCTAGTACCTTCCAAATCAAGCTTCAACA**G**A	dCAPS for *ccs-3*	
ubiqitin-F	TGTGTCTCAACATTCTTCGTGA	RT-PCR	Stellari *et al.* (2010) [27]
ubiqitin-R	ATACAGCAGCTGCGTCGT	RT-PCR	Stellari *et al.* (2010) [27]

[a]Names in the parenthesis are used in Lang *et al.* (2004) [13]. [b]The artificial mismatch site to create restriction site is shown in bold and underlined.

To distinguish *ccs-3* that is another mutated allele of *Ccs* rediscovered in this study, a dCAPS marker was developed. At first the region including single base indel polymorphic site at the 3' end was amplified by PCR using primers ccs3dCAPS F and R developed in this study. Reverse primer contains an artificial SNP changing A to G at the second nucleotide position from 3' end to create a recognition site of restriction enzyme *Nde*II (GATC). PCR was conducted in 20 μL volume with 40 ng template DNA. The condition of PCR amplification was 1 cycle at 94˚C for 1 min, 30 cycles at 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 20 sec, followed by a final extension at 72˚C for 3 min. Takara Taq (Takara Bio) that does not possess 3' to 5' exonuclease activity was used as DNA polymerase. Restriction enzyme reaction was carried out using 3 μL PCR product and 30 units of *Nde*II in 20 μL volume. The mixture was incubated at 37 ˚C overnight. The expected band size of the PCR product is 180 bp for wild type and 179 bp for *ccs-3*. It is expected that wild type is not digested with *Nde*II but *ccs-3* is digested to produce a 40 bp shorter band. Electrophoresis was performed using 3% agarose gel with 1 × TBE buffer.

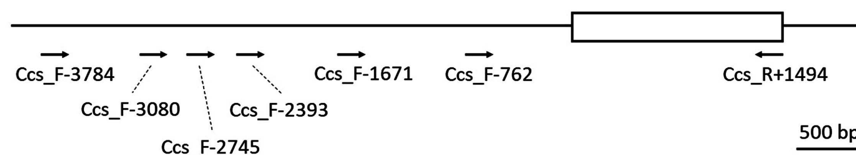
2.4. RT-PCR

To investigate the expression of *ccs-3*, RT-PCR was performed for "Kiiro Togarashi", a yellow fruit cultivar possessing *ccs-3* (**Figure 1(c)**) and "Takanotsume", a red fruit cultivar, as positive control. Total RNA was extracted from pericarp of fruits at color turning stage, using RNeasy Plant Mini Kit (QIAGEN). Reverse transcription reaction was carried out using PrimeScript II Reverse Transcriptase (Takara Bio). Two primer sets were applied, that is, Ccs F+174 and Ccs R+352 for upstream part of exon and Ccs F+1284 and Ccs R+1494 for downstream part of exon. Expression of ubiquitin was also examined as positive control. For all the primer sets, RT-PCR was carried out in 20 μL of reaction volume and the condition was 1 cycle at 94˚C for 1 min, 30 cycles at 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, followed by a final extension at 72˚C for 30 sec.

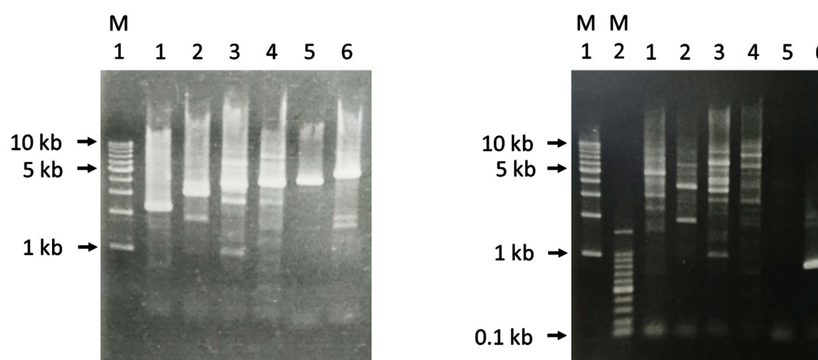
3. Results

3.1. Identification of the Large Deletion of the *Ccs* Gene in Non-Red Fruit Color Pepper

To identify the large deletion in upstream region of the *Ccs* gene, diagnostic PCR was carried out using six forward primers designed at 300 - 900 bp intervals with a fixed reverse primer (**Figure 2(a)**). At first, to verify the amplification accuracy, PCR was performed for the red pepper variety "Takanotsume" having the intact *Ccs* gene. As expected, a stepwise amplification of intensive bands with different sizes was observed for the six primers, although some minor non-specific bands were shown (**Figure 2(b)**). Then diagnostic PCR using these six primers was conducted for the yellow pepper cultivar "Sonia Gold" (**Figure 2(c)**). Five downstream primers did not show clear single band amplification



(a)



(b) (c)

Figure 2. Diagnostic PCR to identify large deletion of the *Ccs* gene. (a) Primer position used for diagnostic PCR. Box and line indicate exon and untranslated region of the *Ccs* gene, respectively. (b) Profile for "Takanotsume" and (c) profile for "Sonia Gold". Lanes 1, 2, 3, 4, 5 and 6 are amplicons using Ccs_F-762, Ccs_F-1671, Ccs_F-2393, Ccs_F-2745, Ccs_F-3080 and Ccs_F-3784 as forward primers, respectively. M1 and M2 are DNA size marker 1 kb ladder and 100 bp ladder, respectively.

and only the most upstream primer Ccs5up_F-3784 amplified an intensive single band. Since the second upstream primer was located at −3080 bp position from the first nucleotide of exon, it was deduced that at least 3000 bp was lack in the *Ccs* gene of "Sonia Gold".

The approximately 800 bp fragment obtained by using the most upstream primer Ccs5up_F-3784 was sequenced and aligned with the published genome sequence of red pepper. As a result, it was revealed that 4430 bp from −3234 bp position in upstream untranslated region to 1196 bp position in exon was deleted in the *Ccs* gene of "Sonia Gold" (**Figure 3**). Since the accurate size and position of the large deletion could be successfully identified, hereafter we called this allele *ccs-del*. The size of the deletion of *ccs-del* was different from but near to *ccs-p1* having 4879 bp deletion recently reported by Jeong *et al.* (2019) [14]. Especially the end of the deletion of *ccs-del* and *ccs-p1* can be regarded to be identical depending on alignment.

3.2. Development of the Large Deletion Distinguishable PCR Marker

Based on the results described above, we tried to develop PCR marker to distinguish *ccs-del* (**Figure 4**). Using the sequence information obtained, the 4430 bp large deletion specific primer Ccs5del F2 was designated astride the deletion

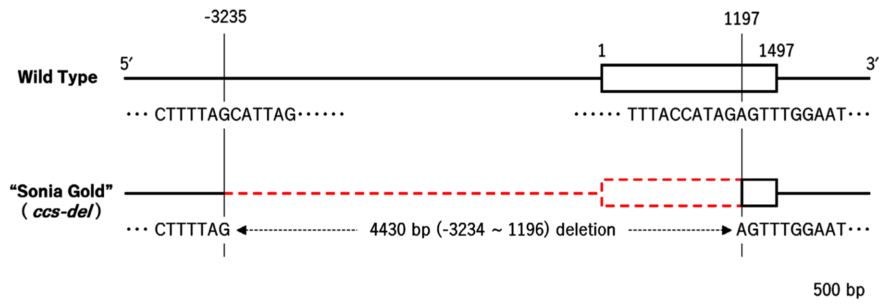


Figure 3. Schematic structure of the *Ccs* gene with a large deletion identified in "Sonia Gold". Deleted region in "Sonia Gold" is shown as red dotted lines. Numbers above the schematized gene structure indicate nucleotide position defined the first nucleotide of the exon as 1.

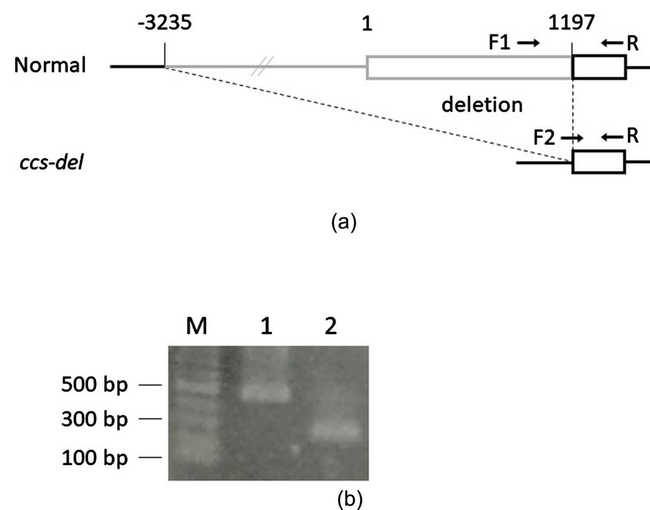


Figure 4. PCR marker developed to distinguish *ccs-del*. (a) Primer position. F1, F2 and R mean Ccs5del F1, Ccs5del F2 and Ccs5del R listed in Table 2, respectively. Deletion region is shown in gray. (b) Profile of multiplex PCR using primers F1, F2 and R. Lanes 1 and 2 are amplicons using "Takanotsume" and "Sonia Gold" as template, respectively. M is DNA size marker 100 bp ladder.

position (Figure 4(a)). In addition to this primer, normal allele specific primer Ccs5del F1 was designed inside the deleted region. Using these two primers at a time as forward with a single reverse primer Ccs5del R, multiplex PCR was carried out. According to the sequence information, it was expected that a 491 bp band was amplified for the normal allele and a 282 bp band was amplified for *ccs-del*. As shown in Figure 4(b), an approximately 500 bp single band was amplified for "Takanotsume" and an approximately 300 bp single band was amplified for "Sonia Gold" as expected. Thus, we successfully developed the PCR marker to distinguish the 4430 bp large deletion.

3.3. Genotyping of the *Ccs* Gene for the Non-Red Fruit *C. annuum* Cultivars

Using the developed PCR marker for *ccs-del*, the *Ccs* genes in 16 non-red fruit

color cultivars of *C. annuum* were genotyped (**Figure 5**). As a result, among 16 cultivars, 14 had *ccs-del*. Out of them, msGTY-1 (lane 10) is the orange fruit cultivar that has been used and revealed to have a large deletion in Lang *et al.* (2002) [13]. This result indicates that the 4430 bp deletion is a common cause of non-red fruit color in *C. annuum*.

Two cultivars "Cayenne Pepper Golden" (lane 5) and "Kiiro Togarashi" (lane 16) showed normal allele bands. "Cayenne Pepper Golden" and "Kiiro Togarashi" are European and Japanese traditional cultivars, respectively, and both are hot pepper having yellow and long type fruit. The sequences of the *Ccs* gene of the two cultivars were determined. Then, it was clarified that the sequences of both the cultivars were identical, having a single base deletion at 1265 bp position from the start codon that results in a frameshift and generates a stop codon (**Figure 6**). This was the allele *ccs-3* that have been previously reported in other *C. annuum* cultivars [14] [24]. Expression of *ccs-3* in "Kiiro Togarashi" was investigated by RT-PCR with two primer sets, that is, upstream and downstream parts of exon (**Figure 7(a)**. Both the upstream and downstream primer sets showed positive amplification in RT-PCR (**Figure 7(b)**). This result suggested that almost entire region of *ccs-3*, including even the region downstream of stop codon generated by frameshift due to the 1 bp deletion, was transcribed in the yellow cultivars. Therefore, the fruit color change might have been caused not by expressional change but by loss or weakening of function of *ccs-3* due to the frameshift. A dCAPS marker was developed to distinguish *ccs-3* (**Figure 7(a)** and **Figure 7(c)**). This is a codominant marker that can recognize heterozygous samples.

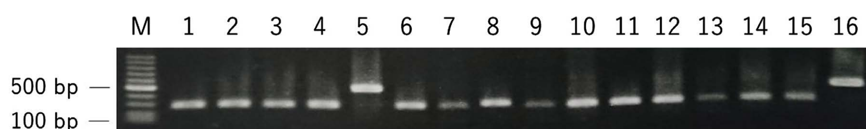


Figure 5. Profile of multiplex PCR to recognize *ccs-del* in 16 cultivars of *C. annuum*. Lanes 1 - 16 correspond to No. 1 - 16 listed in **Table 1**.

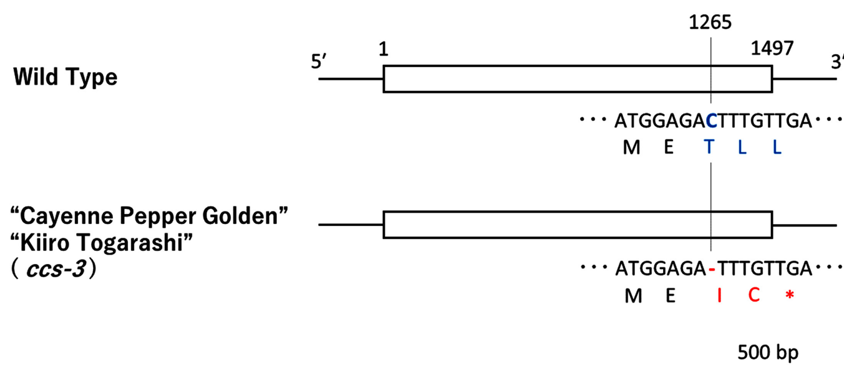
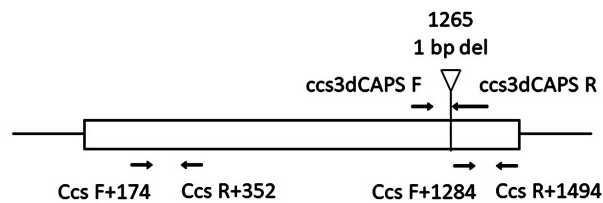
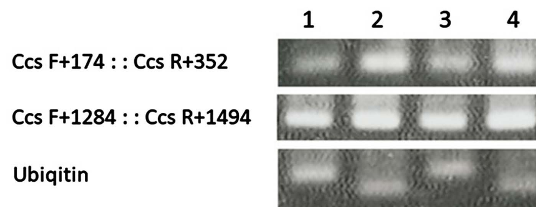


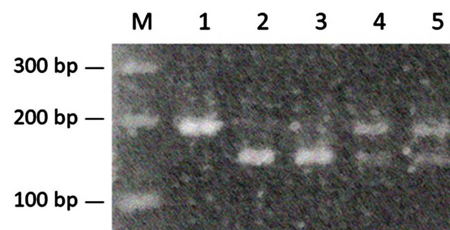
Figure 6. The gene structure of *ccs-3*, containing 1 bp deletion at 1265th nucleotide position found in "Cayenne Pepper Golden" and "Kiiro Togarashi". Nucleotide and translated amino acid sequences around the deletion position are shown below the schematized gene structure. Changed nucleotide and amino acid are shown in blue (before mutation) and red (after mutation). Asterisk means stop codon.



(a)



(b)



(c)

Figure 7. Expressional analysis and dCAPS marker development for *ccs-3*. (a) Primer position used for RT-PCR (shown below the schematized gene structure) and dCAPS (shown above the schematized gene structure) analyses. (b) Profile of RT-PCR. Lanes 1, 2, 3 and 4 are amplicons using genomic DNA of "Takanotsume", RNA of "Takanotsume", genomic DNA of "Kiiro Togarashi" and RNA of "Kiiro Togarashi" as template, respectively. (c) Confirmation of availability of dCAPS marker developed in this study. Profile of PCR products amplified using primers ccs3dCAPS F and ccs3dCPAS R digested with *Nde*II is shown. Lanes 1, 2 and 3 are profiles for "Takanotsume", "Cayenne Pepper Golden" and "Kiiro Togarashi", respectively. Lanes 4 and 5 are dCAPS profiles using 1:1 mixed DNA of "Takanotsume" and "Cayenne Pepper Golden" (lane 4) and "Takanotsume" and "Kiiro Togarashi" (lane 5) as template. M is DNA size marker 100 bp ladder.

3.4. Genotyping of the *Ccs* Gene for the Non-Red Fruit *C. chinense* Cultivars

Sixteen non-red fruit cultivars of *C. chinense*, the second major pepper species, were genotyped by the PCR marker for *ccs-del* (Figure 8(a)). Among 16 cultivars, 13 showed normal allele bands and a cultivar PI 485593 (lane 13) exhibited the 4430 bp large deletion specific band. No amplification was observed in two cultivars PI 224448 and CGN 22854 (lanes 10 and 16), suggesting that these cultivars had unknown mutation in the *Ccs* gene. When using the dCAPS marker for *ccs-3*, it was found that a cultivar PI 670898 (lane 14) had *ccs-3* (Figure 8(b)). In Figure 8(b), PI 485593 (lane 13) that was revealed to have the 4430 bp deletion did not show band, which is as expected since the forward primer used

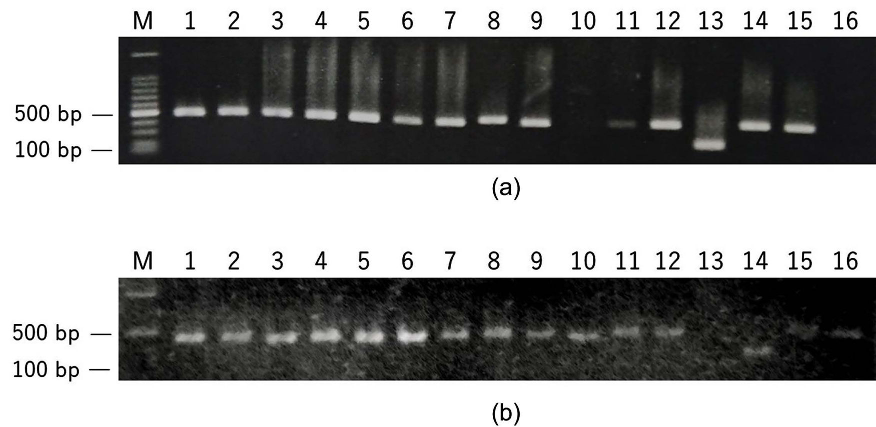


Figure 8. Genotyping of the *Ccs* gene of 16 cultivars of *C. chinense*. Lanes 1 - 16 correspond to No. 1 - 16 listed in **Table 1**. (a) Profile of multiplex PCR to recognize *ccs-del*; (b) Profile of dCAPS to distinguish *ccs-3*.

for dCAPS was included in the deleted region. These results indicated that the mutated alleles of the *Ccs* genes generating non-red fruit color, *ccs-del* and *ccs-3*, were shared in two major *Capsicum* species, *C. annuum* and *C. chinense*.

4. Discussion

In this study, we could successfully identify the large deletion causing non-red fruit color of pepper that has been known for more than 20 years but whose substance has not been identified [11] [12] [13]. It was revealed to be a 4430 bp deletion from −3234 bp position in 5' upstream untranslated region to 1196 bp position in exon. We named the allele having this deletion *ccs-del*. The size of the deletion of *ccs-del* was different from but near to that of *ccs-p1* recently reported by Jeong *et al.* (2019) [14]. The size of the deletion of *ccs-p1* was 4879 bp. Since the sequence of *ccs-p1* could not be obtained, the identity of *ccs-del* determined in this study and *ccs-p1* is obscure, but it is possible that these two alleles are the same because the length of the deletion region is depending on the reference sequence compared. The most part of the deleted region was promoter region of the *Ccs* gene. Since the length in promoter region is not so conservative, it is considerably possible that the two alleles are substantially the same.

We also succeeded to develop the PCR marker for *ccs-del*. Using this PCR marker, we clarified that most of the non-red fruit *C. annuum* cultivars investigated (14 of 16) possess *ccs-del*. Jeong *et al.* (2019) reported that 23 *C. annuum* accessions had *ccs-p1*. Although the identity of the *ccs-del* and *ccs-p1* is ambiguous, these results indicate that more than 4 kb large deletion of *Ccs* is the most common cause to non-red fruit color in *C. annuum* and is shared by various sweet bell pepper. This is probably resulted from modern pepper breeding aiming colorful fruit color in which genetically limited preferable alleles were introduced intensively. The yellow or orange sweet bell pepper possessing *ccs-del* have nearly identical genetic traits such as flavor and productivity to the red sweet bell pepper that does not have *ccs-del*, indicating that *ccs-del* does not affect

traits other than fruit color. Jeong *et al.* (2019) have reported another allele *ccs-p2* that had 2968 bp deletion in the promoter region [14]. This allele, however, was found only in *C. baccatum*. Considering this fact and our result that all the cultivars having large deletion possess *ccs-del*, it is considered that *ccs-p2* is not a source of non-red fruit color of *C. annuum*. In our present study, in addition to *ccs-del*, another allele *ccs-3* was found in two traditional hot pepper cultivars. This allele is the most common among the alleles with frameshift of the *Ccs* gene and reported in other hot pepper cultivars. [14]. These results suggest that traditional non-bell type peppers possibly have alleles of *Ccs* other than *ccs-del*.

The genotyping of *C. annuum* and *C. chinense* revealed that *ccs-del* and *ccs-3* were also present in *C. chinense* although they were minor in the species. As far as we know, this is the first report that the large deletion of *Ccs* that is the major cause for non-red fruit color in *C. annuum* is present in other *Capsicum* species. Also with respect to *ccs-3*, although it has been found in *C. baccatum* [14], presence in *C. chinense* has not been reported to date. These results evidently indicate that major alleles responsible for non-red fruit color in *C. annuum* were shared across species throughout interspecific introgression. Such introgression may contribute to expansion of pepper fruit color variation.

In this study, regardless the fruit color is orange or yellow, non-red fruit color cultivars had *ccs-del*. Several studies indicated that carotenoid amount was not necessarily determined by *Ccs* alleles [24] [25]. Furthermore, recent study clarified that besides *Ccs* and *Psy*, the major genes responsible for pepper fruit color, other genes included in the carotenoid synthesis pathway or regulatory genes are involved in pepper fruit color determination [14] [26]. Thus, further analysis is necessary for understanding genetic mechanism of pepper fruit color. In this study, we developed two DNA markers to distinguish *Ccs* mutant alleles, *ccs-del* and *ccs-3*. DNA markers are powerful tool for breeding, especially, for selection of the traits that can be discerned only at the later growth stage such as fruit color. Even now it can be considered that demands to the vibrant color of pepper fruit are potentially high. The DNA markers developed in this study will be helpful for acceleration of pepper breeding targeting fruit color.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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