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Research Paper

Development of IP and SCAR markers linked to the yellow seed color gene in *Brassica juncea* L.

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Previous studies showed that the yellow seed color gene of a yellow mustard was located on the A09 chromosome. In this study, the sequences of the molecular markers linked to the yellow seed color gene were analyzed, the gene was primarily mapped to an interval of 23.304 to 29.402M. Twenty genes and eight markers' sequences in this region were selected to design the IP and SCAR primers. These primers were used to screen a BC_8S_1 population consisting of 1256 individuals. As a result, five IP and five SCAR markers were successfully developed. IP4 and Y1 were located on either side of the yellow seed color gene at a distance of 0.1 and 0.3 cM, respectively. IP1, IP2 and IP3 derived from *Bra036827*, *Bra036828*, *Bra036829* separately, co-segregated with the target gene. BLAST analysis indicated that the sequences of newly developed markers showed good collinearity with those of the A09 chromosome, and that the target gene might exist between 27.079 and 27.616M. In light of annotations of the genes in this region, only *Bra036828* is associated with flavonoid biosynthesis. This gene has high similarity with the *TRANSPARENT TESTA6* gene, *Bra036828* was hence identified as being the gene possibly responsible for yellow seed color, in our research.

Key Words: *Brassica juncea*, yellow seed color gene, fine mapping, IP and SCAR.

Introduction

Rapeseed is one of the most important oil crops in China. The total growing area of rapeseed exceeds 100 million acres in China every year, but it still cannot satisfy the demands of the Chinese market. Every year the Chinese government imports millions of tons of vegetable oil from other countries (Wang 2006). So the main goal of rapeseed breeding in China is to improve oil production per unit area. Oil content is one component of oil production, many studies have shown that the oil content of yellow seeded rapeseed is higher than that of black seeded rapeseed with the same genetic background (Abraham and Bhatia 1986). Therefore, yellow seed breeding is considered as one effective way to improve oil content. However, few studies of pure yellow seeded materials in *B. napus* have been reported, due to limited sources of yellow seeded rapeseed germplasm in *B. napus* worldwide. *B. juncea*, an allotetraploid species, possesses some pure yellow seed types. A yellow mustard is the main rapeseed variety in the northwest of China. The yellow seed color of this mustard was controlled by a single recessive gene, which was mapped to the A09 chromosome in *B. rapa* (Huang *et al.* 2012).

Some studies have been conducted on the inheritance and mapping of the yellow seed color genes in *Brassica* (Rahman *et al.* 2007, Xiao *et al.* 2007). Xiao *et al.* (2012) identified 17 AFLP and SSR markers linked to a yellow seed color gene from "Dahuang", which is a *B. rapa* landrace in Qinghai-Tibetan plateau, China. Liu *et al.* (2005) constructed a genetic map around the yellow seed color gene in a re-synthesized *B. napus*, lines No. 2127-17, the yellow seed color gene was mapped in a region of 7.0 cM. However, all of the yellow seed color genes above were derived from the A genome in *Brassica*. Research about yellow seed color genes in the B genome can be rarely found. Padmaja *et al.* (2005) studied a *B. juncea* yellow seeded line, the yellow seed color was controlled by two independent loci (*BjSc1* and *BjSc2*), the two yellow seed color genes were mapped to two linkage groups, LG A9 and B3, respectively.

Before the publication of the *B. rapa* genome sequence, gene mapping and cloning in *Brassica* were mainly based on the published genome of *Arabidopsis*, which is a member of the *Brassica* family and has been serving as a model species. High levels of synteny and remarkably conserved genome structure have been found between *Arabidopsis* and *Brassica* genomes (Mun *et al.* 2009). In *Arabidopsis*, there

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are some *TT* (*TRANSPARENT TESTA*) or *TTG* (*TRANSPARENT TESTA GLABRA*) genes that are involved in seed coat pigmentation, a process involving flavonoid biosynthesis (Debeaujon *et al.* 2001, Routaboul *et al.* 2006). It is suggested that the information of these *TT* or *TTG* genes is helpful for studying yellow seed in *Brassica*. *TTG1* and *TT8* in *B. rapa*, *TT10* in the eight *Brassica* lines that have been cloned successfully (Li *et al.* 2012, Zhang *et al.* 2009, 2013).

Thanks to the power of next generation sequencing technology, the *B. rapa* genome has been completely sequenced and published in the public domain (Wang *et al.* 2011). The sequence information can be employed freely in developing markers linked to genes of interest, and in fine mapping or cloning target genes in *Brassica*. Developing Intron Polymorphism (IP) markers is a very effective method for gene mapping, which has already exhibited its powerful application specifically in narrowing the chromosome region of a target gene. Lei *et al.* (2007) developed a dominant amplified consensus genetic marker (ACGM, equivalent to IP marker), which was more closely linked to *BnMs2*, and this marker reduced the candidate chromosome interval. Ban *et al.* (2013) developed three IP markers based on the sequences of *Arabidopsis* and *B. rapa* to narrow down the mapping region of the yellow seed color gene.

In this paper, we studied a yellow seeded *B. juncea* line popular in northern Shaanxi, China. A previous study (Huang *et al.* 2012) reported that the yellow seed color gene was located on the A09 chromosome in *B. rapa*. In order to fine map the yellow seed color gene, we made use of the genome sequences of *B. rapa* to develop the IP and SCAR markers. We then fine mapped the yellow seed color gene and predicted the possible genes responsible for yellow seed color. This study will provide a useful clue for cloning the yellow seed color gene.

Materials and Methods

Plant materials and population construction

A BC_8S_1 population consisting of 1256 individuals derived from a yellow seeded landrace 'Wuqi mustard' and a brown seeded landrace 'Wugong mustard' was developed for gene mapping. The 'Wuqi mustard" was used as the recurrent parent to cross with the brown seeded individuals of the BC population in every generation. Finally, a brown seeded plant in the BC_8 population was selected for selfing to produce a BC_8S_1 population. Every individual in BC_8S_1 was selfed to produce BC_8S_2 seeds and each BC_8S_2 line was sown in field to produce BC_8S_3 seeds. The genotypes underlying seed coat color of BC_8S_2 individuals were determined according to the phenotypes of the BC_8S_3 seeds. Similarly, the genotypes of every BC_8S_1 individual were inferred. Three genotypes in the BC_8S_1 population (homozygous brown seeded, heterozygous brown seeded and yellow seeded plants) were found and distinguished. All of the field experiments were carried out in the field of Northwest A&F University, Yangling, Shaanxi, China.

DNA extraction and bulked segregant analysis

Genomic DNA was extracted from young leaves of BC_8S_1 individuals at the seedling stage by the CTAB method (Doyle and Doyle 1990). Equal quantities of DNA from twelve yellow seeded BC_8S_1 plants and twelve brown seeded BC_8S_1 plants were pooled to form the yellow bulk (BY) and brown bulk (BB), respectively. The final DNA concentration was adjusted to 50 ng/μl.

Gene location analysis on the A genome

Huang *et al.* (2012) identified 23 SSR and AFLP markers linked to the yellow seed color gene, which was mapped to the A09 chromosome in *B. rapa*. In our research, we first used the markers identified by Huang *et al.* (2012) to screen a partial mapping population of 96 BC_8S_1 individuals. The AFLPs and SSRs amplifications were performed as described by Vos *et al.* (1995) and Lowe *et al.* (2002), respectively. The PCR products were separated on a 6% denaturing polyacrylamide gel. Silver staining was carried out using the method reported by Yi *et al.* (2006). Mapmaker 3.0 software (Lander *et al.* 1987, Lincoln *et al.* 1992) was used to analyze the linkage between markers and the gene. Secondly we sequenced AFLP and SSR markers used in this study and BLAST searched the *B. rapa* genome (http://brassicadb.org/brad, version 1.5).

Development of IP and SCAR markers linked to the yellow seed color gene

Through analyzing previous markers' sequences, we found that the gene was located between 23.304 and 29.402M on the A09 chromosome in *B. rapa* (**Fig. 1** and **Table 1**). We randomly selected genes in this region and designed primers according to the sequences of these genes. At the same time, we also used the markers' sequences of this region published on the website: http://brassicadb.org/brad to develop new SCAR markers. Finally, we selected 20 genes and 8 markers' sequences to design 28 pairs of primers. These primers were used to screen a small segregated population comprised of 12 yellow seeded individuals (*yy*) and 12 homozygous brown seeded plants (*YY*) in BC_8S_1 population. The PCR products were separated on a 6% denaturing polyacrylamide gel to test whether or not there was polymorphism between the phenotypes. The specific fragments that showed reproducible polymorphism between brown seeded and yellow seeded individuals were regarded as markers linked to the yellow seed color gene.

Linkage analysis

The newly developed markers in this experiment and the previously identified markers (Huang *et al.* 2012) were used to amplify BC_8S_1 populations (1256 individuals), the results were analyzed using MAPMAKER/EXP 3.0 program (Lander *et al.* 1987, Lincoln *et al.* 1992). A minimum LOD score of 3.0 was used for map construction. Map distances were calculated using Kosambi's (1943) mapping function.

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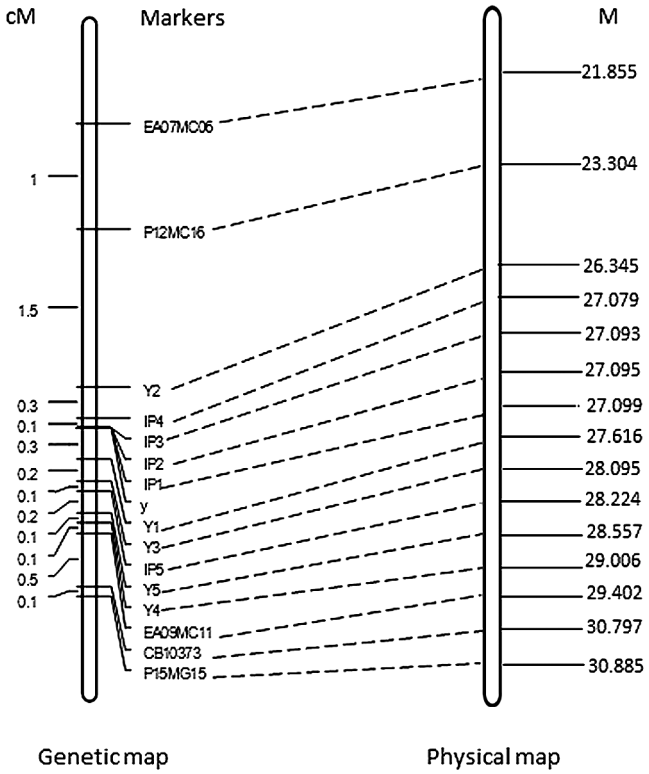

Fig. 1. The left map is a genetic map surrounding the yellow seed color gene (y) from the BC_8S_1 population; the right map is a partial physical map of the A09 chromosome of *B. rapa* showing the homologues of mapped markers sequences. Dotted lines indicate the relationship of the two maps.

Construction of the physical map around the yellow seed color gene

Mapdraw 2.9 (Liu *et al.* 2003) was used to construct a high resolution genetic map around the target gene. All of the markers' sequences were BLAST analyzed against the *B. rapa* genome (http://brassicadb.org/brad, version 1.5). The collinearity between the sequences of the genetic markers and those of the A09 chromosome was compared using the BLAST tool found on the website: http://brassicadb.org/brad. All of the genes' annotations within the mapping region were retrieved from the publicly accessible *Brassica* genetic database (http://brassicadb.org/brad). The genes related to flavonoid biosynthesis were selected for assumption as the possible genes responsible for yellow seed color in *B. juncea*.

Results

Genetics of yellow seed color in B. juncea

There were 1256 individuals in the BC_8S_1 population, which included 305 homozygous brown seeded individuals, 661 heterozygous brown seeded and 290 yellow seeded plants. This segregation of 3 genotypes was consistent with the expected ratio of 1:2:1 ($\chi^2 = 3.83$, and $p > 0.05$), indicating that yellow seed color is controlled by a single Mendelian recessive gene.

Preliminary analysis of the gene's location on the A genome

In order to confirm the primary location of the yellow seed color gene on the A genome, five molecular markers (P15MG15, CB10373, EA09MC11, EA07MC06 and P12MC16) located on the either side of gene (Huang *et al.* 2012) were sequenced and used to screen 96 plants from the BC_8S_1 population. The results showed that P12MC16 and EA09MC11 were the two closest markers linked to the yellow seed color gene. Through comparing the markers' sequences with the *B. rapa* genome, these five markers' sequences showed good collinearity with the A09 chromosome of *B. rapa* (**Fig. 1**). The homologues of P12MC16 and EA09MC11 were located between 23.304 and 29.402M on the A09 chromosome. Therefore, we primarily justified that the gene responsible for yellow seed color might also exist between 23.304 and 29.402M on the A09 chromosome.

Table 1. Characterization of molecular markers linked to yellow seed color gene

Markers	Sequences (5′-3′)	Homologous *B. rapa* gene and *E* value	Locations on A09
Y1	TAAAGGGGTGGACAATAACA/GGTCAGAAGTGTTACGGGT	Bra006909, 2e-33	27616367..27616462
Y2	TCTACCCCATAACTGCATTC/CAGCTAATGAAGCCAAAACT	Bra036036, 1e-72	26345808..26345945
Y3	GGTGGCGTATCCGTAAAGGTAGA/CGCCGTCGCTGCTCCACT	Bra006970, 1e-67	28095119..28095357
Y4	TCCCGTATCAATGGCGTAACAG/CGATGGTGACATTATTGTGGCG	Bra007133, 3e-08	29006078..29006215
Y5	TCAAGCTACTACCTTTCAAGC/TTGACTCACTTCGAGTCTGA	Bra007049, 2e-45	28557476..28557567
IP1	GGCTTAAGAGGGGAAACGAG/ACCGAACCAGTGATGAGTCC	Bra036827,7e-47	27099538..27099895
IP2	CTTACCTCCTGAGGAGAAACTCA/CCGTGAGTAGTCTCTGTTTC	Bra036828, e-38	27095848..27096349
IP3	GAGGCTGTCACTCTCTTCGG/CGAGGAGCTGAGACAAAACC	Bra036829, 1e-63	27093092..27093399
IP4	ATCAAATGGAAACGACCTGC/ CCAACGGATTTTGCTTGTTT	Bra036832, 4e-74	27079608..27079959
IP5	GAATCAAGTGCTAAGAGAGATGGTC/CATCTGAACCATCATATGCGAAC	Bra006991, 5e-25	28224201-28224487
CB10373	CGGTCAGATTCCAACAGA/GCCATCTCAGAGACGACA	Bra007453, 2e-65	30797156..30797514
EA09MC11	GACTGCGTACCAATTCACA/GATGAGTCCTGAGTAACCC	Bra007194, 2e-32	29402040..29402207
EA07MC06	GACTGCGTACCAATTCATC/GATGAGTCCTGAGTAACTT	Bra032392, 7e-56	21855926..21856106
P15MG15	GACTGCGTACATGCAGACA/GATGAGTCCTGAGTAAGGC	Bra007469, 7e-71	30885797..30885935
P12MC16	GACTGCGTACATGCAGTGA/GATGAGTCCTGAGTAACGG	Bra032885, 2e-55	23304264..23304372

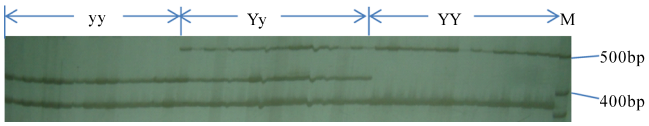

Fig. 2. Analysis of the PCR products obtained using IP2 on BC_8S_1 plants. The BC_8S_1 individuals are represented as yellow seeds (yy), brown seeds (Yy, heterozygous and YY, homozygous). M: 100 bp DNA ladder.

Development of IP and SCAR markers linked to the yellow seed color gene

28 pairs of primers were designed according to the sequences of the region from 23.304 to 29.402M on the A09 chromosome. Five pairs of primers IP1, IP2, IP3, IP4 and IP5 derived from *Bra036827*, *Bra036828*, *Bra036829*, *Bra036832* and *Bra006991*, respectively, could show polymorphisms between 12 yellow seeded individuals (*yy*) and 12 homozygous brown seeded plants (*YY*). All of them were co-dominant markers that could distinguish the heterozygous brown seeded (*Yy*) and homozygous brown seeded plants (*YY*) (**Fig. 2**). Five pairs of primers Y1, Y2, Y3, Y4 and Y5 derived from BrID10539, BrID10955, 8C0462, BRMS016 and BrID10541 respectively (http://brassicadb.org/brad) could also amplify the polymorphic bands in the small population comprised of 12 yellow seeded individuals (*yy*) and 12 homozygous brown seeded plants (*YY*). These five pairs of primers were verified as the SCAR markers linked to the yellow seed color gene. All of the newly developed markers' information is in **Table 1**.

Linkage analysis

The two farthest markers, P15MG15 and EA07MC06, were used to screen 1256 individuals in the BC_8S_1 population, resulting in identification of 18 recombinants (**Fig. 3**). Next, all of the markers, including the newly developed markers and the other three AFLP and SSR markers previously used in our study, were used to screen these recombinants. As a result, a high resolution genetic map around the yellow seed color gene was constructed. There were 15 molecular markers on this map, spanning a region of 4.5 cM, with an average genetic distance of 0.3 cM between the two adjacent markers. Three IP markers derived from *Bra036827*, *Bra036828* and *Bra036829* were co-segregated with the target gene. IP4 and Y1 were located on either side of the gene, and the genetic distance was only 0.1 and 0.3 cM, respectively (**Fig. 1**).

Construction of the physical map around the yellow seed color gene

All of the molecular markers' sequences on the genetic map around the yellow seed color gene showed good collinearity with those of the A09 chromosome, and the gene was located between 27.079 and 27.616M on the A09 chromosome. This region was approximately 0.54M with only 95 genes residing. The locations of IP1, IP2 and IP3 were in 27.099M (*Bra036827*), 27.095M (*Bra036828*) and 27.093M (*Bra036829*), respectively, these three markers co-segregated with the target gene (**Fig. 1**). The functions of 95 genes in the gene region were analyzed according to the annotations published on the website: http://brassicadb.org/brad. Because *Bra036828* is associated with pigment formation such as flavones, and it is highly homologous with *F3H* (*TRANSPARENT TESTA 6*, *tt6*, *E* value, 0.0), it is possible that *Bra036828* is one of the genes responsible for yellow seed color in *B. juncea* in our research.

Discussion

IP markers in gene fine mapping

Mapping the yellow seed color gene to the A09 chromosome was the key step to fine mapping the yellow seed color gene (Huang *et al.* 2012). Through analyzing the sequences of previously published molecular markers linked to the target gene, the yellow seed color gene was mapped in a region of 6M on the A09 chromosome. However, it was extremely challenging to narrow down the mapping region due to the unavailability of the *B. rapa* genomic sequences

Recombinants	EA07MC06	P12MC16	Y2	IP4	IP3	IP2	IP1	Y1	Y3	IP5	Y5	Y4	EA09MC11	CB10373	P15MG15	Phenotypes
489																y
36																y
1211																y
265																y
1011																y
52																y
368																b
829																b
993																b
982																b
310																b
498																b
1026																b
901																b
1165																b
1071																b
779																b
72																b

Fig. 3. Genotypes and phenotypes of recombinants selected from the BC_8S_1 population. Recombinants and phenotypes (y for yellow seed, b for brown seed) are denoted on the left and right, respectively, with marker names at the top. The yellow seeded alleles are denoted in white and brown seeded alleles in gray. IP1, IP2, and IP3 have no recombinant.

before publication of the mapping results. Utilization of the *Arabidopsis* genome as a reference didn't improve the situation on account of the differences in genome size and genome sequences between the two species. Fortunately, the whole genome sequence of *B. rapa* (Chiifu-401) was published by the multinational *Brassica* genome project (BrGSP) (Wang *et al.* 2011), which provided vital information for gene mapping on the A genome. Some studies showed that the A genome had retained a high degree of collinearity between *Brassica* species (Parking *et al.* 2005, Suwabe *et al.* 2008). Therefore, marker development or candidate gene prediction in *B. juncea* could likely be carried out according to the whole genome sequence of *B. rapa*. IP marker discovery was a very effective way to develop the linked markers. Sometimes, the sequence of an exon in a gene is conserved, but its intron sequence varies. If a pair of primers whose sequences were located in two different exons, were used to amplify the introns, the products may display differences in band size between the yellow seeded and the brown seeded individuals. This approach has been successfully employed by some researchers. Xia *et al.* (2012) developed nine IP markers linked to a sterile gene in *B. napus* using the sequences of *Arabidopsis* and *B. rapa*. In previous research, the yellow seed color gene was mapped to a region of 6M on the A09 chromosome through analyzing the AFLP and SSR markers' sequences. Now that the sequence of the gene region has been completely published, it becomes practically possible to develop dense markers to narrow down the genomic sequence region where the yellow seed color gene is localized. Eventually, five IP markers and five SCAR markers were developed successfully. The gene region was reduced to approximate 0.54M and three co-segregated IP markers in this region were developed. Through analyzing a bigger population, it is believed that we can confirm linkage between these three co-segregated IP markers and the yellow seed color gene, therefore our next work is to use different genetic populations or a bigger population to determine the gene region.

Co-dominant markers in MAS

Because of the rarity and instability of yellow seeds in *B. napus*, it is essential to transfer yellow seeded germplasms from *B. juncea* or *B. rapa* to *B. napus*. Because conventional breeding in field is complicated and time-consuming, it takes a long time and a large space to improve the yellow seed in *B. napus*. The co-dominant markers linked to the yellow seed color gene are helpful for the selection of the yellow seed color gene in a segregated population. IP1, IP2, IP3, IP4 and IP5 identified in this study are co-dominant markers linked to the yellow seed color gene and three of them are co-segregated with the yellow seed color gene. These markers can distinguish three genotypes in yellow seed locus (*yy*, *Yy* and *YY*), which can help breeders accurately select yellow seed individuals in separated populations. The utilization of these molecular markers combined with comprehensive trait observation in field will not only save time and space for breeders, but also the accuracy of selection will be increased substantially.

Prediction of possible genes responsible for yellow seed color in B. juncea

In *Arabidopsis*, mutants of the genes for flavonoid biosynthesis produce transparent testa (*tt*) and yellow seed. Some genes regulating flavonoid biosynthesis have been found in *Brassica* species and close allies of *Arabidopsis*. Zhang *et al.* (2009) reported on the *TTG1* gene responsible for the yellow seeded trait in Chinese cabbage; there was a 94-base deletion for this gene in the yellow seeded line. Li *et al.* (2012) found that a large insertion of transposable elements in the *BrTT8* gene in yellow sarson caused the yellow seed. It is possible that some *TT* genes are also responsible for yellow seed synthesis in this study. Through analyzing the annotations of genes in the mapping region, a gene, *Bra036828*, is related to the flavones pathway and there is high homologue with the *F3H* gene (*TT6*). Therefore it was tempting to predict that *Bra036828* was one gene responsible for yellow seed color formation. However, this prediction should be confirmed by a functional complementation experiment, which is currently an on-going project in our lab.

Analysis of yellow seed color gene in Brassica genomes

B. juncea is an *allotetraploid* species containing genome A and B. It is reasonable to predict that multiple genes from both genomes are responsible for yellow seed color in *B. juncea*. Padmaja *et al.* (2005) mapped two yellow seed color genes to A9 and B3 through QTL mapping. However, our meticulous study on the inheritance of yellow seed color in *B. juncea* through classical Mendel statistical analysis revealed that yellow seed color was controlled by a single gene, which was consistent with a previous publication (Xu *et al.* 2010). In order to investigate the location of the underlying gene in *Brassica* genomes, the sequences of the newly developed markers for yellow seed color gene were submitted to the BRAD website for BLAST searches in a hope to estimate the location of the gene. All the BLAST hits with high similarity turned out to fall in a region of 0.54M on chromosome A09. Due to the unavailability of B genome sequences in the public domain, it is unlikely to perform BLAST analysis of these marker sequences against the B genome, hence the possibility of the presence of orthologous genes in the B genome cannot be ruled out at this moment. However, numerous orthologous genes were identified on different chromosomes of *B. oleracea* through BLAST search. For instance, *Bra036828* in *B. rapa* exhibits high similarity with a sequence (34.01M) on the C08 chromosome (*E* value = 109, $4e^{-22}$), indicative of a homologous gene present in the C genome. It is tempting to assume that multiple genes located in the A and B genomes contribute to the formation of yellow color, and identification and characterization of the genes and subsequent investigation on their interaction becomes key to shedding light on the

mechanism behind yellow color formation. A tremendous effort is still directed to addressing this in our lab right now.

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