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Localization of a New Gene for Bitterness in Cucumber

Shengping Zhang*, Han Miao*, Rifei Sun, Xiaowu Wang, Sanwen Huang, Todd C. Wehner, and Xingfang Gu

From the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 10081 China (Zhang, Miao, Sun, Wang, Huang, and Gu); and the Department of Horticultural Science, North Carolina State University, Raleigh, NC 27695-7609 (Wehner).

*These authors contributed equally to this work.

Address correspondence to Xingfang Gu at the address above, or e-mail: guxf@mail.caas.net.cn.

Abstract

Bitterness in cucumber fruit and foliage is due to the presence of cucurbitacins. Several genes have been described that control the trait, with *bi (bi-1)* making fruit and foliage bitter free and *Bt (Bt-1)* making the fruit highly bitter. Previous studies have reported the inheritance and molecular markers linked to *bi-1* or *Bt-1*, but we were interested in studying the inheritance of fruit bitterness in the progeny of 2 nonbitter fruit inbred lines. The objective was to determine the inheritance of cucumber fruit and foliage bitterness and to locate them on a current linkage map using a recombinant inbred lines (RILs) population derived by crossing 9110Gt and 9930. It was concluded from the inheritance analysis that there were 2 loci controlling fruit bitterness in the population. One locus was in the same position as the location previously identified for *bi-1*, and another locus was for *bi-3*. Using a simple sequence repeat (SSR) linkage map, 2 loci for fruit bitterness in this RILs population were mapped. The locus of *bi-1* was located at the region between SSR0004 and SSR02309 within the genetic distance of 5.2 cM on chromosome 6. The locus of *bi-3* was placed in the region of SSR00116–SSR05321 within the genetic distance of 6.3 cM on chromosome 5. The physical distances for the regions of *bi-1* and *bi-3* were 11,430.94 Kb with 160 predicted genes and 1528.23 Kb with 198 predicted genes, respectively. Among 160 predicted genes for *bi-1*, there is a terpene synthase gene named Csa008595, which was speculated as the candidate gene of *bi-1*.

Key words: Cucumis sativus L., *gene prediction, inheritance, Marker-assisted selection, molecular marker*

Most cucurbit species have bitter compounds called cucurbitacins in their foliage (Rehm et al. 1957). Cucurbitacins are believed to be toxins produced by these plants as a defense against insects and herbivores (Balkema-Boomstra et al. 2003). Most cucurbit cultivars have been selected by breeders to have low cucurbitacin content in the fruit. Bitterness in cucumber fruit is also due to the presence of cucurbitacins, and the trait has a complex inheritance mechanism. As well as genetic factors, bitterness is also related to environmental conditions (Kano and Goto 2003). Some cucumber cultivars are nonbitter in their foliage, as well as their fruit. The Dutch researchers Andeweg and DeBruyn (1959), selected a nonbitter cucumber line from an improved American cultivar, "Long Green," and reported that nonbitterness in the foliage was controlled by the recessive gene, *bi* (*bi-1*). When the *bi-1* gene is present, cucumber foliage is nonbitter, and the fruit do not develop bitterness under environmental stress. Nearly, all Dutch cultivars released for greenhouse use have nonbitter foliage and fruit. Some cucumber cultivars have bitter foliage but nonbitter fruit. Most Northern Chinese type (Chinese Long) cucumbers belong to this type.

In addition to *bi-1* controlling foliage nonbitterness in cucumber, other genes are known to be involved with the presence or absence of cucumber bitterness, including *bi-2* (Wehner et al. 1998), *Bt*(*Bt-1*) (Barham 1953), and *Bt-2* (Walters et al. 2001). The *bi-2* gene for bitter-free foliage in cucumber was found in the germplasm named NCG-093. It fit a recessive, single-gene model. *Bt-1* is the dominant gene responsible for the extremely bitter flavor found in the cucumber line PI173889 and is linked to *bi-1* (Walters et al. 2001). *Bt-2* is proposed as the gene controlling bitterness of fruit in LJ90430. It is a separate locus from *Bt-1* and it was inherited as a single dominant gene. However, there are few reports on the interaction among genes controlling bitterness in fruit and foliage. During our study of single-gene phenotypes, it was found that F_1 fruit were unexpectedly

bitter (not extreme bitter like the fruit of *Cucumis sativus* var. *hardwickii*) when crossing 2 fruit nonbitter inbreds, the Dutch *bi-1bi-1* genotype and the Chinese line with foliage bitterness genotype, indicating that there was another gene different from *bi-1* in the Chinese line that interacted with *bi-1* causing fruit bitterness.

Bitter cucumber fruit are unpleasant to eat, so genetically bitter cultivars can cause losses for cucumber producers if the plants are grown under stress, even in greenhouse or other protected culture systems. Fruit bitterness can be avoided by using genetically nonbitter cultivars. However, phenotypic selection for nonbitterness is difficult due to the influence of environmental factors and the effort required for taste testing. Given these difficulties, cucumber breeders would benefit from an efficient and reliable detection method for bitterness such as marker-assisted selection (MAS) using molecular markers rather than taste. There are few reports of molecular markers linked to bitterness genes in cucumber. The cucumber *bi-1* gene was mapped to chromosome 6 (Chl.6) using flanking simple sequence repeat (SSR) markers SSR02309 and SSR00004 with genetic distance of 1.7 and 2.2 cM (Li et al. 2010). The physical distance for this region was 35 Kb (Huang et al. 2009). We found an amplified fragment length polymorphism (AFLP) marker linked to the *bi-1* gene with a genetic distance of 6.43 cM and 2 flanking AFLP markers E23M66-101 and E25M65-213 linked to the *Bt-1* gene with genetic distance of 5 and 4 cM (Chi et al. 2007; Gu et al. 2006).

The objective of this study was to determine the inheritance and to map the loci for cucumber fruit bitterness in a recombinant inbred lines (RILs) population derived from 2 inbred lines having nonbitter fruit. We were interested in understanding the interaction between 2 genes causing fruit bitterness and in screening SSR markers for MAS for fruit nonbitterness. The work might also benefit studies on fruit bitterness in other cucurbits.

Materials and Methods

Plant Materials

A set of 148 F_9 RILs was used as materials in this study. The RILs were developed from the cross between 2 cultivated cucumber inbred lines 9110Gt (P_1) and 9930 (P_2). 9110Gt was derived from the cross between a European greenhouse cultivar named BRUNEX and 3 Northern Chinese lines GANCHAO, WUQING, and JINYAN No.6. Inbred line 9930 selected from BEIJINXIAOCI is a Northern Chinese fresh-market cucumber for which the genome has been sequenced (Huang et al. 2009). Foliage and fruit of 9110Gt are nonbitter. The trait of foliage nonbitterness in 9110Gt was derived from BRUNEX. Allelism test verified that the genotype of 9110Gt was *bi-1bi-1*. Foliage of 9930 is bitter but fruit is nonbitter. A single F_1 plant from 9110Gt × 9930 mating was self-pollinated to produce F_2 progeny, which were then advanced to F_9 by single-seed descent to generate 148 RILs.

Foliage and Fruit Bitterness Evaluation

Foliage and fruit bitterness evaluation was conducted in 4 greenhouse seasons in Beijing, China (2006 spring and autumn, 2007 autumn, and 2009 spring). Plants from the 2 parental lines, F_1 and 148 RILs, were grown in the greenhouse to study bitterness. For each growing season, the experiment was a randomized complete block design, consisting of 3 blocks with 5 plants per plot for each RIL (in total, 15 plants per RIL), 10 plants per plot for parental lines, and the F_1. Seeds of the test materials were sown in pots in the greenhouse, and the seedlings were transplanted into the greenhouse at the 3-leaf stage. Foliage and fruit bitterness were evaluated using tasting method described by Andeweg and DeBruyn (1959). Evaluation of foliage bitterness was conducted by tasting the cotyledons of seedlings, mature leaves, or tendrils 3 times. The tasting of the fruit was conducted from the first fruit to the end of trial. Three people trained to detect bitterness tasted the same plant every time to ensure proper results. Each taste-tester rinsed orally and ate a soda cracker after tasting a bitter plant in order to maintain their palate for further tasting.

SSR Marker Analysis

DNA was extracted from young leaf tissue of the parental lines, F_1, and each line in the population of RILs using a CTAB (Cetyltriethylammonium bromide) extraction procedure (Staub et al. 1996). DNA concentration was estimated on a 1% agarose gel with 1× TEA buffer, stained with ethidium bromide.

Each 15 μL of the polymerase chain reaction (PCR) mix contained double distilled water (ddH_2O) 8.02 μL, 10× buffer 1.5 μL, dNTPs (10 mM) 0.2 μL, Taq DNA polymerase (10 U/μL) 0.08 μL, primer F (50 ng/μL) 0.6 μL, primer R (50 ng/μL) 0.6 μL, DNA (10 ng/μL) 4.0 μL. The PCR amplifications were performed using a GeneAmp PCR system 9700 (Applied Biosystems incorporated, Foster City, California) as follows: 94 °C/4 min, 35 cycles of 94 °C/15 s, 55 °C/15 s, 72 °C/30 s, and 72 °C/5 min, 16 °C. Subsequently, 3 μL of the PCR product was employed for electrophoresis in 6% polyacrylamide gel according to the method used by Sambrook and Russell (2001).

A total of 2416 pairs of SSR primers were screened to identify polymorphisms between the parental lines (9110Gt and 9930) of the RILs population. The development of SSR primers used in this study was described by Ren et al. (2009). PCR using identified polymorphic SSR primers were conducted on DNA from each line of the RILs to collect data for gene locus detection.

Genetic Analysis and Locus Detection

Mendelian theory was employed to analyze the inheritance of fruit bitterness according to the segregation ratio in the RILs population. JoinMap version 3.0 (Van Ooijen and Voorrips 2001) was used to develop linkage maps. Marker segregation was analyzed for conformation to Mendelian ratios expected in the RILs using a chi-square test. A minimum logarithm of

Table 1 Segregation ratios of plants with bitter or nonbitter foliage and fruit in 9110Gt×9930 RILs population pooled over the 4 seasons in spring 2006, autumn 2006, autumn 2007, and spring 2009

Generation	Total plants tested (No.)	Plants with bitter fruit (No.)	Plants with nonbitter fruit (No.)	Plants with bitter foliage (No.)	Plants with nonbitter foliage (No.)	Tested segregation	Chi-squared statistic
9110Gt P_1	30	0	30	0	30	not tested	
9930 P_2	30	0	30	30	0	not tested	
9110Gt × 9930 F_1	30	30	0	30	0	not tested	
9110Gt × 9930 RILs	148	41	107	—	—	1:3	0.58
9110Gt × 9930 RILs	148	—	—	81	67	1:1	1.32

The segregation of plants with foliage bitterness and nonbitterness fit a ratio of 1:1.

odds (LOD) of 4.0 was set as a threshold to relegate marker loci into linkage groups, to order markers, and to estimate interval distances (Kosambi function). An interval mapping analysis (Lander and Botstein 1989; Van Ooijen 1992) was conducted using the MapQTL 4.0 package (Van Ooijen et al. 2000) to detect quantitative trait loci (QTLs).

Sequence Annotation and Gene Prediction in Genomic Region Harboring Locus Controlling Fruit Bitterness

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 the matches with the 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

Fruit Bitterness Inheritance of the RILs

The assessment of foliage and fruit bitterness for parental lines and their F_1 generation were consistent over the 4 runs in spring 2006, autumn 2006, autumn 2007, and spring 2009. In total, 120 plants of 9110Gt, 120 plants of 9930, and 120 plants of F_1 were investigated in the 4 seasons. Foliage and fruit of 9110Gt were nonbitter, the foliage of 9930 was bitter but the fruit was nonbitter, and the foliage and fruit of the F_1 were bitter.

For the population of 148 RILs, pooled over the 4 seasons, there were 107 lines with nonbitter and 41 lines with bitter fruits. The segregation fits a ratio of 3:1 (Table 1). There were 81 lines with bitter foliage and 67 lines with nonbitter foliage. The segregation fits a ratio of 1:1 (Table 1). In 148 RILs, there were 39 lines with both fruit and foliage bitterness, 67 lines with both fruit and foliage nonbitterness, and 42 lines with fruit nonbitterness but foliage bitterness.

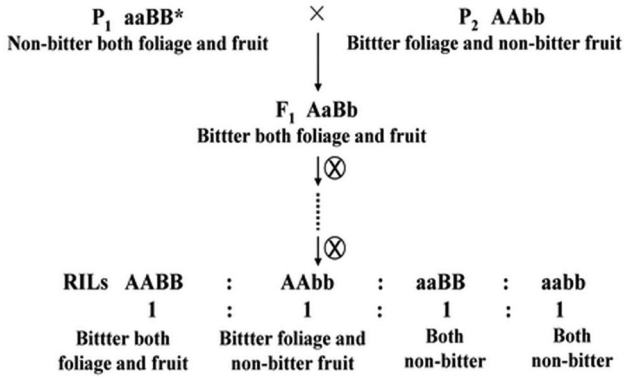

Figure 1. Genetic model of fruit bitterness in the RILs derived from 9110Gt × 9930. P_1:9110Gt; P_2:9930; a:*bi-1*; b:*bi-3*; A:*Bi-1*; B:*Bi-3*. *means homozygous *a* had epistatic recessiveness on *B*. Namely, cucumber plants with nonbitterness foliage must have nonbitter fruit.

They accounted for 25%, 50%, and 25% of the total number of RILs, respectively. From the above data, it was concluded that there were 2 loci controlling fruit bitterness in this RILs population, and one locus was at the same position as the *bi-1* gene. We named the gene harboring the second locus as *bi-3* (Figure 1). The inheritance model was as Figure 1.

Locus Detection using SSR Markers

Molecular analysis performed on 9110Gt and 9930 using the SSR method resulted in identification of 320 primers generating polymorphic amplicons from the total of 2416 pairs of SSR primers. A total of 248 SSR markers showed polymorphism in the RILs population and were employed for linkage analysis and map construction (Miao et al. 2011). Using this SSR linkage map, 2 loci (*bi-1* and *bi-3*, Table 2) were detected for fruit bitterness in this RILs population in each of 4 seasons. Both *bi-1* and *bi-3* were mapped at same loci under 4 environments.

The locus of *bi-1* was located at the position between SSR0004 and SSR02309 within the genetic distance of 5.2 cM on Chr.6. It explained 24.3–25.8% of the phenotypic

Table 2 Characteristics of the 2 loci controlling fruit bitterness obtained from the RILs population

Season	Gene locus	Chr.	Position (cM)	Marker interval	LOD	R2%	Additive effect
Spring 2006	*bi-1*	6	21.5	SSR00004-02309	10.01	24.4	0.22
Autumn 2006	*bi-1*	6	21.5	SSR00004-02309	13.15	26.5	0.22
Autumn 2007	*bi-1*	6	21.5	SSR00004-02309	12.76	24.3	0.21
Spring 2009	*bi-1*	6	21.5	SSR00004-02309	14.50	25.8	0.23
Spring 2006	*bi-3*	5	15.8	SSR00116-05321	8.17	19.2	−0.19
Autumn 2006	*bi-3*	5	15.8	SSR00116-05321	13.4	27.5	−0.23
Autumn 2007	*bi-3*	5	15.8	SSR00116-05321	14.27	27.9	−0.23
Spring 2009	*bi-3*	5	15.8	SSR00116-05321	15.39	27.8	−0.24

R^2 = proportion of phenotypic variance explained by the locus.

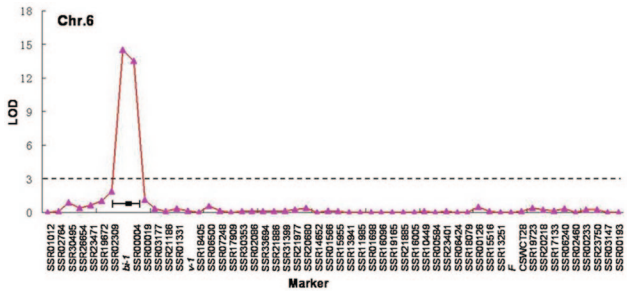

Figure 2. Detecting the locus of *bi-1* controlling fruit bitterness in 9110Gt × 9930 RILs population. LOD scores are shown on the *y*-axis. The LOD threshold of 3.0 is indicated by a horizontal dotted line.

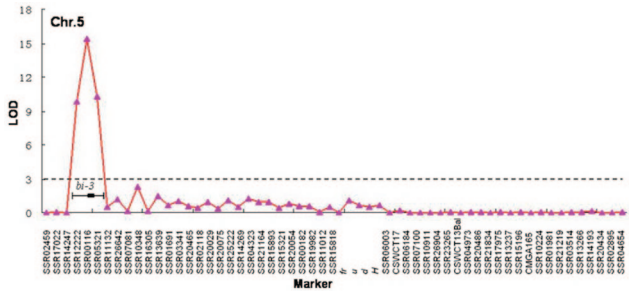

Figure 3. Detecting the locus of *bi-3* controlling fruit bitterness in 9110Gt × 9930 RILs population. LOD scores are shown on the *y*-axis. The LOD threshold of 3.0 is indicated by a horizontal dotted line.

variation with LOD score of 10.01–14.50 in 4 seasons (Figure 2). In previous studies, we had mapped *bi-1* gene to Chr.6 using flanking markers SSR0004 and SSR02309 with genetic distance of 1.9 and 3.3 cM, respectively (Li et al. 2010; Miao et al. 2011). The results for the *bi-1* locus screened by SSR linkage map in the present study and in previous study were consistent with our inheritance analysis. The locus of *bi-3* was placed in the region of SSR00116-SSR05321 within the genetic distance of 6.3 cM on Chr.5. It explained 19.2–27.8% of the phenotypic variation with LOD score of 8.17–15.39 in 4 different seasons (Figure 3).

Annotation and Gene Prediction in the Locus Controlling Fruit Bitterness

The physical distance of the region between SSR00116 and SSR05321 that harbored *bi-3* was 1528.23 Kb based on the whole genome sequence of cucumber, and there were 198 annotated genes in this region. The SSR marker SSR00116 was located on the Scaffold000049 within the region of 78–826257. There were 98 genes annotated in this region. SSR05321 was in the region of 346273–498741 on the Scaffold000047, and there were 100 predicted genes in this region. No cucurbitacin-related candidate gene was identified.

The physical distance between SSR00004 and SSR02309 was 11,430.94 Kb, and there were 160 annotated genes in this region. The SSR marker SSR00004 was located on the Scaffold000028 with 99 annotated genes. SSR02309 was on the Scaffold000058, and there were 61 predicted genes in this region. Among these predicted genes, there is a terpene synthase gene Csa008595, which we previously speculated as the candidate gene for *bi-1* (Huang et al. 2009).

Discussion

It was reported that fruit bitterness in cucumber was controlled by *Bt-1 (Bt)* and *Bt*-2 (Barham 1953; Walters et al. 2001). Further, *bi-1 (bi)* and *bi*-2 control foliage bitterness in cucumber (Andeweg and DeBruyn 1959; Wehner et al. 1998). The genes *Bt-1*, *Bt*-2, *bi-1,* and *bi*-2 were inherited in single-locus fashion (Barham 1953; Andeweg and DeBruyn 1959; Wehner et al. 1998; Walters et al. 2001). The linkage relationships of *Bt-1*, *Bt-2*, *bi-1,* and *bi-2* to other genes have been studied (Bar-Nun and Mayer 1990; Walters and Wehner 1998; Gu et al. 2005; Wehner 2006; Miao et al. 2011). *Bt-1* was not linked to the genes of *F*, *D*, *u*, *dg*, *v-1*, and *pm* (Bar-Nun and Mayer 1990; Gu et al. 2005), but *Bt*-2 was linked to *u*, *D* and *ss* genes (Wehner et al. 1998; Walters and Wehner 1998). There were weak linkages between *bi-1* gene and *dvl*, *de*, *v-1*, *F* (Gu et al. 2005; Miao et al. 2011; Wehner 2006), but there were no linkages between *bi-1* gene and *D*, *u*, *dg* (Gu et al. 2005). However, there are few reports on the interaction among the genes controlling bitterness in fruit and foliage. The inheritance for fruit bitterness in the progeny of 2 parental lines with fruit nonbitterness was

not clear before this study. Here, we concluded that there were 2 loci, *bi-1* and *bi-3*, controlling fruit bitterness in the RILs population derived from 2 parental lines without fruit bitterness. The *bi-1* gene is epistatically recessive to the *bi-3* gene, such that cucumber plants with nonbitter foliage also have nonbitter fruit. However, if the foliage is bitter, its fruit can be bitter or nonbitter. This finding explains the observation that fruit of progeny can be bitter even if the fruits of both parents were nonbitter (Gu et al. 2007). In our study, the cross the *bi-1* and the proposed *bi-3* genotype produced F_1 plants with bitter fruit. Our results indicate that *bi-3* is unique and not allelic to *bi-2* since F_1 plants derived from a cross between *bi-2* and *bi-1* genotypes all produce nonbitter fruit (Wehner et al. 1998). Similarly, *bi-3* is not attributed to *Bt-1* or *Bt-2* since the parental lines utilized in our study produce nonbitter fruit, unlike the bitter fruit phenotype conditioned by *Bt-1* and *Bt-2*.

There have been several studies on the construction of cucumber linkage maps using molecular markers (e.g., Kennard et al. 1994; Park et al. 2000; Young et al. 2000; Bradeen et al. 2001; Fazio et al. 2003; Ren et al. 2009; Weng et al. 2010; Miao et al. 2011). Several important qualitative traits (litteleaf, gynoecious, determinate, small spines, dull fruit skin, fruit ribbing, heavy netting of fruit, foliage bitterness, tuberculate fruit, scab resistance, and monoecious) and some QTLs for quantitative traits (powdery mildew resistance, lateral branching, parthenocarpy, plant height) have been placed on molecular linkage maps (Staub and Serquen 2000; Fazio et al. 2003; Sakata et al. 2006; Sun et al. 2006; Matthew et al. 2008; Heang et al. 2008; Li et al. 2009; Li et al. 2010; Zhang et al. 2010a, 2010b; Kang et al. 2011).

In this study, we developed a SSR linkage map in order to identify loci for fruit bitterness using a RILs population. Consistent with prior results (Li et al. 2010; Miao et al. 2011), the flanking SSR markers (SSR0004 and SSR02309) linked to *bi-1* within the genetic distance of 5.2 cM. Two flanking SSR markers (SSR00116 and SSR05321) linked to *bi-3* within the genetic distance of 6.3 cM are described in this present study. These SSR markers will assist in MAS breeding for nonbitter fruit in cucumber breeding programs.

The *bi-1* gene controls foliage bitterness in cucumber because of the presence of cucurbitacin (Da Costa and Jones 1971). Cucurbitacins are bitter cucurbit triterpenoid compounds and are toxic to most organisms, but attract specialized insects, such as cucumber beetle (Da Costa and Jones 1971; Balkema-Boomstra et al. 2003). Oxidosqualene cyclase (OSC) catalyzes the formation of the triterpene carbon framework in plants (Phillips et al. 2006). An OSC gene (CPQ) in squash (*Cucurbita pepo* L.) is the first confirmed enzyme in the cucurbitacin biosynthesis pathway that has been reported (Shibuya et al. 2004). In our previous study of cucumber, we identified 4 OSC genes, and the CPQ ortholog (Csa008595) resides in a genetic interval that defines *bi-1* (Huang et al. 2009), suggesting that *bi-1* is responsible for cucurbitacin synthesis. Additional research is required to ascribe function to *bi-3*.

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