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Molecules and Cells

A Splicing Mutation in the Gene Encoding Phytoene Synthase Causes Orange Coloration in Habanero Pepper Fruits

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Peppers (*Capsicum* spp.) display a variety of fruit colors that are reflected by the composition and amount of diverse carotenoid pigments accumulated in the pericarp. Three independent loci, *c1*, *c2*, and *y,* are known to determine the mature color of pepper fruits by their allelic combinations. We examined the inheritance of fruit color in recombinant inbred lines (RILs) derived from an interspecific cross between *C. annuum* cv. TF68 (red) and *C. chinense* cv. Habanero (orange). The *c2* gene encodes phytoene synthase (PSY), a rate-limiting enzyme in the carotenoid biosynthesis pathway. TF68 has a dominant *c2*+ allele whereas Habanero is homozygous for the recessive *c2* allele, which determined RIL fruit color. Here we report that the recessive *c2* allele has a point mutation in the *PSY* gene that occurs at a splice acceptor site of the fifth intron leading to both a frame shift and premature translational termination, suggesting that impaired activity of PSY is responsible for orange fruit color. During ripening, *PSY* is expressed at a significantly high level in orange colored fruits compared to red ones. Interestingly, the *PSY* gene of red Habanero has a conserved splice acceptor dinucleotide AG. Further analysis suggests that red Habanero is a wild type revertant of the *PSY* mutant orange Habanero.

INTRODUCTION

Carotenoids are C_{40} hydrocarbon compounds found in many photosynthetic bacteria, algae, and plants. The pathway and essential enzymes of carotenoid biosynthesis are conserved among photosynthetic organisms. Consecutive condensation of C_5 isopentenyl pyrophosphate produces C_{20} geranylgeranyl pyrophosphate (GGPP), and the subsequent head-to-head condensation of two GGPP molecules gives rise to colorless C_{40} phytoene, a universal precursor of colored carotenoids (Armstrong and Hearst, 1996; Cunningham and Gantt, 1998; Hirschberg, 2001). Through a series of enzymatic desaturation, cyclization, and isomerization processes, phytoene is converted to a variety of carotenoid compounds, each of which has a unique light-absorbing property with distinct color.

Pepper (*Capsicum* spp.) fruits display a wide range of colors which are determined by both composition and the amount of carotenoid pigments stored in the fruit pericarp. In pepper, three independent loci (*c1*, *c2*, and *y*) are known to control mature fruit color, and their allelic combinations yield eight different colors ranging from white (*y c1 c2*) to red (*y*+ *c1*+ *c2*+) (Hurtado-Hernandez and Smith, 1985). Genetic linkage analyses between the genes encoding carotenogenic enzymes and fruit color loci revealed that the *c2* and *y* loci correspond to genes that encode phytoene synthase (PSY) and capsanthin-capsorubin synthase (CCS), respectively (Huh et al., 2001; Lefebvre et al., 1998; Thorup et al., 2000). CCS catalyzes the last step of carotenoid biosynthesis in the pepper chromoplast converting antheraxanthin to capsanthin and violoaxanthin to capsorubin (Bouvier et al., 1994). Both capsanthin and capsorubin are two major red pigments in pepper, and fruits homozygous for recessive *y* alleles fail to accumulate these pigments producing white to orange-yellow colors depending on the allelic configuration at the *c1* and *c2* loci (Hurtado-Hernandez and Smith, 1985; Lefebvre et al., 1998). Previous work has shown that the *CCS* gene in yellow pepper has a deletion in the coding region, presumably causing loss of enzyme activity associated with a loss of pigmentation (Lefebvre et al., 1998). Another study reported that a variation in the promoter region of *CCS* might determine the level of pigmentation expression in diverse pepper cultivars (Ha et al., 2007).

Despite the previous finding of the association of *PSY* with the *c2* locus, the underlying cause that distinguishes between red (*c2*+) and orange (*c2*) fruit colors is not clearly understood (Huh et al., 2001; Thorup et al., 2000). The recessive *c2* allele is associated with both orange fruit color and reduced carotenoid content in the segregating population (Huh et al., 2001). The lower level of total carotenoids in orange than red colored fruits suggests reduced activity of PSY in homozygous *c2* fruits (Huh et al., 2001). As a rate-limiting enzyme in the carotenoid biosynthesis pathway, mutations in *PSY* often cause a severe reduction in the amount of total carotenoids with less coloration

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in plants and algae (Buckner et al., 1996; Fray and Fray and Grierson, 1993; McCarthy et al., 2004). Therefore, we determined whether a mutation occurred in the *PSY* gene of orange colored peppers by investigating *PSY* structure and its expression.

Here we report that the recessive *c2* allele of orange-colored Habanero peppers carries one mutation at a splice acceptor site in *PSY* that causes less pigmentation during ripening. In addition, our data suggest that a recently developed red Habanero cultivar is a wild type revertant of the orange Habanero *PSY* splicing mutant.

MATERIALS AND METHODS

Plant materials

The F_1 hybrid from a cross between *C. annuum* cv. TF68 and *C. chinense* cv. Habanero was self-pollinated to generate the F_2 population (Kang et al., 2001). Plants were grown under greenhouse conditions at the National Horticultural Research Institute in Suwon, Korea. By repeated self-pollination, the population was advanced to the F_9 generation. The Joongbu Breeding and Research Station, Monsanto, Korea provided other plant materials.

Cloning of the *PSY* gene

The *PSY* coding region, including introns, was PCR-amplified with primers psy5′ (5′-ATGTCTGTTGCCTTGTTATGG) and psy3′ (5′-CCTGATTTCATGTTCTTGTAGAAGG) using genomic DNA as template with initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 67°C for 1 min, followed by a 10 min extension at 72°C. The *PSY* cDNA was obtained by RT-PCR using the psy5′ and psy3′ primers and 2 μg of total RNA extracted from TF68 and Habanero fruit pericarps at the mature red stage with the same thermocycling parameters.

Allele-specific PCR amplification

To distinguish between wild type and mutant *PSY* alleles, the genomic region spanning the 5th intron-6th exon junction was PCR-amplified with primers psySNP5′ (5′-GTTGAGACGAA AGGGTTTTGAAGTT) and psySNPA (wild-type-specific; 5′-CAACAGCAGAGATGCCAACACCT) or psySNPC (mutant-specific, 5′-ACAGCAGAGATGCCAACACCG) using genomic DNA as template. PCR amplification was conducted with initial denaturation at 94°C for 5 min, 28 cycles of denaturation at 94°C for 1 min, and annealing and extension at 67°C for 1 min, followed by a 10 min extension at 72°C. To detect the wild type *PSY* transcript, primers psy5′ and psyPM3′ (5′-CGGTACAAC AGAGATGCCAACCT) were used for RT-PCR amplification with the procedure described above.

Expression analysis

Total RNA was prepared from the fruit pericarp at five different ripening stages: immature green (IG), mature green (G), breaker (B), immature red (IR), and mature red (R) according to the degree of coloration (Supplementary Fig. S1). For northern blot analysis, 10 μg of total RNA was separated on a 1% agarose gel containing 0.67 M formaldehyde and blotted onto Hybond N+ membrane (Amersham Pharmacia Biotech). Probe preparation and hybridization were carried out as described by Huh et al. (2001). Expression levels were also measured by semi-quantitative RT-PCR using the conditions described above.

RESULTS

Inheritance of fruit color in recombinant inbred lines derived from red TF68 and orange Habanero

In this study, 100 F_9 RILs were generated by successive self-pollination of an F_1 hybrid that was derived from an interspecific cross between *C. annuum* cv. TF68 and *C. chinense* cv. Habanero (Kang et al., 2001). TF68 and Habanero have red and orange fruit colors, respectively (Supplementary Fig. S1A). The RILs displayed a range of phenotypes for both qualitative and quantitative traits, most of which fell between the two parental values. Among the many traits examined, mature fruit color was the only apparent qualitative trait observed in the F_9 RILs, showing red and orange with a 65:35 ratio, albeit with the presence of subtle variations in depth of color and brightness (Supplementary Fig. S1B). Our previous work showed that the segregation ratio approximated the expected 3:1 in the F_2 popula-

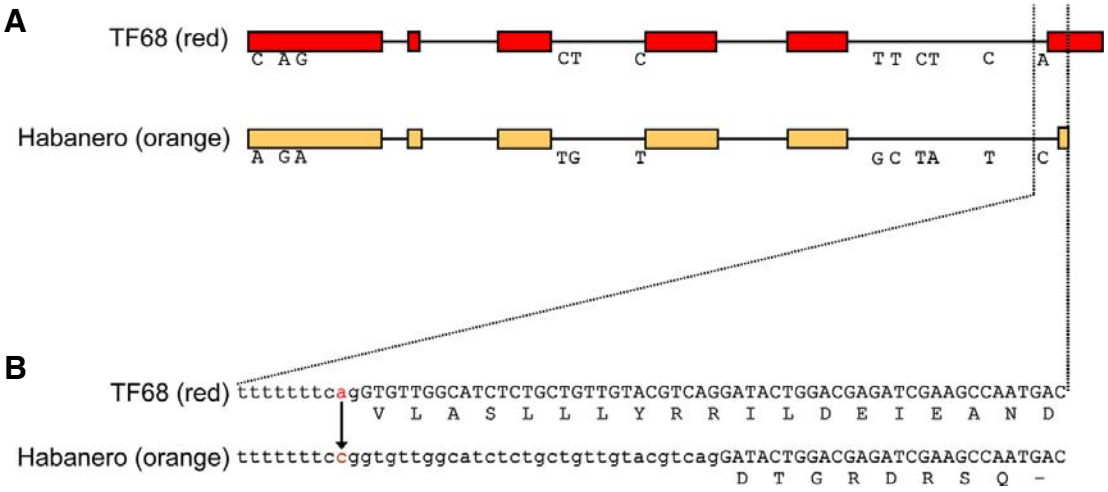

Fig. 1. Schematic representation of *PSY* gene structure. (A) The *PSY* genomic region consists of six exons (boxes) and five introns (solid lines). Twelve SNPs exist between TF68 and Habanero *PSY*. The last SNP occurs at a splice acceptor site at the fifth intron. (B) Nucleotide sequences of *PSY* at the fifth intron (lowercase) and sixth exon (uppercase) junction. The A-to-C transition in Habanero *PSY* results in the utilization of a cryptic splice site, causing a frame-shift accompanied with premature termination of translation. Deduced amino acids are shown below the nucleotide sequence. The arrow indicates the A-to-C transition at the splice receptor site.

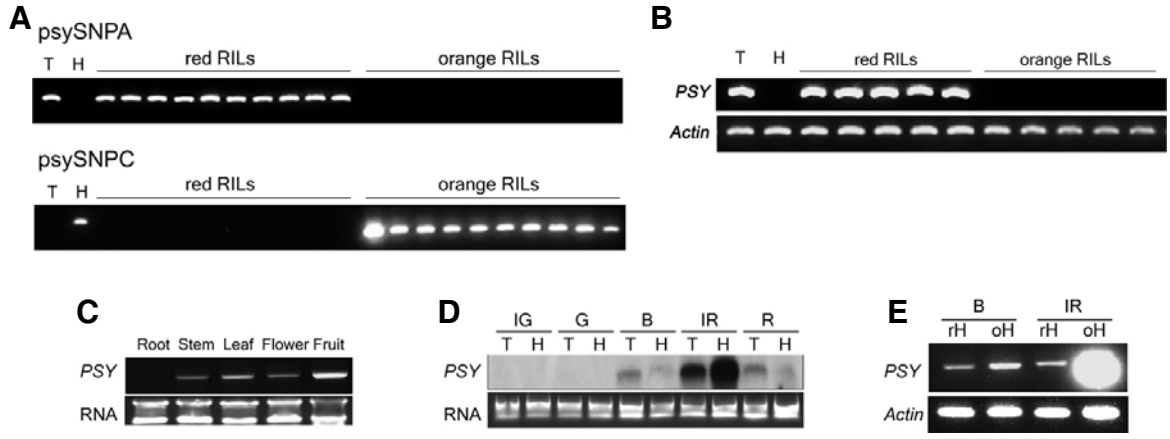

Fig. 2. Genomic and RT-PCR amplification of the *PSY* gene. (A) Allele-specific PCR amplification in RILs. psySNPA or psySNPC primers were used to selectively amplify the *PSY* sequence derived from TF68 (T) and Habanero (H). psySNP5′ was used as an upstream primer for PCR amplification. The TF68-specific *PSY* fragment was amplified in red-colored RILs and the Habanero-specific fragment in orange RILs. (B) TF68-specific *PSY* expression in red RILs. The downstream psyPM3′ primer was derived from a normal *PSY* transcript which is absent in the abnormal splice product. Actin was used as a control for RT-PCR. (C) Tissue-specific expression of *PSY* in TF68. Total RNA is shown as a loading control. (D) *PSY* expression in TF68 and Habanero fruits during ripening. *PSY* was upregulated during ripening with highest expression at IR. *PSY* is expressed at much higher level in orange Habanero than in red TF68. IG, immature green; G, mature green; B, breaker; IR, immature red; R, mature red. (E) Upregulation of *PSY* in orange Habanero at the IR stage. *PSY* expression in orange Habanero (oH) was dramatically increased at IR, whereas that of red Habanero (rH) fruits was relatively constant.

tion (Huh et al., 2001), and such segregation distortion might be due to inadvertent selection of the lines (Fu et al., 2006). The F_9 RILs continued to display discrete red and orange mature fruit colors, suggesting its stable inheritance over generations.

Orange Habanero has a mutation at a splice acceptor site in *PSY*

Previous work determined that the *c2* locus is associated with the *PSY* gene (Huh et al., 2001; Thorup et al., 2000). As a rate-limiting enzyme, PSY catalyzes the production of phytoene, a precursor of diverse carotenoid species. We previously showed that red-colored TF68 fruits contain approximately 6 times more carotenoids than orange-colored Habanero fruits, particularly for the two major red pigments capsanthin and capsorubin in the fruit pericarp (Huh et al., 2001). This finding led us to speculate that fruit color is determined by differential activities of PSY in red- and orange-colored fruits.

To identify polymorphisms between the two cultivars, we analyzed the *PSY* genomic regions from TF68 and Habanero. Both *PSY*s consist of 2,853 bp from start to stop codon with six exons and five introns (Fig. 1A). The mature transcript is predicted to encode a protein of 419 amino acids. Twelve single nucleotide polymorphisms (SNPs) were revealed between TF68 and Habanero *PSY* (Fig. 1A, Supplementary Table S1) with 99.6% identity. The first exon contains three SNPs (positions 39, 150, 184), two of which result in amino acid transitions between TF68 and Habanero *PSY* (Asp13Glu and Gly62Arg). The remaining SNPs occur in introns. Notably, the 12th SNP (position 2683) occurs at a predicted splice acceptor site at the 3′ end of the 5th intron (Fig. 1A, Supplementary Table S1). This SNP produces an unusual CG dinucleotide for Habanero *PSY* instead of a canonical AG splice acceptor sequence. A transition from AG to CG at the acceptor site causes aberrant splicing of Habanero *PSY* (Fig. 1B). The cDNA sequence revealed that the Habanero *PSY* utilizes a cryptic splice acceptor site located 29 bp downstream of the original dinucleotide (Fig. 1B). Consequently, a deletion of 29 bp occurs, accompanied with a premature stop codon and, in turn, a truncated PSY is produced (Fig. 1B). We therefore conclude that the Habanero species is a splicing mutant of the *PSY* gene, and such a difference in gene structure would explain why Habanero fruits are orange-colored with less carotenoid content than red TF68 fruits (Huh et al., 2001).

Orange-colored RILs inherit a splicing mutation in *PSY*

To confirm inheritance of the *PSY* mutation associated with orange fruit color, SNP-specific primers were designed and allele-specific PCR amplification was performed. The downstream primers PsySNPA and PsySNPC differ only at the 3′ end of the oligonucleotide that corresponds to the 12th SNP between TF68 and Habanero *PSY*. As shown in Fig. 2A, all red RILs inherited the TF68-derived *PSY* sequences, whereas orange RILs inherited only Habanero-specific sequences. Both red- and orange-specific amplifications were detected in F_1 (data not shown); however, this biallelic amplification was not observed in the RILs (Fig. 2A), which implies fixed homozygosity of the *PSY* alleles at the *c2* locus through consecutive self-pollinating processes. To examine the splicing patterns of *PSY* in red and orange RILs, RT-PCR was performed with oligonucleotide psySNPA, which would exclusively amplify the wild type *PSY* transcript. Amplification was obtained in red but absent in orange RILs (Fig. 2B). This suggests that all *PSY* transcripts in orange RILs perform aberrant splicing due to a mutation at the splice acceptor site.

Expression of mutant *PSY* is highly upregulated during ripening

Expression of *PSY* was predominant in the fruit pericarp, whereas a lower level of expression was observed in stems, leaves, and flowers (Fig. 2C). In both TF68 and Habanero fruits, expression of *PSY* starts at the breaker stage with the highest level of expression at the IR stage (Fig. 2D). *PSY* expression decreased as fruits fully matured. Interestingly, Habanero fruits showed significant upregulation of *PSY* at the IR stage com-

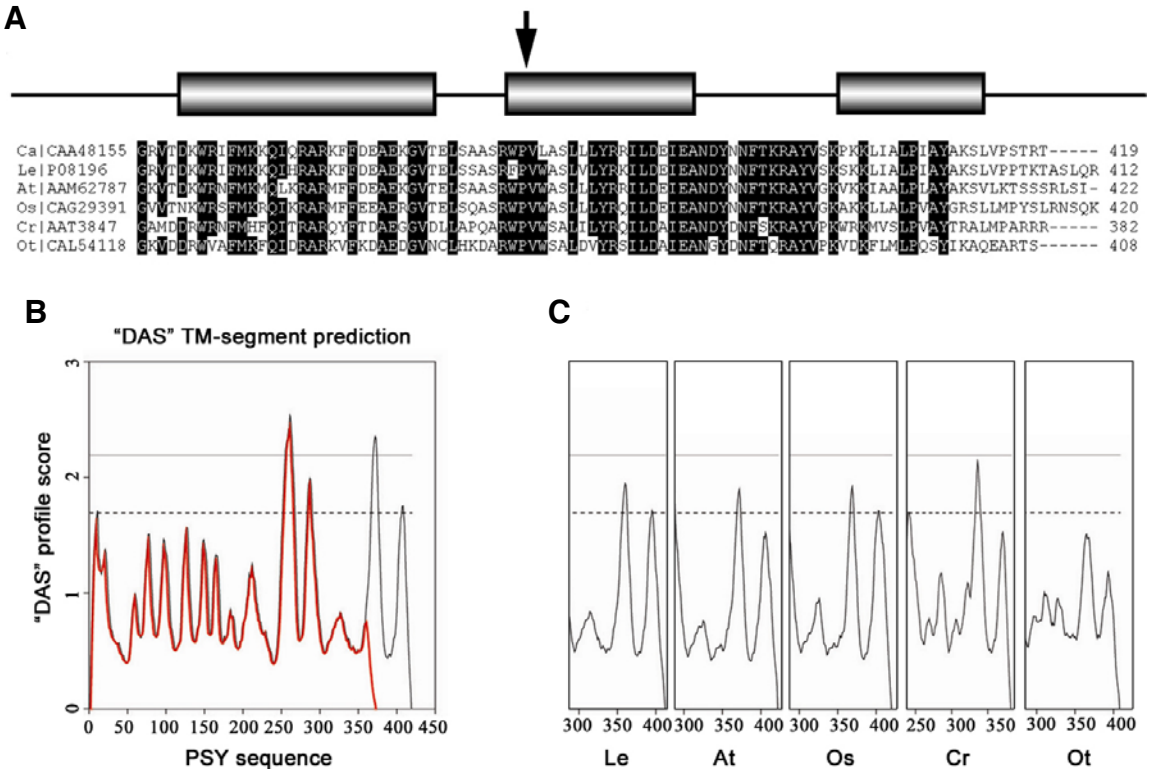

Fig. 3. Transmembrane region at the carboxy-terminus of PSY. (A) Multiple sequence alignment of PSY C-termini among various organisms. Gray cylinders above the primary sequence indicate α-helices. The arrow indicates the junction between the 5th and 6th exons. Ca, *Capsicum annuum*; Le, *Lycopersicon esculentum*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Cr, *Chlamydomonas reinhardtii*, Ot, *Osterococcus tauri*. GenBank Accession Numbers are given next to their origin. (B) Prediction of PSY transmembrane regions using DAS. Transmembrane probability plot of orange Habanero PSY is colored in red and overlapped with that of *C. annuum* PSY (CAA48155). The x-axis represents amino acid position. The solid horizontal line indicates a strict cutoff of the DAS profile score, and a dashed line indicates a loose cutoff. (C) Prediction of PSY transmembrane regions in other organisms shown in (A).

pared to expression in TF68 (Fig. 2D). We assumed that the different TF68 and Habanero genetic backgrounds might influence *PSY* expression patterns. To rule out this possibility, *PSY* expression was examined in ripening fruits of two near-isogenic cultivars, the orange Habanero and the Red 'Savina' Habanero. At the breaker stage, expression of *PSY* was slightly higher in the orange Habanero than in the Red 'Savina' (Fig. 2E). However, upregulation of *PSY* during the IR stage was more dramatic in orange Habanero fruits (Fig. 2E). This suggests that a feedback control exists to enhance *PSY* expression to compensate for decreased enzyme activity when an insufficient amount of phytoene is supplied for carotenoid biosynthesis.

The C-terminus of PSY is important for function

PSY is an essential enzyme in the carotenoid biosynthesis pathway and is evolutionarily conserved from photosynthetic bacteria to higher plants. Yeast and mammals utilize squalene synthase (SQS) that catalyzes the similar head-to-head condensation of two molecules of C_{15} farnesyl diphosphate to form squalene, a C_{30} triterpene (Tansey and Shechter, 2000). Even though human SQS shares little overall homology with PSY, amino acids comprising the catalytic cleft are well conserved in both enzymes, and thus, these two proteins are predicted to fold in similar ways as they are functionally related (Pandit et al., 2000). Despite the lack of significant similarities, the C-terminal regions of both SQS and PSY are thought to be membrane-anchored, presumably to facilitate the release of lipophilic squalene and phytoene products closer to the membrane (Pandit et al., 2000). The C-terminus of *Capsicum* SQS was predicted to serve as a membrane anchor (Lee et al., 2002). In *Narcissus pseudonarcissus*, the majority of active PSY forms were also found associated with the chromoplast membrane (Schledz et al., 1996). The C-termini of PSY from diverse organisms share common structural characteristics (Fig. 3A). Based on Dense Alignment Surface (DAS) Transmembrane Prediction analysis (Cserzo et al., 1997), two C-terminal α-helices of TF68 PSY are predicted as transmembrane regions and could serve as membrane anchors in the chromoplast (Fig. 3B). However, Habanero PSY is devoid of a C-terminal transmembrane region due to premature translational termination caused by the aberrant splicing (Fig. 3B). Such PSY transmembrane regions with two α-helices are conserved in both photosynthetic prokaryotes and eukaryotes (Figs. 3A and 3C). Thus, it is likely that Habanero PSY is either less tightly or not tethered to the chromoplast membrane, thereby decreasing immediate accessibility of the substrate GGPP to the enzyme, since the production of GGPP by geranylgeranyl pyrophosphate synthase (GGPS) is also known to occur in the same multimeric complex including both GGPS and PSY (Fraser et al., 2000). Unbound Habanero PSY may retain moderate catalytic activity, as orange Habanero fruits are still capable of producing capsanthin and capsorubin, albeit the level of each carotenoid pigment is significantly decreased (Huh et al., 2001).

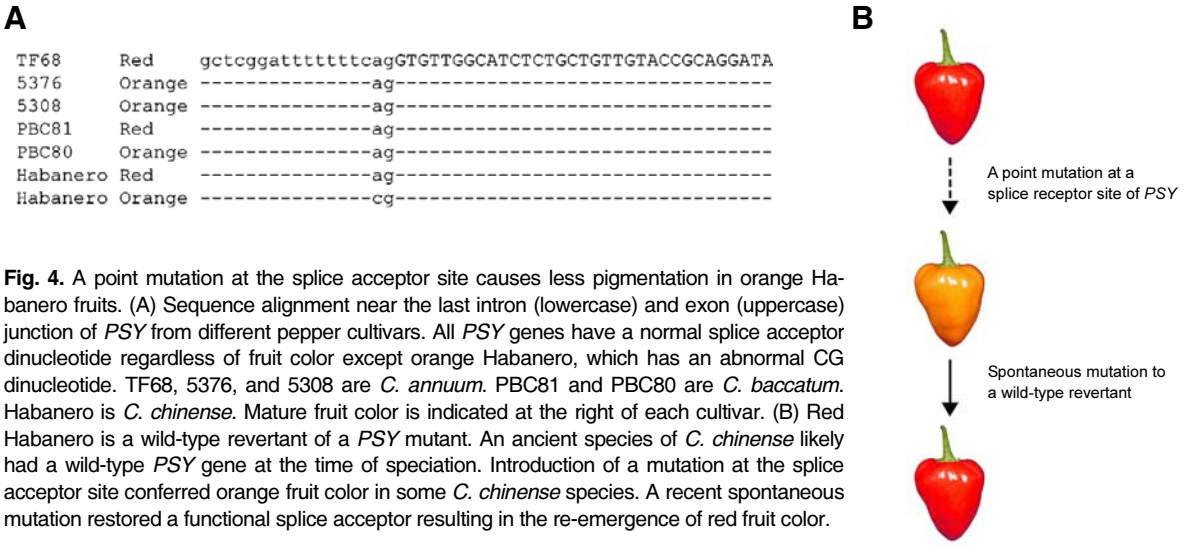

Fig. 4. A point mutation at the splice acceptor site causes less pigmentation in orange Habanero fruits. (A) Sequence alignment near the last intron (lowercase) and exon (uppercase) junction of *PSY* from different pepper cultivars. All *PSY* genes have a normal splice acceptor dinucleotide regardless of fruit color except orange Habanero, which has an abnormal CG dinucleotide. TF68, 5376, and 5308 are *C. annuum*. PBC81 and PBC80 are *C. baccatum*. Habanero is *C. chinense*. Mature fruit color is indicated at the right of each cultivar. (B) Red Habanero is a wild-type revertant of a *PSY* mutant. An ancient species of *C. chinense* likely had a wild-type *PSY* gene at the time of speciation. Introduction of a mutation at the splice acceptor site conferred orange fruit color in some *C. chinense* species. A recent spontaneous mutation restored a functional splice acceptor resulting in the re-emergence of red fruit color.

Red Habanero has a wild type PSY gene

We next investigated whether the point mutation at the splice acceptor dinucleotide was solely responsible for the fruit color phenotype in other orange pepper cultivars. We examined the genomic regions around the 5th intron-6th exon junction of the *PSY* gene in seven pepper cultivars of three different species *C. annuum*, *C. baccatum*, and *C. chinense*. Interestingly, except for the orange Habanero, all *PSY* genes were found to have the same normal AG dinucleotide at a splice acceptor site regardless of the fruit color (Fig. 4A). This implies that the recessive *c2* allele accompanied with abnormal splicing does not account for all orange fruit color phenotypes in the genus *Capsicum*. More strikingly, the two *PSY* genes from Red 'Savina' Habanero and orange Habanero were entirely identical to each other with only one exception: the AG → CG transition at a splice acceptor site (Fig. 4A). This strongly suggests that both red and orange Habanero cultivars diverged very recently. Considering the sequence similarity of the *PSY* genes between these two cultivars, it is unlikely that the wild type *PSY* gene was introduced to the Red 'Savina' Habanero from other red pepper species advertently or inadvertently via crossing, because a moderate level of sequence variations exists for *PSY* among the different cultivars (data not shown). It was reported that, in contrast to the low level of carotenoid content in orange Habanero (Huh et al., 2001), Red 'Savina' Habanero fruits have a comparably high level of carotenoids such as capsanthin, one of the end products of the *Capsicum*-specific carotenoid biosynthesis pathway (Howard et al., 2000).

DISCUSSION

Peppers have a variety of carotenoid-dependent mature fruit colors, and *PSY* and *CCS* are two major genes implicated in this pigmentation diversity (Ha et al., 2007; Huh et al., 2001; Hurtado-Hernandez and Smith, 1985; Lang et al., 2004; Lefebvre et al., 1998; Popovsky and Paran, 2000; Thorup et al., 2000). *CCS* has been identified as a *y* locus gene discriminating yellow and red colors in mature fruits (Lefebvre et al., 1998), and the yellow color is caused either by a deletion (Lefebvre et al., 1998) or by structural mutations including a premature stop codon and a frame-shift (Ha et al., 2007). Several studies revealed that a mutation in *CCS* is also responsible for the orange fruit color due either to an absence of expression (Ha et al., 2007; Lang et al., 2004) or to deletions or mutations in the coding sequences (Popovsky and Paran, 2000). Despite the complexity of the mature fruit color phenotype, few genes other than *PSY* and *CCS* have been reported to affect fruit color in peppers. Other components in the carotenoid biosynthesis pathway might have been under strong selective pressure, with very little sequence divergence allowed, because their mutations might greatly compromise the synthesis of some essential xanthophylls such as zeaxanthin, which is crucial for light harvesting and photoprotection in the chloroplast reaction center (Holt et al., 2005).

Many carotenogenic genes are thought to have undergone gene duplication, establishing parallel carotenoid biosynthesis pathways (Galpaz et al., 2006). For example, a second copy of the *PSY* gene (*PSY2*) has been found in many species including maize, rice, and tomato, and its expression is prevalent in leaf tissues (Bartley et al., 1992; Gallagher et al., 2004). In tomato, *PSY2* is constitutively expressed in leaf tissues while *PSY1* expression is confined to flowers and/or fruits (Bartley and Scolnik, 1993; Bartley et al., 1992; Fraser et al., 1999). Because orange Habanero displays no defects in green tissues, it is conceivable that another *PSY* gene is present in the *Capsicum* genome, which might function independently of chromoplast-specific carotenoid biosynthesis.

Given the fact that a total of twelve SNPs exist between red TF68 and orange Habanero *PSY* (Fig. 1A, Supplementary Table S1), the presence of only one SNP between Red 'Savina' Habanero and orange Habanero *PSY* is quite surprising. The most plausible scenario is that *C. chinense* originally carried the *c2*+ allele with a red fruit color phenotype, and a later mutation gave rise to the orange fruit color in Habanero. However, in 1989 a natural red Habanero plant variant was found in a field of orange Habanero peppers. Seeds from the single plant were grown, and through selective breeding the Red 'Savina' Habanero was developed. In 1994, the Red 'Savina' Habanero set a world record for heat at 577,000 Scoville units (Krebs, 1995). This story signifies that orange Habanero appeared first, and red Habanero emerged from it very recently. We thus hypothesize that reintroduction of a spontaneous mutation into a once-mutated splice acceptor sequence of the orange Habanero *PSY* resulted in the recovery of normal splicing patterns (Fig. 4B). Consequently, Red 'Savina' Habanero displays red fruit color by restoring the normal carotenoid level owing to reacqui-

sition of the PSY activity once lost.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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