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Molecular mapping and candidate gene analysis for yellow fruit flesh in cucumber

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Abstract Vitamin A deficiency is a major health problem in many countries around the world. Efforts to alleviate this have included adding supplements to wheat and maize flour. China produces over 70 % of the world production of cucumbers, and any attribute that could increase the β-carotene content of the fruit has the potential to have a major impact on world health. Cucumbers with yellow flesh contain larger amounts of β-carotene than those with white and green flesh. In this study, yellow fruit flesh cucumber inbred line PI200815 and white fruit flesh inbred line 931 were used as parents to construct a population for genetic analysis of cucumber fruit flesh color. The F_2 segregating population was analyzed by the bulked segregant analysis method and SSR analysis with 2,112 pairs of SSR primers to build a genetic map using JoinMap 4.0 to map the yellow fruit flesh gene. The results showed that the yellow fruit flesh trait of PI200815 was controlled by a single recessive gene, namely *yf*. A total of 12 SSR primers and five Indel markers were used to build a molecular marker linkage group. The *yf* gene was mapped to cucumber chromosome 7 (Chr. 7). The closest flanking markers linked to *yf* were yfSSR108 and yfIndel29 with genetic distances of 0.6 and 0.3 cM, respectively. The physical distance for the region harboring *yf* was 149.0 kb with 21 predicted candidate genes. The accuracy of marker-assisted selection breeding using the molecular markers, yfSSR108 and yfIndel29, was 92.3 and 84.6 %, respectively.

Keywords *Cucumis sativus* L. · Fruit flesh color · SSR marker · Indel marker · Gene mapping

Introduction

Vitamin A deficiency is a major worldwide health problem. It is estimated that it affects 190 million preschool-aged children and 19 million pregnant and lactating women globally, and 83 million adolescents in Southeast Asia (Klemm et al. 2010). Attempts to alleviate this have included adding supplements of vitamin A to wheat and maize flour in countries where the deficiency is considered to be a major health issue.

Cucumber is one of the world's top ten most widely produced vegetables, so it is understandable that fruit quality is a major focus in breeding programs. Fruit

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quality, including the color of the mesocarp, has an important impact on the marketability of cucumber. The yellow color of the fruit mesocarp of some cucumber varieties depends on the level of β-carotene (Simon and Navazio 1997; Cuevas et al. 2010). This pigment is the precursor for the biosynthesis of vitamin A, which is essential for human health. Therefore, cucumbers with yellow mesocarp would have a greater nutritional value than white- or green-colored varieties. In 2010, Chinese production of cucumber exceeded 40 million tonnes, or more than 70 % of world production (http://en.wikipedia.org/wiki/Cucumber). Therefore, any improvement in the quality of cucumber that would lead to an increase in vitamin A could potentially have a major effect on world diet. Thus, the cultivation of new yellow-fleshed varieties would have the potential to improve the nutritional quality of cucumbers. Chinese landrace cucumbers such as xishuangbanna cucumber (*Cucumis sativus* L. var. xishuangbannanesis Qi et Yuan) exhibit orange flesh when mature, and these have been shown to be cross-compatible with cultivated cucumber (Simon and Navazio 1997).

In 1971, Kooistra (1971) analyzed the inheritance of yellow fruit flesh in cucumber and suggested that flesh color (including orange, yellow, dingy white, and intense white) was determined by two genes. The ratio of dingy white (V_W_): intense white (V_w_): yellow (v_W_): orange (v_w_) was shown to be 9:3:3:1. In the publication of the gene list 2005 for cucumber (Wehner 2005), the two genes were named *wf* and *yf*. Shen (2009) reported that the orange flesh in xishuangbanna cucumber followed quantitative inheritance rules, although some gene interaction may be present. Using β-carotene content as an indicator, Cuevas et al. (2010) classified cucumbers into three grades: low β-carotene content (white, light green, green), moderate (yellow-green, yellow), and high (light orange, orange) in the ratio of 9:6:1, indicating that two pairs of allelic genes were responsible for the yellow mesocarp.

However, there are few reports on cucumber flesh color at the molecular level. Shen (2009) identified three quantitative trait loci (QTLs) related to β-carotene content (βCC4a, βCC4b, and βCC7) and two QTLs related to flesh color (FCG5 and FCG7). Song (2009) constructed two genetic maps and analyzed the QTLs for yellow mesocarp and endocarp. Four and two QTLs for flesh color, accounting for a total of 52 and 38 % of phenotypic variation, and five and three QTLs for endocarp, accounting for 76 and 47 % of phenotypic variation, were identified, respectively. In addition, one QTL for mid-mesocarp and two QTLs for β-carotene content were identified, which accounted for 37 and 43 % of phenotypic variation, respectively. Some of these loci influenced both endocarp and mesocarp, whereas some impacted only one of the two, but all failed to identify a location on any chromosome. Bo et al. (2012) used orange flesh cucumbers that were either the xishuangbanna variety or its derived strains and reported that the gene responsible for orange endocarp of cucumber was located on chromosome 3 (Chr. 3) with a genetic distance of 6 cM. However, the location ranges of the QTL were relatively large, and more research is needed to identify molecular markers tightly linked to endocarp color (<1 cM) for application in breeding. In the present study, yellow flesh inbred line PI200815 and white flesh inbred line 931 were used to construct a genetic population for inheritance analysis and chromosomal mapping of the *yf* gene. The results will provide the foundation for fine mapping, gene cloning, and marker-assisted selection (MAS) breeding of cucumber yellow fruit flesh.

Materials and methods

Experimental materials

Cucumber inbred lines PI200815 and 931 were used as the parental materials for the F_1, reciprocal F_1 (F_1'), and F_2 populations. PI200815 (P_1), a widely known accession, was preserved by Prof. Todd C. Wehner, Department of Horticultural Science, North Carolina State University. It has dark green, oval-shaped fruit, sparse black spines, and yellow fruit flesh (Fig. 1a). 931 (P_2) is a Xintaimici homozygous line, which has strong growth ability, fewer branches than P_1, dark green, long rod-shaped fruit, dense white spines, and white flesh (Fig. 1c). 2,112 pairs of simple sequence repeat (SSR) primers were used to map the *yf* gene (Ren et al. 2009).

Experimental design

All experiments were conducted in the greenhouse of the Institute of Vegetables and Flowers, Chinese

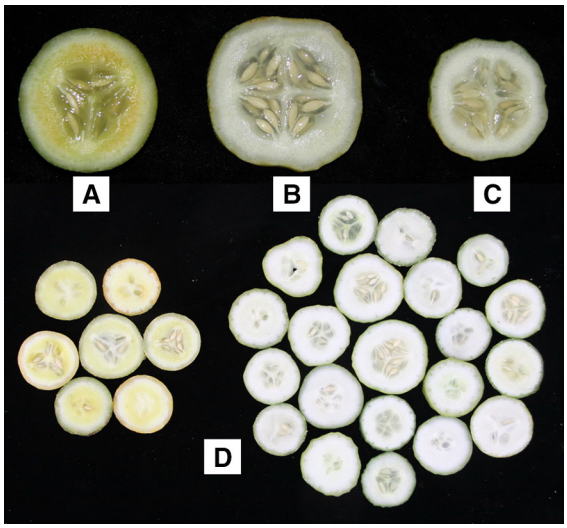

Fig. 1 Phenotype pictures of the parental lines, F_1 and F_2 population. **a** P_1 (PI 200815 with *yellow* fruit flesh); **b** F_1 (*white* fruit flesh); **c** P_2 (931 with *white* fruit flesh); **d** F_2 population with segregation fruit flesh. (Color figure online)

Academy of Agricultural Sciences, during 2012 and 2013. PI200815 (P_1) and 931 (P_2) were used as the parents to develop the F_1, F_1', and F_2 populations. Each of ten plants of P_1, P_2, F_1, F_1' was planted in an experimental greenhouse in spring 2013 and replicated three times in a randomized complete block arrangement. A total of 190 plants of F_2 were also planted. The distances between plants and lines were 25 and 55 cM, respectively.

Trait investigation and statistics

The flesh color of all fruits that had reached physiological maturity (35 days after blooming) on each plant was determined for at least two fruits for each plant. Fruit color was assessed by two investigators. The segregation ratio was analyzed using SAS 9.2 and Microsoft Excel 2003 software.

Construction of SSR linkage groups and preliminary chromosomal mapping for the *yf* gene

The modified CTAB method was used for the isolation of genomic DNA from each of the ten plants used for P_1, P_2, F_1, F_1', and for each plant of the F_2 population. The SSR reaction system was the same to Zhang et al. (2013b). Statistical method for codominant markers: According to JoinMap 4.0, the band pattern of the maternal parent (PI200815) is denoted as a, that of the paternal parent (931) as b, and the heterozygous band as h. Statistical method for dominant marker: If the maternal parent is dominant, then the separated population with the same band pattern as the maternal parent is denoted as d, and that with the same band pattern as the paternal parent is denoted by b. If the paternal parent is dominant, then the separated population with the same band pattern as the maternal parent is denoted as a, and that with the same band pattern as the paternal parent is denoted by c.

Construction of the linkage map: Firstly, screening of SSR markers that showed polymorphisms between paternal and maternal parents; secondly, according to the BSA method (Zhang et al. 2013b), DNA from seven cucumbers with yellow or white flesh in the F_2 generation was used to construct the gene pool for primer screening; thirdly, using the primers that had been screened for polymorphisms, the genotypes of each plant in the F_2 populations were analyzed, and the linkage map was constructed using JoinMap 4.0. The calculate command was first used to calculate the relevant parameters, followed by grouping of the linkage groups with the Groupings (tree) command and LOD $\geq$ 3.0. Finally, the map was drawn with Create Groups for the Mapping and Map commands, and the map distance was calculated.

The linkage map obtained in this study was compared with a previous integration map (Zhang et al. 2012), and the linkage groups and chromosomal location of the *yf* gene were identified.

Development of new molecular markers and second mapping of the *yf* gene

In the preliminary mapping region harboring the *yf* gene, new SSR primers were designed using the full sequence information of the cucumber genome (Huang et al. 2009). The new primers were first used to screen for polymorphisms in the parents. Selected primers were then used in the F_2 population to determine whether or not they were linked to the *yf* gene.

Using BLAST, sequence alignment was conducted between the genome sequence of 115 core germplasms with yellow and white flesh fruits. Insertion and deletion (Indel) markers were designed based on the insertion and deletion points and used in the Indel reaction system described by Zhang et al. (2011).

Validity of the closest marker for MAS breeding

A total of 13 accessions of xishuangbanna cucumber with orange fruit flesh (Table 2) were used to validate the flanking markers closely linked to the *yf* gene, to determine the accuracy of the markers for MAS breeding.

Sequence annotation and gene prediction in the genomic region harboring the *yf* gene

The sequences were aligned with the cucumber genome sequences (Huang et al. 2009) using BLASTN at an *E* value cutoff of 1×10^{-20}. Only matches with an identity of more than 95 % were retained. Gene prediction was performed with the computer program BGF (http://bgf.genomics.org.cn) and verified by FGENESH (http://sunl.softberry.com/) (Salamov and Solovyev 2000), GENESCAN (http://genes.mit.edu/GENSCAN.html) (Burge and Karlin 1997), TwinScan (http://mblab.wustl.edu/software/twinscan) (Korf et al. 2001), and lastly checked manually. InterProScan (http://www.ebi.ac.uk/InterProScan) (Zdobnov and Apweiler 2001) was used for gene annotation.

Results

Inheritance of the yellow fruit flesh trait in cucumber

Fruit flesh colors of the parental lines, PI200815 and 931, were assessed as yellow and white, respectively (Fig. 1a, c). Fruit flesh colors of the F_1 and reciprocal F_1 of PI200815 $\times$ 931 were white (Fig. 1b). In the F_2 population, there were 143 plants with white fruit flesh and 47 plants with yellow fruit flesh (Fig. 1d). Chi-square analysis ($\chi^2 = 0.933 < 3.841$) indicated a segregation ratio of yellow to white of 3:1. Therefore, the yellow fruit flesh trait in PI200815 is controlled by a single recessive nuclear gene, namely *yf*, and is recessive to white.

Construction of SSR linkage groups and preliminary chromosomal mapping for the *yf* gene

From the total of 2,112 pairs of SSR primers tested, 275 (13 %) showed distinct polymorphisms between the parental lines P_1 (PI200815) and P_2 (931). These primers were further used to analyze for polymorphisms in the genomic DNA bulks from the yellow and white fruit flesh plants. Six pairs (2 %) were selected to analyze the DNA from 190 plants in the F_2 population. The resulting data were used to construct a linkage group using JoinMap 4.0 (LOD = 10). The total length of the linkage group was 16.4 cM, and the average genetic distance was 2.7 cM. The *yf* gene was located between SSR17292 and SSR13188 with a genetic distance of 3.1 and 1.9 cM, respectively (Fig. 2a). Combined with the integrated genetic map of cucumber reported by Zhang et al. (2012), the present linkage group had six common markers on cucumber Chr. 7. So the *yf* gene was mapped putatively to the cucumber Chr. 7.

Second mapping of the *yf* gene using new developed molecular markers

Based on the genome sequence at the preliminary mapping region of the *yf* gene, 120 pairs of new SSR primers were designed, among which seven pairs showed polymorphisms among the parents. Using re-sequencing information, the sequence differences in the *yf* mapping region were compared by BLAST analysis, and 40 pairs of Indel markers were designed, five of which showed polymorphisms among the parents. The 12 pairs of SSR primers or Indel markers that were selected were used to analyze the F_2 mapping population. The linkage group was constructed with 13 SSR primers, five Indel markers, and a total length of 17.9 cM (the names and sequences of the primers are shown in Table 1). Among these, the markers most closely linked to the *yf* gene were yfIndel29 and yfSSR108, with genetic distances of 0.3 and 0.6 cM, respectively (Fig. 2b).

Validity of the linked molecular markers for MAS breeding

The veracity of the Indel marker yfIndel29 was tested using 13 accessions of xishuangbanna cucumbers with orange fruit flesh. Two accessions, namely CG9187 and CG9203, did not match with PI200815 (Fig. 3), suggesting that the accuracy of this marker for using in a breeding program would be 84.6 %. And the accuracy of yfSSR108 is 92.3 % with only one accession not matching (Table 2).

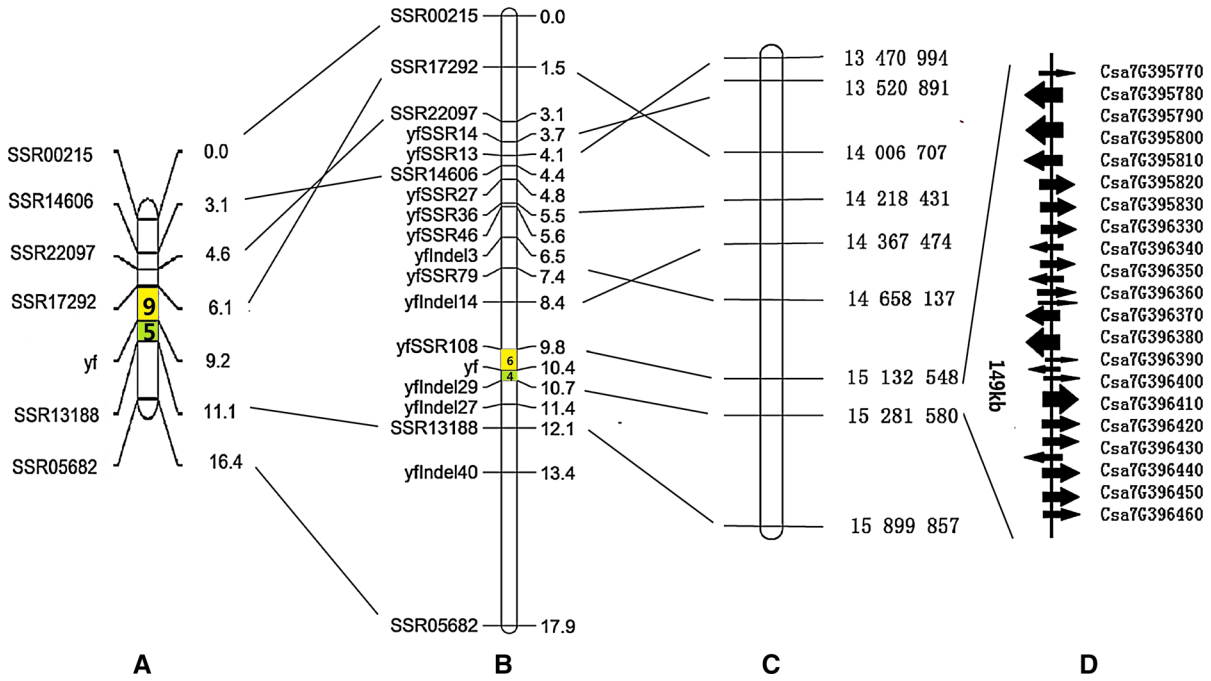

Fig. 2 Molecular marker linkage and chromosomal mapping of the *yellow* fruit flesh gene in cucumber. **a** SSR linkage of the *yellow* fruit flesh gene for preliminary mapping; **b** linkage of molecular markers to the *yellow* fruit flesh gene for second mapping; **c** physical map of partial SSR markers; **d** predicted genes among the flanking markers (the number on the bars presented the number of recombinant plants between the *yf* gene and flanking markers.). (Color figure online)

Table 1 Sequence of SSR and Indel primers for the genetic linkage map of the yellow fruit flesh gene in cucumber

Primer name	Forward primer	Reverse primer
SSR00215	GGTCTCGAATTTTGTGTAACATAGG	CCTTTTACGTTTGTGTGTTAGTTGG
SSR17292	CCCTCTTCTTTCCCACATCA	TGGAAGTGCCAGATGAAATG
SSR22097	TGGCAACTTCTACATTGGATACC	TGGCCAACTTTTCCTAACAA
yfSSR14	ACACATTCCTGGCTGATTAGTT	TTCAAACGAAGGTAGAAGGCT
yfSSR13	TTGGAGCCCGAGTCGTAAAT	TATATTTCTCGTAAAAGGGGT
SSR14606	CCATTTCGTTAATGCCCTTC	CAGCTCACAAACAACAACAACA
yfSSR27	CGATTGGAGTCCCTTTGTAAGT	CAACCACTTAAATTTTCGAGCA
yfSSR36	GCTCTTGATCTGGGTTTTGG	CCAGAATCTACACGGGAGGA
yfSSR46	GATTGCTTGCTTTGGCTTTT	TGGTCCAAAAAGGTGTGTGT
yfIndel3	AAACCTACCAAATGGTTCCT	GACGTGCCAACTTTTTGTTA
yfSSR79	AAGGGGCCTACGAAATCAGT	AGCATCCAATGACCAACACA
yfIndel14	GCTTCTCATTCTCCTCTCAA	CTTTGAGGCTGTCCTTGAC
yfSSR108	TTTGAGGGCACTCACAAGC	CATTCGATCGATGGTGGATT
yfIndel29	CGTGATGAGATGATTTCGTA	GTTGGAATGAATGCTGAAGT
yfIndel27	CCTTCTTCCTGGCTTTGTAT	GAAGAGGAGAAGGAAAAACA
SSR13188	AGGTACGAAACAACGGCAAT	TCGCACTCACTCTTTACCGA
yfIndel40	TCAATCCAGGAGTTTACAGG	AATTGTTGACCGTATTGTGG
SSR05682	CGGAAGTTGTAAAAGATTTTGTTAAT	GAAATGAACGGCCACAATTT

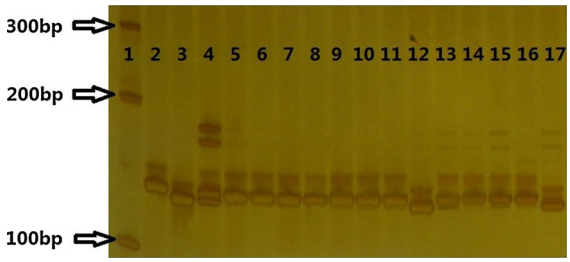

Fig. 3 Marker analysis for 13 accessions of xishuangbanna cucumber with *yellow* fruit flesh using Indel marker yfIndel29. *Lanes 1* to *17*, respectively, represent DNA molecular weight marker, PI200815, 931, F_1, CG9207, CG9142, CG9143, CG9160, CG9164, CG9165, CG9185, CG9187, CG9197, CG9198, CG9199, CG9201, and CG9203. (Color figure online)

Annotation and gene prediction in the genomic region harboring the *yf* gene

The physical distance between yfSSR108 and yfIndel29, two flanking markers tightly linked to the *yf* gene, was about 149 kb. A total of 21 predicted candidate genes are located in the region, and their distribution on the chromosome is shown in Fig. 2. These annotated genes mainly consist of potassium transporters, DNA mismatch repair proteins, protein kinase catalytic domains, glutathione S-transferases, DNA topoisomerases, transcription factors, rapid alkalinization factors, protein kinases, cell signaling factors, and ribosomal proteins (Table S1).

Discussion

Cucumber yellow fruit flesh trait and its inheritance

In the present study, cucumber line 931 with white flesh was crossed with line PI200815 with yellow flesh, and a segregation ratio of white to yellow of 3:1 was determined. This result suggested that the yellow flesh trait in PI200815 was controlled by a single recessive gene, *yf*, which provided a basis for the further study of the *yf* gene on the molecular level.

Development of molecular markers and gene mapping for the *yf* gene in cucumber

The publication of the complete sequence of the Chinese long cultivar (line 9930) provided an invaluable new resource for biological research and rapid breeding of cucurbits. Several SSR genetic maps have been produced, and the integration of a high-density genetic map has been completed. The molecular markers on the high-density genetic map reached 1681 markers (Yang et al. 2010, 2013; Zhang et al. 2012; Ren et al. 2009; Miao et al. 2011; Weng et al. 2010). The genetic mapping of several cucumber genes was finished, including fruit tumor gene (*Tu*) (Zhang et al. 2010b), black spine gene (*B*) (Li et al. 2013), β-carotene content gene (*ore*) (Bo et al. 2012), scab resistance gene (*Ccu*) (Zhang et al. 2010a; Kang et al. 2010), foliage bitterness gene (*Bi-3*) (Zhang et al. 2013a), downy mildew resistance gene (Zhang et al. 2013b), white rind gene (*w*) (Dong et al. 2012), compact plant gene (*cp*) (Li et al. 2011), and leaf color mutant gene (*v-1*) (Miao et al. 2011). However, chromosomal mapping of the yellow flesh gene (*yf*) gene has not been reported.

In the present study, SSR primers were used to map the *yf* gene on Chr. 7 within a region of 4.7 cM. Based on this preliminary mapping information, new SSR primers and Indel markers were used to shorten the region from 4.7 to 0.9 cM, with a physical distance of about 149 kb. These results provided the foundation for fine mapping and molecular cloning of the *yf* gene. The marker position in the linkage group was consistent with previous map (Zhang et al. 2012). For the five QTLs associated with Xishuangbanna orange fruit flesh (β-carotene content is 92.66 mg/kg DW) reported by Shen (2009), βCC7 and FCG7 were very close, which also partially overlaps with the *yf* location in the present study. We speculated that the gene responsible for yellow fruit flesh in PI200815 may be the same as the QTL located on Chr. 7 of xishuangbanna cucumber, but this required further verification.

Application of molecular markers tightly linked to the *yf* gene for MAS breeding

In recent years, DNA molecular marker technology has become widely studied in crop breeding research. Using DNA markers that are closely linked to the quality traits under investigation, MAS can improve breeding efficiency and accelerate the breeding process. Therefore, it was important to identify molecular markers that are tightly linked to the *yf* gene for MAS breeding of new cucumber varieties with a high content of β-carotene.

Table 2 Validity of the Indel marker yfIndel29 tightly linked to the yellow fruit flesh gene was tested using 13 accessions of xishuangbanna cucumber

Material code	Phenotype	yfIndel29	yfSSR108	Material code	Phenotype	yfIndel29	yfSSR108
PI200815	Yellow	a	a	CG9165	Orange	a	a
931	White	b	b	CG9185	Orange	a	a
F_1	White	h	h	CG9187	Orange	b	a
CG9207	Orange	a	a	CG9197	Orange	a	a
CG9142	Orange	a	a	CG9198	Orange	a	a
CG9143	Orange	a	a	CG9199	Orange	a	a
CG9160	Orange	a	a	CG9201	Orange	a	a
CG9164	Orange	a	b	CG9203	Orange	b	a

The 13 accessions of xishuangbanna cucumber had been reported in the article of Qi et al. (2013)

In the present study, one Indel marker and one SSR marker were identified to be tightly linked to the *yf* gene, namely yfSSR108 and yfIndel29, with genetic distances of 0.3 and 0.6 cM, respectively. Using 13 lines of the xishuangbanna cucumber, the accuracy of the two markers for MAS was achieved 92.3 and 84.6 %, respectively. This study identified linked markers, rather than the gene itself, which has some limitations in breeding. Therefore, the development of gene markers or markers that are co-segregating with the *yf* gene will be the object of further research.

Prediction of candidate genes for the *yf* gene

As described above, the *yf* gene was delimited within a region of 149 kb on cucumber Chr. 7, and 21 predicted candidate genes were identified from the genomic map of cucumber. In plants, β-carotene synthesis is mainly governed by several genes: IPP isomerase, GGPP synthase, phytoene synthase (PSY), phytoene desaturase (PDS), zeta-carotene desaturase (ZDS), and lycopene-cyclase (LCYB) (Cunningham and Gantt 1998). None of these genes were found to be located within the region of 149 kb on Chr. 7. It is possible that gene *yf* may be a regulatory factor of a functional gene in the β-carotene synthesis pathway, and the elucidation of this will require further study.

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