Note

Mutations in the CCD4 Carotenoid Cleavage Dioxygenase Gene of Yellow-Flesh Peaches

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Peach trees bear either white- or yellow-flesh fruit. We found that Japanese peach cultivars have two types of mutation in a carotenoid catabolic gene, CCD4: the insertion of a retrotransposon, and a frame shift in the microsatellite sequences of the first exon. CCD4 in yellow-flesh peaches was disrupted by these mutations.

Key words: Prunus persica; carotenoid; long terminal repeat (LTR) retrotransposon; microsatellite; yellow flesh

The color of fruit flesh is an important factor that affects the preferences of customers. Peaches, one of the major fruits on the world market, show two types of flesh color: white and yellow. In Japan, white-flesh peaches are preferred for eating raw and yellow-flesh peaches are mainly processed as canned fruits. Crossing between peach trees bearing white-flesh fruits sometimes produces trees bearing yellow-flesh fruits, causing a decrease in the breeding efficiency of white-flesh peaches. To date, genetic analysis has revealed that the white color is dominant to the yellow color, and that flesh color is governed by a single locus, named Y.1 The Y locus was mapped on the middle of the first linkage group.2,3 Yellow-flesh peaches contain high amounts of carotenoids, major plant pigments that produce the yellow color.4 It has been reported that the yellow color of chrysanthemum flowers and potato buds is also due to carotenoids, and that carotenoid accumulation is caused by mutations in CCD4, a carotenoid cleavage dioxygenase gene.5,6 In peaches, the expression of CCD4 is repressed in yellow flesh as compared to white flesh.7 In the present study, we identified mutations in CCD4 in peach cultivars grown in Japan.

The sequence data of the peach genome were reported recently.8,9 We queried the CCD4 gene against the peach genome using “Arabidopsis CCD4, AT4G19170” as query in the Genome Database for Rosaceae,9 and found that the peach CCD4 homolog (ppa006109m; 25,639,445 to 25,641,500) is located between two SSR markers, MA036a (20,091,449 to 20,091,716) and pchgm3 (27,691,861 to 27,691,861) on scaffold 1. This position exactly matches the Y locus,10 suggesting that CCD4 is responsible for the color of peach flesh. To confirm this, we compared the structure of CCD4 between the yellow-flesh peach Yamate-shimizu ( genotype y/y) and the white-flesh peach Hakuho by genomic PCR. Hakuho might have a Y/Y genotype, since 26 crossings of Hakuho to various cultivars in breeding by us have never produced yellow-flesh peaches. We designed three sets of primers: 5′-TTGCTTAGAGAAAGTGAAGC-3′ and 5′-GTGAGTGTTGGATCA-3′ for amplification of the promoter region, 5′-GGAAACGGAGATATTGCAG-3′ and 5′-AGGCACATCAACCCACCTCA-3′ for the N-terminal half of CCD4, and 5′-GATGCAAACGCGCACAAGCAG-3′ and 5′-TGCACCTCACCTTTG-3′ for the C-terminal half. When we used these primer sets for the promoter region and the C-terminal half, the sizes of the amplified fragments were apparently not different (Fig. 1A). However, when we used the primer set for the N-terminal half, the size of the fragments amplified from the Yamate-shimizu genome was approximately 7 kb longer than that from the Hakuho genome. A comparison of the sequences of the amplified fragments revealed that an extra sequence of 6,253 bp is present in the intron of Yamate-shimizu CCD4 (Fig. 1B). This sequence has tandem repeats of 486 bp at both ends. This indicates that it is a long terminal repeat (LTR)-type retrotransposon, which is widely distributed in the peach genome.10 LTRs have short inverted repeats (SIR, TGTATTA) at both ends, which are known recognition motifs for a integrase and a TATA box sequence (TATATG) in the middle. These motifs are specific to LTR retrotransposons.11 The retrotransposon in Yamate-shimizu CCD4 lacks long open reading frames, indicating that it is a non-autonomous element.12 To determine the distribution of the retrotransposon in the CCD4 gene among peach cultivars, we performed PCR for the genomic DNA of 39 cultivars using three primers: 5′-ACCACCTGGT-TGACGGAGAC-3′ (CCD4-f), 5′-TGTCCTCATGAGAGCCTTGGCA-3′ (CCD4-r), and 5′-TCCTGAGACCTTCTGTCG-3′ (RT-f). This PCR can produce 594-bp fragments from the CCD4 and 729-bp fragments from retrotransposon-containing CCD4 at the same time. The result of PCR was that the 594-bp fragment was transposed only from the genomes of 10 cultivars, including Hakuho (Fig. 1C), all of which bear white-flesh fruits. The 729-bp fragment was amplified only

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Abbreviations: CCD, carotenoid cleavage dioxygenase; LTR, long terminal repeat; SSR, simple sequence repeat
from the genomes of seven cultivars, including Yamate-shimizu, all of which are yellow-flesh peaches. Both 594- and 729-bp fragments were amplified from the remaining 22 cultivars. All of these bear white-flesh fruits, except for Golden peach. Thus many cultivars grown in Japan have the transposon in CCD4, and the presence of the transposon is highly correlated with flesh color.

Golden peach is a yellow-flesh peach having CCD4 of normal size and CCD4 disrupted by the transposon. This suggests the possibility that the former Golden peach CCD4 has another mutation that disturbs the function of the gene. We cloned CCD4 of normal size from the Golden peach genome and compared the sequence with that of Hakuho CCD4. Only one difference was detected: in the microsatellite sequence (seven repeats of TC, 48 to 61 bp from the start codon of Hakuho CCD4) of the first exon, Golden peach CCD4 has an extra TC sequence, which produces eight repeats of TC (Fig. 2A). This causes a frame shift near the N terminus of CCD4 and probably induces a null mutation. Thus Golden peach has the transposon-containing CCD4 and the frame-shifted CCD4. To determine whether other cultivars have the frame-shifted CCD4, a small fragment containing the microsatellite region was amplified from 39 cultivars using primers 5'-CCCATTTTGCAGTGAAAGGGC-3' (SSR-f) and 5'-FAM-GCTGTGGTGCTTTTGTGGAG-3' (SSR-r). The size of the fragment was determined with a 3130xl genetic analyzer and GeneMapper (Life Technologies, Boston, MA). The results indicated that the smaller band (198 bp), containing seven repeats of TC, was detectable in all the

<table>
<thead>
<tr>
<th>LTR retrotransposon</th>
<th>−/−</th>
<th>−/+</th>
<th>+/+</th>
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<tbody>
<tr>
<td>9 cultivars (Hakuho, Kanoiwa-hakuto, Misakakkko, Natugokoro, Benishimizu, Tachibana-wase, Nagasawa-hakuto, Wase-momoyama) and 57 cross-bred lines</td>
<td>21 cultivars (Hanayome, Hikawa-hakuto, Kawanakajima-hakuto, Okayama yume-hakuto, Hakurei, Akaakari, Shimizu-hakuto, Yamato-hakuto, Shimizu-hakuto RS, Hakuto, Setouchi-hakuto, Sakigake-hakuto, Hanashimizu, Asama-hakuto, Chikusa-hakuto, Shanhai suimitsu, Sueki-hakuto, Shin-hakurei, Akizora, Hakuyo, and Yamane-hakuto) and 78 cross-bred lines</td>
<td>7 cultivars* (Yamate-shimizu, Sun gold, Kinto, Nakayama-kinto, Obanto, Tsukikari, and Takinosawa gold) and 29 cross-bred lines*</td>
<td></td>
</tr>
<tr>
<td>−/+</td>
<td>1 cultivar (Hakushu) and 9 cross-bred lines</td>
<td>1 cultivar* (Golden peach) and 8 cross-bred lines*</td>
<td>(none)</td>
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<tr>
<td>+/+</td>
<td>(none)</td>
<td>(none)</td>
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*Yellow-flesh peaches
cultivars, including Hakuho (Fig. 2B). The larger band (200 bp), containing eight repeats of TC, was detectable only in Golden peach and Hakushu, indicating that the frame-shift mutation of \( \text{CCD4} \) is not widely distributed in the peach cultivars grown in Japan.

To confirm the relationship between the genotype of \( \text{CCD4} \) and the phenotype of flesh color, we examined the genotypes of 181 cross-bred lines grown in our field, including 37 lines bearing yellow-flesh fruits. The \( \text{CCD4} \) with transposon was detected heterozygously in 86 lines and homozygously in 29 lines. The frame-shifted \( \text{CCD4} \) was found heterozygously in 17 lines, but was not found homozygously in any line. These results are summarized in Table 1. None of the yellow-peach lines had a normal \( \text{CCD4} \). Twenty-nine of them had the \( \text{CCD4} \) with transposon homozygously, like Yamate-shimizu, whereas the other eight lines had both the transposon-inserted \( \text{CCD4} \) and the frame-shifted \( \text{CCD4} \), like Golden peach. In contrast, all of the white peach lines had functional \( \text{CCD4} \) either homozygously or heterozygously. This suggests that \( \text{CCD4} \) is involved in the flesh color of peach fruits, and that highly efficient selection of white-flesh peaches is possible, before the bearing of fruit, by the use of our PCR primers.

During the preparation of this manuscript, Adami et al.\(^{13}\) and Falchi et al.\(^{14}\) reported on \( ccd4 \) mutations in peach cultivars grown mainly in Europe and the United States. In their reports, the frame-shift mutation was detected more frequently than the transposon-present mutation. In addition, they identified T/A transversion leading to a nonsense mutation, which was not detected by us in the 39 cultivars grown in Japan (data not shown). The distribution of \( ccd4 \) alleles among Japanese cultivars is at variance with the observations.

References