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ORIGINAL ARTICLE

Fine genetic mapping of the white immature fruit color gene *w* to a 33.0-kb region in cucumber (*Cucumis sativus* L.)

Hanqiang Liu[1] · Huanwen Meng[1] · Yupeng Pan[1] · Xinjing Liang[1] · Jianqing Jiao[1] · Yuhong Li[1] · Shuxia Chen[1] · Zhihui Cheng[1]

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Abstract

Key message **The white immature fruit color gene *w* was rapidly mapped to a 33.0-kb region to identify a valuable candidate gene that encodes peroxidase.**

Abstract The skin color of immature fruit is a crucial external trait of cucumbers, and white skin is shared by limited numbers of commercial cultivars. Herein, one BC_1 population and two F_2 segregating populations were constructed using four inbred parental lines (WD3 × B-2-2 and Q30 × Q24) to investigate the inheritance patterns and chromosomal locations of immature fruit color genes in cucumbers. Consequently, a single recessive gene, *w*, was identified that controls white immature fruit color. A total of 526 markers, which were derived from published genetic maps, two reference cucumber genomes ("9930" and GY14), and two parents (Q30 and Q24) for which whole-genome sequence information is available, were used to map the target gene *w* to a 33.0-kb region flanked by two SNP-based markers, ASPCR39262 and ASPCR39229, which are physically located at 39262450 and 39229482 of chromosome 3 ("9930" draft genome assembly), respectively. Gene prediction indicated that four potential genes were located in the target region. One gene that encodes peroxidase is likely to be a valuable candidate gene because quantitative real-time PCR revealed an eightfold difference in its transcriptional level, and several amino acid variations were found when the deduced amino acid sequence was aligned. A co-segregating marker was used synergistically to test its ability to predict the skin colors of 83 dark green/white germplasms, and the validity of its utility in marker-assisted selection was confirmed. Fine mapping of this locus will assist in cloning the gene and in marker-assisted breeding to develop dark green/white cucumber cultivars.

Introduction

Cucumber (*Cucumis sativus* L.; $2n = 2x = 14$) is generally considered to have originated in India (Bisognin 2002; Li et al. 2013; Sebastian et al. 2010), and it is an economically important vegetable crop worldwide (Li et al. 2013). Globally, its total production is 65.1 tons and 48.0 tons (73.7 %) of cucumbers were distributed throughout the Chinese mainland in 2013 (data available at http://faostat.fao.org/). Immature cucumbers can be consumed fresh or processed into other foods and they are even used in cosmetic production. The visual quality of a cucumber is an important commodity characteristic for consumers. There are significant visual differences between the two primary cucumber types found in China: the South China type has long fruit, numerous white spines, thin skin, no fruit ribbing and a green color, and the North China type has short but large fruit, hard skin, spines that are usually black in color, fruit ribbing and a white or green color. Therefore, the targeted improvement of fruit appearance traits, such as fruit size, spine color, skin texture, fruit netting, and immature fruit skin color, is considered to be an important breeding goal for the production of plants with high commercial value.

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✉ Zhihui Cheng
chengzh@nwsuaf.edu.cn

[1] College of Horticulture, Northwest A&F University, Yangling 712100, Shaanxi, China

The skin color of immature fruit is an important agronomic factor that influences consumer choice. Dark green, green, yellow-green (*yg*), light green and white (*w*) skin colors are under genetic control in cucumbers (Dong et al. 2012; Pierce and Wehner 1990; Xie and Wehner 2001). Another two important fruit traits, namely dull/glossy skin (Yang et al. 2014b) and mottled/uniform skin (Yang et al. 2014a), also contribute to the visual appearance of cucumbers. The colors of commercial cucumber cultivars in China differ from north to south. For example, in part of the Gansu and Shandong province area, white-skinned cucumbers are the main commercial cultivars, and the desirability of these cultivars is increasing over time in China. Typically, most desirable cultivars are grown under protected growing environments or in fields only, and only a limited selection of cultivars is available in the market. Compared to conventional breeding, rapid breeding via marker-assisted selection (MAS) is considered to be a more effective strategy for the development of various cultivars (Nie et al. 2015). Thus, the investigation of genetic patterns, the cloning of the gene (s) that control desirable traits and the development of practical markers should be top priorities.

Some early studies have shown that yellow-green (*yg*) was recessive to dark green and was epistatic to light green (Dong et al. 2012; Pierce and Wehner 1990; Xie and Wehner 2001). Wang et al. (2013) determined that the major gene and polygene inheritance of immature fruit skin color in cucumbers was in accordance with the additive-dominance-epistasis pattern. Major gene heritability in the F_2 generation was higher, while polygene heritability and environmental effects were decreased. However, Li (2008) crossed the green-skinned cucumber line WD3 with the white-skinned line B-2-2 and observed 3:1 segregation between green and white plants in the F_2 generation. In another cross between inbred homozygous cucumber lines 1507 (green) and 1508 (white), Dong et al. (2012) found similar segregation ratios, with 3:1 (green:white) in the F_2 and 1:1 (green:white) in the BC_1 population. Both Li and Dong suggested that a single recessive gene (*w*) controlled the white trait and that dark green (*DG*) was dominant over white (*w*). Gene *w* was mapped to chromosome 3 and was located between markers SSR23517 and SSR23141 at genetic distances of 4.9 and 1.9 cM (Dong et al. 2012), respectively; however, this map was constructed with only 189 F_2 individuals. Until now, no other studies have been performed on this topic. Therefore, the inheritance of skin color, identification of flanking markers and cloning of genes associated with white skin color deserve further study.

The present study was designed to verify the inheritance of the immature fruit skin color trait and to determine its genomic location via fine genetic mapping and candidate gene analysis as well as to investigate the validity of the closest flanking markers for marker-assisted selection (MAS) of the *w* locus. The whole-genome sequence, including two parental lines tested in this study and two reference genome ("9930" and GY14), combined with the published high-density genetic map, were used to develop markers, which made it possible to rapidly identify a 33.0-kb region that contained a potential candidate gene encoding peroxidase. Molecular markers closely linked to the white skin color gene were tested for use in MAS breeding with 83 cucumber germplasms. This work will allow for the development of breeding strategies that incorporate traditional and molecular approaches with the aim of developing white-skinned cultivars for the commercial cucumber market.

Materials and methods

Plant materials

Two green-skinned lines, WD3 and Q30, and two white-skinned lines, B-2-2 and Q24 (Fig. 1), were employed in the current study. Both WD3 and Q30 are North China types with thick, white spines and ridged, dull green skin, whereas B-2-2 and Q24 have smooth fruits with fewer spines. Q24 fruits are round, and B-2-2 fruits are long. These four lines were provided by the cucumber research group of the Horticulture College at Northwest A&F University, Yangling, China.

Two crosses, WD3 × B-2-2 and Q30 × Q24, were performed to produce the F_1 generation. F_1 plants were self-pollinated to produce F_2 plants. For the WD3 × B-2-2 group, the F_1 plants were backcrossed with the recessive parent (B-2-2) to obtain the BC_1 population. Four parental lines, along with the F_1, F_2, and BC_1 generations, were used to describe and validate the inheritance pattern of skin color traits in immature fruit. The F_2 fruits were used in linkage analysis and genetic map construction. For each of the primary mapping individuals and recombinant plants detected in the F_2, at least 30 F_2-derived F_3 offspring ($F_{2:3}$) were scored to determine the genotype at the *w* locus. Eighty-three cultivars and cucumber germplasms were obtained from the cucumber research team at the Horticulture College of Northwest A&F University as well as the commercial market to test the validity of the flanking molecular markers. All the plants were hand-pollinated with two staminate flowers and clamped with grafting clips to prevent re-pollination by entomophily or pollen dispersal. All the plants were grown in plastic greenhouses under natural sunlight from spring 2013 to summer 2014 at Northwest A&F University.

Fig. 1 Immature fruit skin *colors* of the parental lines and their progeny. **a** From *left to right* Q30 (*green*), F_1 (*green*) of Q30 × Q24 and Q24 (*white bulb*). **b** From *left to right* WD3 (*green*), F_1 (*green*) of WD3 × B-2-2 and B-2-2 (*white*). **c** F_2 of Q30 × Q24 divided into *green* and *white* skins and different shapes (color figure online)

Phenotypic data collection

Each plant, including the parental lines as well as F_1, F_2, BC_1 and $F_{2:3}$ individuals, was given an identification tag revealing its serial number, pollination date, color and cross combination type. The skin color of the immature fruit was recorded at 7–10 days after pollination, and each fruit was photographed for reassessment. Two or three young leaf samples of each individual were cut from the plant apex, wrapped in aluminum foil, flash-frozen in liquid nitrogen and maintained at −80 °C for further use in experiments.

DNA/RNA extraction, PCR and gel electrophoresis

Genomic DNA was extracted from young leaves by the CTAB method as described by Clark (1997). RNA was extracted from the exocarp of immature fruits (7–10 days after pollination) with Column Plant mRNAout kit (TIANDZ, China) according to the manufacturer's instructions. All of the primers used in this study were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The reaction of 10-μl volume includes 2 μl of DNA template (30 ng/μl), 1 μl of each primer (5 μM) and 6 μl of master mix (Novoprotein, Shanghai, China). The PCR protocol was as follows: 94 °C for 5 min followed by 28–33 cycles of 94 °C for 30 s, 50–58 °C for 30 s, and 72 °C for 30 s with an extension step at 72 °C for 8 min, then held at 4 °C forever. However, allele-specific PCR (ASPCR), which was used for SNP typing, required hot-start PCR for specific amplification. Thus, a PCR thermocycler (S1000™ Thermal Cycler, Bio-Rad) was preheated to 95 °C and was kept running continuously, then the PCR tubes/plates were placed inside, and the instrument was switched rapidly to the normal preset protocol. Starting the PCR thermocycler at a higher temperature would thus reduce nonspecific amplification.

The PCR amplification products were separated by polyacrylamide gel electrophoresis (PAGE) on 9 % non-denaturing or 6 % denaturing gels with 1× TBE buffer at a constant voltage of 180 V for 1.5–2 h (105 × 185 mm gel size, electrophoresis apparatus type DYCZ-30C) or 1000 V for 1.0–1.5 h (300 × 340 mm gel size, electrophoresis apparatus type DYCZ-20E), respectively. The electrophoresis power supply and two electrophoresis apparatus types were supplied by Beijing Liuyi Instrument Factory, China. Both the 9 % non-denaturing and 6 % denaturing gels were silver-stained following Weng et al. (2005).

Marker development and mapping strategy

Earlier studies showed that immature fruit color was controlled by a single gene, *w* (Dong et al. 2012; Li 2008; Xie and Wehner 2001), which was mapped to chromosome 3 (Dong et al. 2012). Thus, 187 published markers on chromosome 3 (Ren et al. 2009) were initially selected to construct the primary genetic map. Based on the mapped location of *w*, a large number of new SSR markers that were described in other published references (Cavagnaro et al. 2010; Miao et al. 2011; Weng et al. 2010; Yang et al. 2012, 2013; Zhang et al. 2012) were selected and screened in the parental lines. The polymorphic markers were then used to identify the genotypes of individuals in the mapping population. When the available published SSR markers in the target region were exhausted, the associated sequences of the Chinese Long cucumber "9930" genome (http://www.icugi.org/cgi-bin/ICuGI/index.cgi) and the GY14 genome (http://cucumber.vcru.wisc.edu/wenglab/gy14-9930/) were used to develop new SSR markers in the target interval using the SSR Locator program (available at http://minerva.ufpel.edu.br/~lmaia.faem/). In addition, InDel markers were also designed by aligning the defined sequences of the two referenced genomes using the DNAMAN program. Furthermore, the genomes of two parental lines, Q30 and Q24, were sequenced with Illumina HiSeq™ 2000 (Biomarker Technologies, Beijing, China) at 24- and 27-fold sequencing depths and were used to develop InDel and SNP markers. Only insertions and deletions that were >3 bp in size were used to design InDel primers. For SNP genotyping, ASPCR, cleaved amplified polymorphic sequences (CAPS) (Neff et al. 1998) and derived CAPS (dCAPS) (Michaels and Amasino 1998) markers were designed based on these SNPs. For ASPCR, two allele-specific primers with a second single-nucleotide mismatch that was introduced at the second or third 3′-terminal base and a publicly available primer were designed such that each primer pair would amplify a specific band in one of parents and no specific band in the other parent (Drenkard et al. 2000) (as shown in Table S1, available as an online resource). Primers for the dCAPS markers were designed with a web-based primer design tool called dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html). Other markers were designed with Primer Premier 5.0 (http://www.premier-biosoft.com/). The physical locations of linkage markers were determined by BLASTing the primer sequences against the "9930" genome (Chinese Long; V2) (known as in silico PCR) spanning a probable size of 100–400 bp (Cavagnaro et al. 2010; Ren et al. 2009).

A total of 158 F_2 plants from WD3 × B-2-2 were initially used for the linkage analysis. Unfortunately, a low level of polymorphism was observed between these two materials. Thus, a new F_2 population derived from Q30 × Q24 was obtained. At first, 93 F_2 individuals were used for primary linkage analysis of the white skin locus *w* with synthesized SSR markers. When no recombination events were observed between the flanking markers, the size of the mapping population was expanded to 353 plants (including the initial 93 plants), which were utilized for further testing. New markers were continuously designed to narrow the interval containing gene *w*. An additional 2618 new plants, which increased the total number of plants to 2971, were employed for fine genetic mapping. The linkage map was constructed using JoinMap 4.0 with a LOD threshold score of 6.0.

Candidate gene prediction and identification

Due to the availability of genome sequence data of parental lines, the target sequence was easily obtained for further genetic analysis. Candidate gene prediction was performed using the cucumber genome browser, version 2 (http://www.icugi.org/cgi-bin/ICuGI/index.cgi), and the online program FGENESH (http://linux1.softberry.com/berry.phtml) (Salamov and Solovyev 2000). The function of the predicted genes was determined with the BLASTP tool from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Quantitative real-time PCR (qPCR) was performed to identify the candidate genes. First-strand cDNA synthesis was performed with a 5× All-In-One Master Mix kit (AccuRT Genomic DNA Removal Kit, ABM, Canada). Primers of all candidate genes were first verified by semi-quantitative real-time PCR to confirm the appropriate annealing temperature. The gene encoding cucumber ubiquitin extension protein was used as the reference gene. Each qPCR included 3 μl of cDNA template (100 ng/μl), 2 μl of each primer (10 μM), and 10 μl of EvaGreen qPCR Master Mix (ABM, Canada), and the volume was adjusted to 20 μl with RNase-free water. The reactions were performed using an iQTM 5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). The values from triplicate reactions were averaged, and the Ct values were determined and compared with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

In addition, the DNA sequences and deduced amino acid sequences of the predicted genes were aligned to detect variations using the DNAMAN program. To develop practical molecular markers for MAS breeding, the molecular marker that co-segregated with locus *w* was tested using 83 cucumber cultivars or pure lines to investigate its utility in marker-assisted selection.

Results

Genetic analysis of the skin color trait in immature cucumber fruit

To determine genetic inheritance patterns, the two green (WD3 and Q30) and two white cucumber lines (B-2-2 and Q24) shown in Fig. 1 were used to generate two F_2 populations and one BC_1 population. None of the F_1 individuals that originated from the two crosses were white. As Table 1 shows, among the 158 and 593 F_2 individuals of WD3 × B-2-2 and Q30 × Q24, the fruit color trait which was separated into green and white, was consistent with a 3:1 ratio. Particularly, out of the 156 $F_{2:3}$ families in Q30 × Q24, 35 were homozygous green, 85 were heterozygous green and 36 were homozygous white (*ww*) at the skin color locus (χ^2 test against 1:2:1 Mendelian segregation ratio). For the backcross of F_1 with B-2-2 in WD3 × B-2-2, 109 had green and 103 had white fruits (χ^2 test against 1:1 segregation ratio). All of the segregation ratios obtained above indicated that a single recessive gene, which was designated as *w*, controlled the white immature fruit skin color.

Preliminary mapping

An earlier report (Dong et al. 2012) describing the construction of a genetic map with 14 SSR markers (partial primer sequences were not available in the article), located locus *w* to chromosome 3 with SSR23517 and SSR23141 as the closest flanking makers. Thus, 187 published markers (serial nos. 1–187, listed in Table S6 of the online supplementary materials) on chromosome 3 (Ren et al. 2009) were selected for parallel chromosome-wide scanning of polymorphic loci in WD3, B-2-2 and their F_1 progeny. Six markers (SSR04628, SSR23177, SSR18640, SSR06210, SSR14307, and SSR15043) (3.2 %) appeared polymorphic, but only SSR04628 and SSR23177 could produce reliable, distinct bands on PAGE gel. To generate a rough genetic map, 43 new SSR markers (serial nos. 188–230 in Table S6) originating from a high-density genetic map (Yang et al. 2013) were synthesized for analysis. However, only one effective polymorphic marker (SSR07137) was detected. The three available markers (SSR04628, SSR23177, and SSR07137) were then used to genotype 158 F_2 mapping individuals (derived from WD3 × B-2-2). A linkage map containing two markers was constructed (Fig. 2a), which supported the earlier study (Dong et al. 2012). Because of the low level of polymorphism observed between WD3 and B-2-2, a new segregating population generated from Q30 × Q24 was selected for genetic mapping.

Initially, 230 synthesized SSR markers were used for a parent survey to identify polymorphic markers. 14 out of 22 (serial nos. 13–26 and 188–195 in Table S6) (9.6 %) polymorphic makers were placed on the primary genetic map using 93 F_2 plants (Fig. 2b). The *w* locus was localized to a 4.3 cM interval that defined by SSR16554 and SSR21652. We tested 109 additional SSR makers (Cavagnaro et al. 2010; Miao et al. 2011; Weng et al. 2010; Yang et al. 2012; Zhang et al. 2012) (serial nos. 231–339 in Table S6) located in this interval and identified 17 polymorphic ones. Consequently, 16 were mapped. Three makers, SSR16554 and UW066738/UW066741 flanked the locus *w* in a 2.0-cM region (Fig. 2b). The physical positions of SSR16554, UW066738 and UW066741 are 39694811, 39236132 and 39240126 (the first base of the 5′-terminus of the forward primer sequence) on chromosome 3 of "9930", and are physically located in the GY14 scaffold02995 as well.

To precisely narrow the genomic region surrounding *w*, we analyzed the distribution of perfect microsatellites with ≥3 repeat units and a minimum total length of 12 bp at 455 kb (between SSR16554 and UW066741) on chromosome 3 in the "9930" cucumber genomic sequence (Huang et al. 2009). A total of 591 SSR markers were detected, and the known amplicon sequences were aligned with scaffold02995 of GY14. The markers that were significantly polymorphic between "9930" and GY14 were synthesized. Consequently, 30 markers (serial nos. 340–369 in Table S6) were selected and three (LH001001, LH001002, and C120-indel) shared the same expected polymorphism between Q30 and Q24. Thus, a genetic map containing 33 polymorphic markers is presented in Fig. 2b. The *w* locus was still localized within the 2.0-cM region flanked by SSR16554 and five co-segregating markers.

Table 1 Segregation of immature fruit skin color in the segregating populations

Population	Plants tested	Green:white	Mendelian expectations	χ^2 value[b]	*P*
WD3 × B-2-2					
F_2	158	113:45	3:1	1.02	0.31
BC_1	212	109:103	1:1	0.17	0.68
Q30 × Q24					
F_2	593	452:141	3:1	0.47	0.49
F_2^a	156	35:85:36	1:2:1	1.27	0.53

WD3 and Q30 are cucumber lines with green fruit color, B-2-2 and Q24 are cucumber lines with white fruit color, BC1 = (WD3 × B-2-2) F1 × B-2-2

[a] F2 population which was identified the genotype of green cucumbers, is included in the 593 F2 of Q30 × Q24

[b] χ^2 (0.05, 1) = 3.84

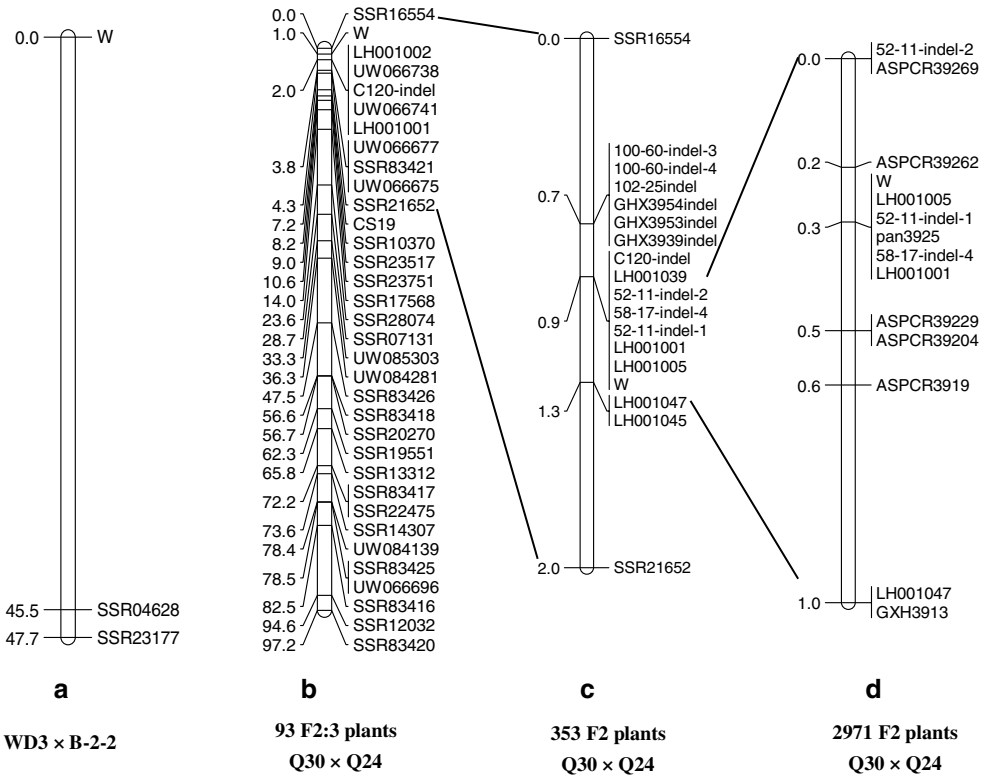

Fig. 2 Fine genetic maps of the white skin locus *w*. **a** An SSR marker-linked genetic map of the white skin locus *w* based on 158 F_2 plants from WD3 × B-2-2. **b** A framework linkage map based on 93 F_2 plants from Q30 × Q24 sharing 33 SSR markers. The white skin color locus (*w*) is localized in the distal region of chromosome 3. **c** A number of InDel markers were developed to localize the *w* locus within a *narrow interval* based on a mapping population of 353 plants. The *vertical bars* delimit the co-segregating marker loci. **d** A fine genetic map of the white skin color gene (*w*) based on 2971 F_2 plants

Fine mapping using genome sequence data

High-throughput InDel and SNP arrays are regarded as high-efficiency approaches for genetic mapping. Therefore, the genomes of Q30 and Q24 were sequenced and 64,007,048 and 86,389,784 reads were obtained (see Table S2 in the online supplementary materials for additional details), respectively. Reads covering the 600-kb target region (SSR16554 to SSR83421) were mapped to the reference cucumber "9930" genome. In total, 444 kb (114 fragments) and 449 kb (106 fragments) of sequence were generated, respectively. Sequence alignments revealed most of the SNPs and InDels. The InDels that varied more than 3 bp in length and partial SNPs were screened and are listed in Table S3 and S5 (online supplementary materials). Initially, 39 InDel markers (serial nos. 370–408 in Table S6) and five SNP markers (converted into dCAPS) (serial nos. 409–413 in Table S6) were synthesized and used for genotype screening of the parents, yielding 15 useful InDels (serial nos. 370–384 in Table S6) that were then applied to a mapping population comprising of 353 plants. After some low-grade markers were excluded, the target locus *w* was localized to a cluster of eight consecutive loci flanked by LH001045/LH001047 and six co-segregating markers (Fig. 2c).

To improve the mapping accuracy, the segregating population was enlarged to 2971 plants, and 23 and 39 recombination events were detected in the two flanking intervals that were defined by C120-indel and LH001045. The other mapped polymorphic markers in Fig. 2c were used to genotype the 62 recombinants, and the markers LH001001, 52-11-indel-1, 58-17-indel-4 and LH001005 still co-segregated with locus *w* (Fig. 2d). The flanking markers 52-11-indel-2 and LH001047 defined two physical gaps that were within 23 kb (52-11-indel-2 to 52-11-indel-1) and 107 kb (LH001001–LH001047) around the target locus, respectively. For the 107 kb region, an analysis of the list (Table S3) of InDels displayed no polymorphic InDels in the unilateral successive 105 kb region but a high density of InDels between "9930" and each of the parents (Q30 and Q24) and between "9930" and GY14. On the basis of these InDels, 103 primer pairs (serial nos. 414–516 in Table S6) were designed for fine mapping. Unfortunately, except for one pleomorphic InDel (GXH3913) derived from supplementary Table S3, no alignment-based InDels were detected from Q30 compared with "9930", from Q24 compared with "9930" or from GY14 compared with "9930". By assessing GXH3913, the location of the causal locus was refined to a physical distance of 139 kb that was defined by 52-11-indel-2 and GXH3913 (Fig. 2d).

Since there were no SSRs and InDels any more, SNP markers were therefore used for the next stage. Upon scanning the 139-kb sequence of the delimited narrow region, a total of 200 SNPs were identified and presented in Table S5. Nine SNP-derived ASPCR makers (see "Materials and methods") were developed (serial nos. 518–526

in Table S6). Moreover, we attempted to map the whole-genome sequence reads of Q30 and Q24 to the GY14 genome, which resulted in an additional InDel, pan3925. This additional InDel together with the SNP markers were used for analysis. Consequently, pan3925 and five SNPs (ASPCR39269, ASPCR39262, ASPCR39229, ASPCR39204 and ASPCR3919; see Table S1 in the online supplementary material for additional details) were mapped on the fine genetic map (as shown in Fig. 2d). Gene *w* clustered with five markers and was flanked by two SNP-type markers, ASPCR39262 and ASPCR39229, which are located at 39262450 and 39229482 (33.0 kb), respectively, on chromosome 3.

Candidate genes for immature fruit skin color

The availability of the parental genome permitted the straightforward identification of candidate genes within the restricted chromosome region. In the 33.0-kb sequence delimited by the flanking markers, four genes were annotated using the online program FGENESH. The putative amino acid sequences were subjected to BLASTP (NCBI), revealing that these genes have functions related to a phosphoribosyltransferase family protein (*PFP*) in *Theobroma cacao*; a peroxidase superfamily protein (*PSP*) in *Theobroma cacao*; a tetraspanin-33 (*T33*) in *Gossypium arboretum*; and a two-component response regulator-like protein (*RRP*) in *Morus notabilis*. Use of the cucumber genome browser to examine the genome of "9930" (http://www.icugi.org/cgi-bin/gb2/gbrowse/cucumber_v2/) revealed five genes (*Csa3G904100, Csa3G904110, Csa3G904120, Csa3G904130 and Csa3G904140*) in this region. By contrast, the *PSP* gene consisted of *Csa3G904110* and *Csa3G904120* according to comparisons of physical positions.

qPCR and comparative analysis of candidate genes

Four primer pairs associated with the four predicted genes were designed and are shown in Table S4 of the online supplementary material. The expression levels in both parents were measured (Fig. 3c). *PSP* expression exhibited an

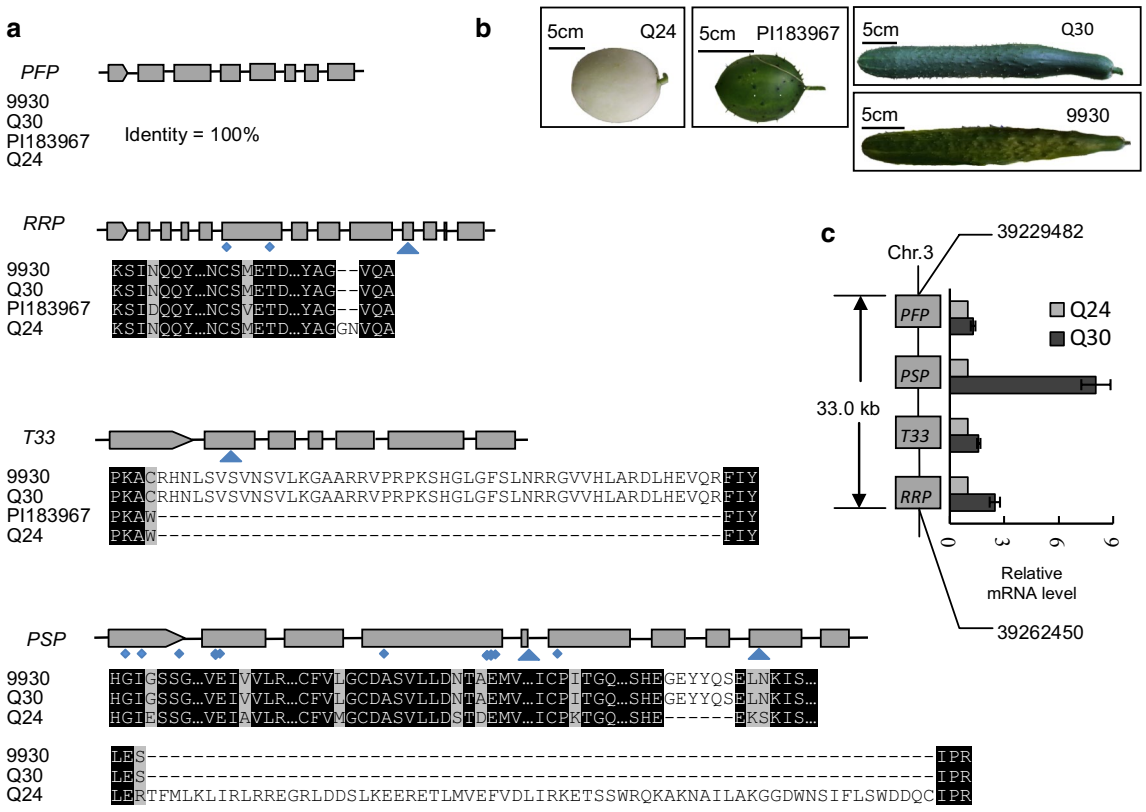

Fig. 3 Relative mRNA transcript levels and amino acid mutations in the deduced coding region of four predicted genes in the delimited 33.0-kb region. **a** Putative exons and divergent amino acid types among four (or three) selected cucumbers. *Blue diamonds* and *triangles* below the exons indicate the positions of non-synonymous SNPs and deletions, respectively. *Apostrophes* indicate omitted amino acid sequences. **b** Four cucumbers were selected for analysis; Q24 is *white* and the others are *green*. **c** Transcript levels of four predicted genes in *white* and *green* fruits detected by real-time quantitative PCR analysis. The data are presented as the average values of three replicates (±SD). The relative transcript level is presented with Q24 as the reference (color figure online)

eightfold increase in Q30 (green color) compared with Q24 (white color), and it participates in the protection of chloroplasts in *Arabidopsis* (Chang et al. 2004); thus, *PSP* maybe a good candidate gene for the *w* locus.

To test this conjecture, the genome sequence of a new cucumber cultivar with green skin called PI183967 (Fig. 3b) was downloaded from the Cucurbit Genomics Database (ftp://www.icugi.org/pub/genome/cucumber/). PI183967, "9930", Q30 and Q24 were then used for the analysis of putative amino acid (aa) sequences deduced from the genomic intervals containing these four candidate genes, as depicted in Fig. 3a. The DNA sequences were also analyzed and are described in Table S7 (Online resource). Annotation of *PFP*, *PSP*, *T33* and *RRP* from "9930" indicated that there were eight, ten, seven and thirteen exons in the coding regions, respectively. The alignment of *PFP* DNA sequences revealed multiple mutations, but the aa sequences were consistent among the four cucumber lines. Significant differences in the aa sequence of *T33* were observed among the three green materials, and the second exon was missing in PI183967 and Q24 compared with "9930" and Q30. This finding indicates that this deletion is not likely to be associated with the skin color trait. Simultaneously, DNA sequence alignment of the *RRP* homologs revealed two vital SNP mutations (A–G) and a 1-bp deletion among the tested cucumbers. Both SNPs were located in the sixth exon and caused aa changes in the three green cucumbers. Conversely, the 1-bp deletion, which was located in the tenth exon of the DNA sequence, resulted in two aa deletions in green cucumbers compared with white cucumbers (see Table S7 in the online supplementary material; Fig. 3a). This result appeared to be in line with the target trait; however, qPCR revealed only a 2.5-fold difference in the expression level of *RRP* between Q30 and Q24 (Fig. 3c). Among the four predicted genes, *PSP* exhibited a considerably high level of variation. In particular, annotation of the *PSP* ortholog in PI183967 revealed two open reading frames (ORFs) with four and six exons. We aligned the *PSP* orthologs of the other three cucumbers and found that Q24 carried nine single aa variants and two deletion variants that likely disrupted the function of the allele. The above results indicate that *PSP* likely defined the *w* locus.

MAS testing in 83 dark green/white cucumber germplasms using pan3925

Flanking markers with small marker/trait mapping intervals have the potential to increase MAS efficacy by reducing errors during selection. On the other hand, accurate target trait selection can validate the genetic distance between the markers and the gene. Thus, 58 dark green and 25 white-skinned cucumber germplasms (as shown in Table S8 of the online supplementary materials) were selected to determine the consistency between pan3925 marker genotypes and fruit phenotypes. The results showed that 56 dark green and 25 white fruits (97.6 %) were predicted correctly. This finding indicates that the co-segregating marker pan3925 is very close to gene *w* and could be used for MAS breeding of the dark green/white fruit trait in cucumbers.

Discussion

Fruit skin color is an essential agronomic trait of cucumbers that influences cucumber planting area and consumption. Ensuring specific genetic patterns promotes more effective plant breeding. To date, a number of skin-related genes have been identified, including uniform immature fruit color (*u*), dull/glossy fruit skin (*D/d*), numerous spines (*ns*), black spine color and mature orange fruit color (*B*) (Kooistra 1971; Li et al. 2013; Meglic and Staub 1996; Yang et al. 2014a, b), which were mapped to a cluster on chromosome 5 (Weng 2014) or chromosome 4. Previous studies indicated that five different genes (Xie and Wehner 2001) contributed to the development of skin color in immature fruit, which ranges from dark green to green, light green, yellow-green and white (*w*). To investigate the genetic inheritance patterns of the white color of immature fruit skin, one BC_1 and two F_2 populations were tested. It was found that a single recessive gene controlled the inheritance of white skin color, with the expected 3:1 and 1:1 phenotype distribution ratios in F_2 and BC_1 populations, respectively, in this study.

It is worth mentioning that all the seeds, especially those of the F_2 generation, should be mixed together and selected randomly in the case of segregation distortion (Phadnis and Orr 2009). When 230 SSR markers were selected initially for the primary mapping using the F_2 progeny of WD3 × B-2-2, 19 out of 26 polymorphic markers displayed segregation distortion to one of the parents. Segregation distortion skews the genotypic frequencies from their Mendelian expectations. This phenomenon is often observed in the progeny of interspecific and intraspecific hybrids and may result from competition among gametes or from the abortion of the gamete or zygote (Faris et al. 1998). Segregation distortion was first reported in maize by Mangelsdorf and Jones (Lu et al. 2002), and it has subsequently been found in other species including rice (McCouch et al. 1988), tomatoes (Paterson et al. 1988), coffee (Ky et al. 2000), *Arabidopsis* (Howden et al. 1998) and *Hordeum* (Zivy et al. 1992). In cucumbers, segregation distortion appeared to be a common phenomenon in genetic mapping studies (Miao et al. 2011; Ren et al. 2009; Weng et al. 2010; Yuan et al. 2008). Plant genomes often harbor hot-spot segregation distortion regions (SDRs), and the marker loci are often clustered on the chromosome (Lu

et al. 2002; Miao et al. 2011). In a cross between GY14 and PI183967 (*C. sativus* var. *hardwickii*) cucumbers, three SDRs were detected on chromosomes 1, 4 and 6. All SSR marker loci within these SDRs were distorted to parent PI183967 (Ren et al. 2009). Distorted markers did not present clear information, and they exhibited greater distortion in the linkage distance and the order of markers compared with co-dominant markers Lorieux et al. (1995a, b).

The release of the cucumber draft genome assemblies and the rapid development of the cucumber genomic infrastructure, including molecular markers and high-density genetic maps, promote the progress of gene mapping. Published markers listed on genetic maps were generally more polymorphic, and out of the 252 SSR markers that were synthesized initially in this study, 34 (13.5 %) were polymorphic between Q30 and Q24. Nevertheless, of the 87 SSRs derived from Cavagnaro et al. (2010) that had not been mapped, only five (2.7 %) exhibited subtle differences in fragment length between parents. This finding indicates that SSR markers that were polymorphic between the tested cucumber materials could potentially also be polymorphic between other materials. The genomes of Q30 and Q24 were sequenced to develop more efficient molecular markers, which led to the rapid development of high-throughput sequencing-based InDel and SNP markers in the target genomic block. SSRs and InDels are easily detected by amplifying a small region of the genome and determining the sizes of the amplicons. However, low sequencing depths may result in incorrect InDel polymorphisms. For example, the InDel markers GXH3926indel and GXH3925indel were polymorphic when the DNA sequences of Q30 and Q24 were aligned; however, polymorphic bands were not observed on the gel. SNPs compose the largest set of sequence variants in most organisms. For the present sequenced cucumber materials, there was one SNP per kb on average in the target 600-kb region between SSR16554 and SSR83421. Cleaved amplified polymorphic sequences (CAPS), derived CAPS and sequencing of amplified products are widely used for SNP genotyping in cucumbers (Hwang et al. 2014; Li et al. 2013); however, these procedures are time consuming and expensive. In this case, using allele-specific PCR (ASPCR) with two allele-specific primers and a publicly available primer was a more desirable method for SNP genotyping. Each allele-specific primer contains one artificially introduced mismatched nucleotide at the second or third 3′-terminal base; thus, the primer can anneal properly to the specific allele but has two 3′ mismatches (the first and second or third 3′-terminal base) with the nonspecific allele. Therefore, a two-nucleotide mismatch between the 3′-terminus of primer and the DNA template leads to much less efficient extension by DNA polymerases compared to a single-nucleotide mismatch between the primer and the DNA template, which results in distinct electrophoretic bands (as shown in Table S1 in the online supplementary materials) and is a much more accurate and fast methodology. Furthermore, the appropriate annealing temperature and cycle threshold also played critical roles in controlling specific amplification in ASPCR genotyping. Based on the adjacent available SNP markers, gene *w* was rapidly mapped to a chromosomal region of 33.0-kb in this study, which suggested that the combined use of published genetic maps and whole-genome sequence data of the tested cucumbers could noticeably accelerate the efficiency of gene mapping and subsequent cloning.

Chlorophylls are responsible for both harvesting solar energy in photosynthetic systems and the green appearance of leaves and immature fruits in plants. Chlorophyll degrades during natural fruit ripening because of a negative balance between synthesis and breakdown (Jacob-Wilk et al. 1999), resulting in chlorotic plant tissues. The white color of immature fruit skin in cucumbers may not be associated with a decrease in chlorophyll but rather a lack of chlorophyll synthesis, which would be maintained over a period spanning from ovary formation to fruit maturity. The phenotypic variations among individuals are significantly influenced by both genetic and environmental factors. In particular, light and temperature play critical roles in controlling the coloration of cucumber fruit. For example, cucumber fruits were observed to be greener in autumn than during the summer in this study. Moreover, in a trial, we wrapped the developing cucumber fruits with aluminum foil and found that the skin of the fruits was white at the 10th day after pollination; thereafter, the fruits gradually turned green under natural light conditions. This trial indicated that light is a vital factor for chlorophyll synthesis and that the skin colors of immature cucumber fruits commonly differed with respect to their chlorophyll content. In angiosperms, 15 genes are required for the 15 steps in the chlorophyll biosynthetic pathway (Beale 2005). Most of the biosynthetic genes encode specific enzymes that function together to contribute to the synthesis process (Bollivar et al. 1994). Previous investigations indicated that the R2R3-MYB transcription factor, which is a member of one of the largest families of transcription factors in plants (Feller et al. 2011), is a valuable candidate gene that controls fruit color in several species (Feller et al. 2011; Li et al. 2013; Motamayor et al. 2013; Singh et al. 2014). Although a similar gene was not observed in the target genomic block in our research, a predicted gene encoding peroxidase, which exhibited an eightfold increase in its expression level in Q30 (green color) compared with Q24 (white color) and contained nine single aa variants and two deletions in Q24, seemed to be a promising candidate gene. For the other two predicted genes, *PFP* and *T33*, the deduced aa sequences were the same between PI183967

(green skin) and Q24 (white skin), and no significant expression difference was found between Q30 and Q24; thus, it is not likely that either of these genes is the target gene. There was some confusion associated with the candidate gene *RRP* because of its variation in aa sequences and 2.5-fold expression difference between green and white cucumber materials; however, a possible role for *RRP* in plants had not been investigated.

The expression levels of peroxidases often increase in response to stress, as these enzymes appear to provide cellular protection from the oxidative stress imposed on all photosynthetic plants (Siegel 1993). To date, no studies have examined the relationship between peroxidases and chlorophyll anabolism. By contrast, many studies have shown that peroxidase is involved in chlorophyll degradation during senescence or postharvest storage (Abeles and Dunn 1989; Funamoto et al. 2003; Yamauchi et al. 2004), during which phenolic compounds are oxidized with hydrogen peroxide (H_2O_2) to form phenoxy radicals, which then oxidize chlorophyll and its derivatives to colorless low-molecular-weight compounds (Yamauchi et al. 2004). The white color of cucumbers is a visible result of a lack of chlorophyll synthesis, rather than chloroplast degradation, during fruit development. We examined the function of various peroxidases and found that ascorbate peroxidase encodes a key enzyme required for the removal of H_2O_2 to protect chloroplasts (Chang et al. 2004; Tripathi et al. 2009), and we felt that this role was likely to be associated with the appearance of fruit skin. We thus hypothesize that the gene responsible for the target trait encodes an enzyme that is not directly involved in the synthesis of chlorophyll but rather has a role in the protection of chloroplasts. When the dark green gene is mutated into white gene *w*, the related enzyme cannot be synthesized, which leads to a reduction in chlorophyll and results in the white skin color of immature fruit. Continued efforts toward understanding the molecular genetics and physiological mechanisms of fruit color development should lead to additional studies. The findings and resources generated herein will provide a basis for genetic research and a tool for MAS breeding of cucumbers.

Author contribution statement H. L. constructed the mapping populations, performed genetic analysis, marker development, genome sequencing, and mapping analysis, and wrote the paper. H. M. provided the cucumber materials, performed some of the field work, and provided advice. Y. P. assisted with construction of the mapping populations, performed the primary mapping work, and performed some of the in silico PCR analysis. X. L. and J. J. extracted DNA and performed MAS testing. S. C. and Y. L. provided valuable research ideas. Z. C. designed and supervised the study.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that this study complies with the current laws of the countries in which the experiments were performed.

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