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ORIGINAL PAPER

Identification and mapping of molecular markers linked to the tuberculate fruit gene in the cucumber (*Cucumis sativus* L.)

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Abstract Warty fruit is one of the highly valuable external quality traits related to the market values of cucumber. Genetic analysis has shown that a single dominant gene, *Tu* (Tuberculate fruit), determines the warty fruit trait in the cucumber plant. An F_2 population (247 individuals) from the cross of S06 × S52 was used for the mapping of the *Tu*/*tu* locus. By combining bulked segregant analysis with the sequence-related amplified polymorphism (SRAP) and simple sequence repeat (SSR) markers, 15 markers (9 SRAPs and 6 SSRs) linked to the *Tu*/*tu* locus were identified. Of nine SRAP markers, three closely linked to the *Tu*/*tu* locus were successfully converted into sequence characterized amplified region (SCAR) markers. The *Tu*/*tu* locus was mapped between the co-dominant SSR marker SSR16203 and the SCAR marker C_SC933, at a genetic distance of 1.4 and 5.9 cM, respectively. Then the linked SSR markers in the study were used as anchor loci to locate the *Tu*/*tu* locus on cucumber chromosome 5. Moreover, the validity analysis of the C_SC69 and C_SC24 markers was performed with 62 cucumber lines of diverse origins, showing that the two SCAR markers can be used for marker-assisted selection (MAS) of the warty fruit trait in cucumber breeding. The information provided in this study will facilitate the map-based cloning of the *Tu*/*tu* gene.

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Introduction

Cucumber (*Cucumis sativus* L.; $2n = 2x = 14$), which belongs to the Cucurbitaceae, is one of the most important vegetable plants grown widely in the world (FAO 1993). Recently, quality requirements for the cucumber have received more and more attention, especially external quality, which is a direct factor in stimulating the purchase desire of consumers (Wang et al. 2007). Warty fruit is one of the highly valuable external quality traits (spine size and color, fruit colors, dull and uniform color, etc.), which are related to the market values of cucumber. Usually, certain cucumber varieties produce warty fruit, while others produce smooth fruit. Cucumber fruits are consumed as the fresh eaten vegetable or processing. Compared with the warty fruit trait, however, smooth and non-warty fruit trait is more important to the fresh eaten cucumber types in cucumber breeding and advantageous in that the maintenance of smooth fruit produces less pollution, they are easy to clean and pack, and have higher resistance during transportation and storage, etc. (Wang et al. 2007). Therefore, studying the warty fruit trait will promote quality breeding of the cucumber. To date, little information on the warty trait has been reported.

Early genetic studies of the warty fruit trait in the cucumber demonstrated that a single gene is responsible for phenotype segregation. This gene was named *Tu* (Tuberculate fruit); the warty fruit phenotype (*Tu*) is dominant to the smooth, non-warty fruit phenotype (*tu*) (Strong 1931; Poole 1944; Andeweg 1956; Walters et al.

2001). Fanourakis and Simon (1987) performed a genetic analysis of 15 loci conditioning morphological and disease resistance characteristic and showed that the *Tu* gene is closely linked to the *D* gene (dull fruit skin) and the *u* gene (uniform immature fruit color). On the classical genetic linkage map, *Tu* is in linkage group IV with the *Te* gene (tender skin of fruit), *ss* gene (small spines), and *pm* gene (powdery mildew resistance) (Pierce and Wehner 1990). Previous reports on the warty fruit trait have been limited to the analysis of the genetic law; however, in 2007, there was a report on molecular markers linked to the *Tu*/*tu* locus. Wang et al. (2007) obtained two simple sequence repeat (SSR) markers linked to the *Tu*/*tu* locus by bulked segregant analysis (BSA); the genetic distance was 20.0 and 14.1 cM, respectively. Although the warty fruit trait has been known, so far there has been little advancement in the study of the *Tu*/*tu* gene.

The main purpose of this work was to determine the genetic mode of inheritance of the warty fruit trait and to identify the molecular markers that are tightly linked to the *Tu*/*tu* locus by combining the BSA method (Michelmore et al. 1991), the sequence-related amplified polymorphism (SRAP) marker technology (Li and Quiros 2001), and the SSR technology. This information gained from this study will facilitate marker-assisted selection (MAS) of warty fruit trait in cucumber breeding and further the map-based cloning of the *Tu*/*tu* gene. Moreover, to further increase our knowledge of the warty fruit trait, we observed the warty fruit trait at morphological and cytological levels. In so doing, we elucidated the cytological mechanism of fruit tumor formation on the cucumber fruit surface. Our results indicate a new direction for future research on the warty fruit trait.

Materials and methods

Plant materials

Three cucumber warty fruit lines—S52, S94, S110—and three smooth fruit lines—S06, S42, S46—were used as parents (Table 1). To determine the inheritance pattern of the warty fruit trait, five cross groups were constructed: between a warty fruit parent and a smooth fruit parent (S06 × S52 and S46 × S110), between two warty fruit parents (S52 × S94), and between two smooth fruit parents (S06 × S42 and S46 × S42). F_1 plants were self-pollinated to produce F_2 plants in each group. For two groups (S06 × S52 and S46 × S110), the F_1 plants were also backcrossed with their recessive parent (S06 and S46) to obtain a BC_1 population. F_1, F_2, and BC_1 plants, as well as the parents, were used in the genetic analysis. The F_2 population of S06 × S52, including 247 plants, and a recombinant inbred line (RILs) population of S94 × S06, comprised 224 lines, were then used for molecular mapping of the *Tu*/*tu* locus. All plants were grown in a greenhouse under natural sunlight at Shanghai Jiaotong University and visually scored twice for the warty fruit trait during the periods of the 7th–10th day after flowering (data not shown). In addition, molecular markers were tested over a range of 62 cucumber lines of diverse origins (Table 1).

Cytological analysis

Fruits of the warty fruit line S52 and the smooth fruit line S06 were collected and immediately fixed in a formalin–acetic acid–alcohol (FAA) solution at room temperature for 48 h or longer. Samples collected were stained with Ehrlich's hematoxylin at 4 days after fixation, then dehydrated with ethanol, infiltrated with xylene, and embedded in paraffin. Serial 8-μm-thick sections were cut with a LEICA RM2126 rotary microtome and affixed to microscope slides. The histochemical staining method described by Zheng and Gu (1993) was followed with minor modifications. The sections were observed and photographed with Olympus microscopes BX51.

DNA extraction and BSA

Genomic DNA was extracted from young leaves with the CTAB method (Clark 1997). Extracted DNA samples were dissolved in TE buffer (pH 8.0) and visualized after electrophoresis on 0.8% agarose gels in 1× TAE. DNA purity and concentration was measured with a UV spectrophotometer. The DNA was adjusted to a final concentration of 30 ng/μl and stored at −20°C until use. Equal amounts of DNA from ten warty fruit (*TuTu* or *Tutu*) and ten non-warty fruit (*tutu*) plants randomly selected from the F_2 population (247 individuals of S06 × S52) were pooled to construct two DNA bulks for BSA (Michelmore et al. 1991).

SRAP and SSR analyses

A total of 736 primer combinations, including SRAP primers from the studies of Li and Quiros (2001), Ferriol et al. (2003), Li et al. (2003), and Wang et al. (2005), and AFLP primers from the studies of Vos et al. (1995) and Xu et al. (2000), were used to screen polymorphisms in the two parents and two bulks. The AFLP primers were used in the SRAP method in the present study, and the resulting polymorphic bands were considered to be SRAP markers. The primers that could amplify polymorphic bands between the BSA pools were tested in the 20 individual plants that made up the two bulks and then further checked

Table 1 Cucumber lines tested with the SCAR markers C_SC69 and C_SC24 for the *Tu/tu* locus

	Lines	Origin	W/S	Markers		Lines		Origin	W/S	Markers	
				C_SC69	C_SC24					C_SC69	C_SC24
1	S106	America	W	A	A	32	S23	Holand	S	B	B
2	S107	America	W	A	A	33	S46	Holand	S	B	B
3	S35-2	China	W	A	B[a]	34	S46-2	Holand	S	B	B
4	S50-3	China	W	A	B[a]	35	S49-1	Holand	S	B	B
5	S52	China	W	A	A	36	S49-2	Holand	S	B	B
6	S57	China	S	B	B	37	S51-2	Holand	S	B	A[a]
7	S58	China	W	A	B[a]	38	S55-1	Holand	S	B	B
8	S59-3	China	S	B	B	39	S05	Israel	S	B	B
9	S60	China	W	A	A	40	S06	Israel	S	B	B
10	S61-2	China	W	A	A	41	S36	Israel	S	B	B
11	S78-2	China	W	A	A	42	S43	Israel	S	A[a]	B
12	S80	China	S	B	B	43	S45	Israel	S	B	B
13	S82	China	S	B	B	44	S47	Israel	S	A[a]	B
14	S94	China	W	A	A	45	S48-1	Israel	S	B	B
15	S98	China	W	A	A	46	S48-2	Israel	S	B	B
16	S99	China	W	A	A	47	S48-4	Israel	S	B	B
17	S100	China	W	A	A	48	S54-2	Japan	S	B	B
18	S103	China	W	A	B[a]	49	S66	Japan	W	A	A
19	S105	China	W	A	A	50	S67	Japan	W	A	B[a]
20	S110	China	W	A	A	51	S69-2	Japan	W	A	A
21	S115-3	China	W	A	A	52	S70	Japan	W	A	A
22	S122-2	China	S	B	B	53	S73-3	Japan	W	A	A
23	S122-16	China	S	B	B	54	S74	Japan	W	A	A
24	H34	China	S	B	B	55	S109-6	Japan	W	A	A
25	S33-1	European	S	B	B	56	S118-1	Japan	W	A	B[a]
26	S34	European	S	B	B	57	S119-17	Japan	W	A	A
27	C19	France	S	A[a]	B	58	S53	Korean	W	A	A
28	C21	France	S	A[a]	B	59	S112-7	Korean	W	A	A
29	C17-1	Holand	S	B	B	60	S42	Spain	S	B	B
30	C17-2	Holand	S	B	B	61	S75	Spain	S	B	B
31	S17-2	Holand	S	B	B	62	S76	Spain	S	B	B

W warty fruit, *S* smooth fruit

Marker genotype designation: *A* warty fruit allele, *B* non-warty fruit allele

[a] Disagreement between marker and phenotype

for their linkage to the *Tu/tu* locus in the F_2 population (247 individuals). The PCR for SRAP was carried out in a 10 µl mixture. The reaction conditions were as follows: 94°C for 3 min, followed by eight cycles at 94°C for 30 s, 35°C for 30 s, and 72°C for 1 min; 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 5 min. The amplification products were separated on 4% denatured polyacrylamide gels with 1× TBE buffer at a constant power of 50 W for 2 h. After electrophoresis, the gel was silver-stained (Bassam et al. 1991) and photographed with a digital camera (Olympus).

All SSR primers used in the study were kindly provided by Professor Sanwen Huang (Chinese Academy of Agricultural Sciences, Beijing, China). The PCR for SSR was carried out in a 10 µl mixture. The reaction conditions were as follows: 94°C for 5 min, followed by 32 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min. The amplification products were separated on 6% denatured polyacrylamide gels with 1× TBE buffer at a constant power of 50 W for 1.5 h. The gels were then silver-stained and photographed, followed by SRAP analysis. Table 2 indicates the polymorphic SRAP and SSR primers used in the study. The primers were synthesized by Sangon Biological Engineering Technology & Service Co. (Shanghai).

Table 2 The primer sequences of polymorphic SRAP and SSR markers used in this study

Marker	Forward primers (5′–3′)	Reverse primers (5′–3′)
SRAP marker		
ME2EM4	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTTGA
ME3SA4	TGAGTCCAAACCGGAAT	TTCTTCTTCCTGGACACAAA
ME6EM9	TGAGTCCAAACCGGTAA	GACTGCGTACGAATTGAT
ME10EM18	TGAGTCCAAACCGGCAT	GACTGCGTACGAATTCCT
ME23EM4	TGAGTCGTATCCGGTAG	GACTGCGTACGAATTTGA
M18EM6	GATGAGTCTAGAACGGCT	GACTGCGTACGAATTGCA
M25OD3	GATGAGTCTAGAACGGTG	CCAAAACCTAAAACCAGGA
M38EM18	GATGAGTCTAGAACGGACT	GACTGCGTACGAATTCCT
M93EM3	GATGAGTCTAGAACGGTTG	GACTGCGTACGAATTGAC
SSR marker		
SSR16203	TCGAGGTAAATCAAAACCGA	ATGTGTCAAACCCACCCATT
SSR07100	CACACCATTTACGGTTATGGG	CATTTGGTTCAGAAAGGGGA
SSR04323	TGGTGGAAAAGAAAAAGGGA	GCTAGGGCACAAGAACGAAG
SSR03943	TTTTTGGTGAAAAGGAACGTG	CACAAAGCAAAATTGAGGGAA
SSR01498	GGCGCCACAAATATTCAACA	CCACAAACGTAAAGAGATTCACA
SSR03529	TGAATTGAATAGACACAACAATATGC	ACATGTTGGGACTCCATGTG

Cloning and sequencing of the SRAP fragment

Sequence-related amplified polymorphism fragments that were found to be linked closely to the *Tu*/*tu* locus in the F_2 population (247 individuals of S06 × S52) were excised from the polyacrylamide gel, washed three times with 20 μl ddH_2O, and boiled at 95°C for 15 min. After centrifugation, the supernatant was used as template for the PCR amplification, using the same SRAP primer combinations as before. Re-amplification reaction was performed in a 50 μl volume; the conditions were as follows: 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and then a final extension at 72°C for 5 min. The re-amplified products were separated on a 1% agarose gel. The DNA bands were purified with the UNIQ-10 Spin Column DNA Gel Extraction Kit (Sangon, Shanghai) and cloned into the pUCm-T vector (Fermentas). Positive colonies bearing DNA of the expected sizes were sequenced with ABI 3700 Sequencer (Sangon, China).

Conversion of SRAP markers to sequence characterized amplified region (SCAR) markers

Sequence characterized amplified region primers were designed from the sequences of the SRAP markers. Then, the SCAR primers were tested in two parental samples, two bulks, and 20 individuals. SCAR amplification was performed in a 10 μl volume; the conditions were as follows: 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, an annealing temperature for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min. The annealing temperature was optimized for the specific pair of the primers. The primer sequences and annealing temperature are listed in Table 3.

For one SRAP marker, the direct conversion into a SCAR marker proved difficult, so a cucumber genomic BAC library was adopted to isolate the DNA sequences flanking the marker fragment. The library, which contained approximately 19,200 *Hin*dIII clones, was constructed with

Table 3 SCAR markers linked to the *Tu*/*tu* locus

SCAR marker	Primers sequence (5′–3′)	Annealing temperature (°C)	Size (bp)	Polymorphism
C_SC69	PF: TTCCGAAAGCAGGAGAGTCAAT	63	218	Dominant
	PR: GCCAGATTTGGTATATCAAACAG			
C_SC933	PF: CTTAAAATCATTTATTTAATGCTTTG	56	368	Dominant
	PR: GTAAGATAAATAACACCAGACCAG			
C_SC24	PF: CCGGAGCTGTGTAATGGAAGAA	55	514/455[a]	Co-dominant
	PR: AATTTGAGATATAACCTACGTGA			

PF Forward primer, *PR* Reverse primer

[a] The size of the PCR product linked to the *Tu* and *tu*, respectively

an inbred line S94 (*TuTu*) according to a previously reported procedure (Guan et al. 2008). After screening the BAC library with PCR, one positive clone containing the specific amplification fragment was sequenced by the Beijing Genomic Institute (Beijing, China). Based on the flanking sequences that were obtained, we concluded that the polymorphic SCAR marker was obtained successfully.

Mapping

An F_2 population of S06 × S52, containing 247 individuals, was used to map the *Tu*/*tu* locus. Data from phenotype survey and SRAP, SCAR, and SSR analyses were combined for linkage analysis using the MAPMAKER/EXP3.0 program (Lander et al. 1987) with a LOD threshold of 3.0 or greater. The recombination percentage was converted to genetic distance by the Kosambi mapping function (Kosambi 1944). A linkage map was drawn using the software MapChart 2.0 (Voorrips 2002).

Results

Morphological–cytological observation of the warty fruit/smooth fruit phenotype

The warty fruit line S52 (Fig. 1a) and the smooth fruit line S06 (Fig. 1b) differed significantly with respect to their fruit phenotypes. Fruit tumors and large fruit spines were observed on the fruit of the S52 line (Fig. 1c), while the fruit of the S06 line was smooth and had small, fine fruit spines (Fig. 1d). To further elucidate the cytological mechanism of fruit tumor formation, we observed the warty fruit trait at cytological levels. Cytological observation showed that a large amount of small tumor cells under the large fruit spine cells existed in the S52 line (Fig. 1e); for the S06 line, however, the fruit tumor was not observed, and the pattern of cells under the fruit spine cells were not distinct from those under the epidermis cells (Fig. 1f). The result indicated that the fruit tumor formation on the cucumber fruit surface arises from an increase in cell number caused by cell division (Fig. 1e). In fact, the fruit tumor was found to be derived from the division of several layers of cells that lie near the fruit spine-base cell by the cytological observations of the entire development of fruit tumor in cucumber (data not shown).

Genetic analysis

The results of the genetic analysis are shown in Table 4. All F_1 plants that were obtained by crossing a warty fruit parent with a smooth fruit parent (S06 × S52 and S46 × S110) had warty fruit; χ^2 tests of the two crosses were consistent with a ratio of 3 warty fruits:1 smooth fruit in each F_2 population and 1 warty fruit:1 smooth fruit in each BC_1 backcross population. Thus, we concluded that a single dominant gene controls the warty fruit trait, which is generally defined as the effect of the *Tu*/*tu* locus. This result is in accordance with previous research reports (Poole 1944; Andeweg 1956; Walters et al. 2001).

F_1 individuals and all F_2 progenies from a cross between two warty fruit parents (S52 × S94) had warty fruit, while F_1 individuals and all F_2 progenies from crosses between smooth fruit parents (S06 × S42 and S46 × S42) had smooth fruit (Table 4). These results show that the warty fruit gene is the same in cucumber lines used in our study.

Identification of SRAP and SSR markers linked to the *Tu*/*tu* locus

Of the 736 SRAP primer pairs that were tested, 348 primer combinations were polymorphic (47.3%) between the two

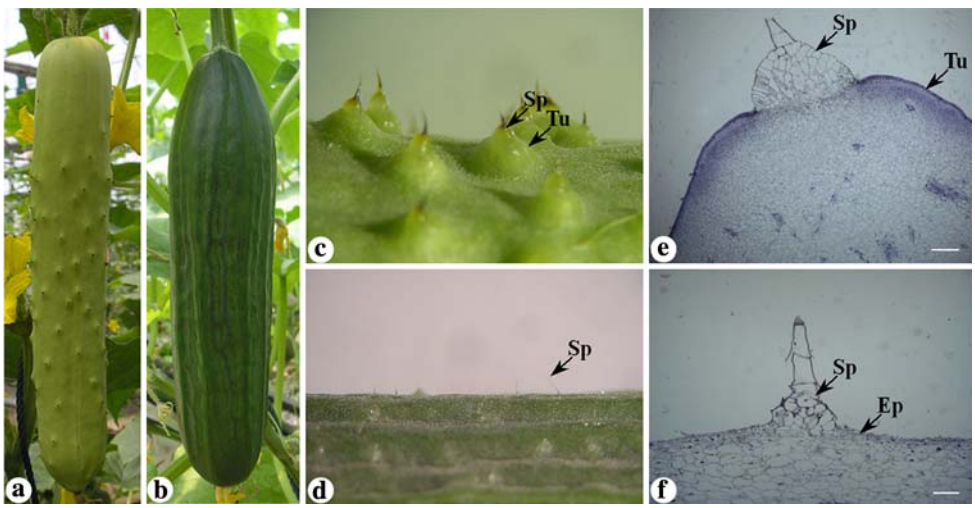

Fig. 1 Morphological and cytological differences of the warty fruit (S52 line) and the smooth fruit (S06 line) phenotype. **a–d** Morphological comparison of the S52 line (**a**, **c**) and the S06 line (**b**, **d**). **e**, **f** Differences in the cytological structure of the S52 line (**e**) and the S06 line (**f**). *Sp* spine, *Tu* tuberculate fruit, *EP* epidermis (*bar* 100 μm)

Table 4 Segregation analysis of the warty fruit trait in the F_1, F_2, and BC_1 progenies of different crosses and the RIL population

Combination	Population	Season (year)	Total	No. of plants		Expected ratio	χ^2 value[a]
				Warty fruit	Smooth fruit		
S06 × S52	F_1		15	15			
	F_2	S (2007)	247	184	63	3:1	0.034
	BC_1	A (2007)	117	58	59	1:1	0.009
S46 × S110	F_1		15	15			
	F_2	A (2007)	230	179	51	3:1	0.980
	BC_1	A (2007)	93	52	41	1:1	1.301
S52 × S94	F_1		5	5			
	F_2	A (2007)	58	58		–	–
S06 × S42	F_1		5		5		
	F_2	S (2008)	62		62	–	–
S46 × S42	F_1		5		5		
	F_2	S (2008)	53		53	–	–
S94 × S06	RIL	A (2007)	224	119	105	1:1	0.875

S spring, *A* autumn

[a] $\chi^2_{(0.05,1)} = 3.84$

parental plants (S06 and S52). Upon further testing with the two DNA bulks and 20 individuals that made up the two DNA bulks, nine SRAP primer pairs (M18EM6, M25OD3, M93EM3, ME6EM9, ME2EM4, ME23EM4, ME3SA4, ME10EM18, and M38EM18) were found to generate polymorphic products in the two bulks and 20 individuals. Using the same approach, six of 240 SSR markers (SSR03943, SSR01498, SSR07100, SSR03529, SSR16203, and SSR04323) were identified linking to the *Tu*/*tu* locus. All potential linkage markers were mapped in the 247 individuals of the F_2 population (S06 × S52). Preliminary linkage analysis was performed with MAPMAKER/EXP3.0.

Conversion of SRAP markers to SCAR markers

The three SRAP markers closely linked to the *Tu*/*tu* locus were converted into SCAR markers, which are more useful for large-scale screens for MAS and further map-based cloning. The polymorphic DNA fragments corresponding to the SRAP markers ME2EM4, ME6EM9, and M93EM3 were cloned and sequenced. Based on the sequencing data, SCAR primers were designed to amplify the corresponding loci from genomic DNA. The SRAP markers ME2EM4 and M93EM3 were converted to co-dominant and dominant SCAR markers directly, designated as C_SC24 (GenBank Accession Number GQ338324, GQ338325) and C_SC933 (GenBank Accession Number GQ338326, GQ338327), respectively. The fragment amplified in the warty fruit parent (S52) and the smooth fruit parent (S06) with C_SC24 primers was 514 and 455 bp long, respectively (Supplementary material S1; Fig. 2a). For the C_SC933 marker, a fragment of 368 bp was found to be present in the warty fruit parent S52 (Supplementary material S2; Fig. 2b).

Unfortunately, the specific primers derived from the ME6EM9 marker could not amplify a polymorphic band in the two parents, indicating that this marker is unable to be converted directly. Therefore, the flanking sequences adjacent to the marker were isolated with a chromosome walking approach using a BAC library. In this way, we successfully obtained approximate 1.0 kb of the flanking sequence and found a one base insertion/deletion and a different of two bases between the two parents (Supplementary material S3, showed in asterisks). Next, the new primers, including the 3′ end of a forward primer that contains the three base differences were designed. The PCR analysis showed that the new SCAR primers could amplify the original polymorphism between the two parents, two bulks, and the 20 individuals that made up the two bulks (Fig. 2c). Eventually, the SRAP marker ME6EM9 was converted into a dominant SCAR marker designated C_SC69 (GenBank Accession Number GQ338328, GQ338329). Its PCR product was a fragment of 218 bp that was linked to the *Tu* allele. Among the 247 individuals of the F_2 population, the segregation patterns of the polymorphic bands amplified by the three SCAR markers were identical to those of their corresponding SRAP markers.

Mapping of the *Tu*/*tu* locus

Based on the six SRAP markers, three SCAR markers, and six SSR markers, a local linkage map of the *Tu*/*tu* locus-encompassing region was constructed (Fig. 3b). The total genetic distance covered by those markers was 47.7 cM. There were 11 markers (SSR03943, M38EM18, SSR01498, ME10EM18, SSR07100, SSR03529, ME3SA4, ME23EM4, C_SC24, C_SC69, and SSR16203) located on

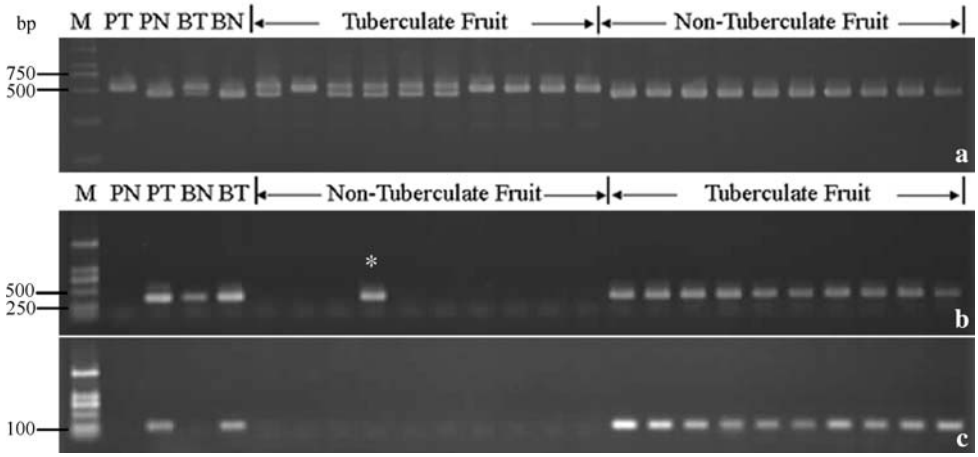

Fig. 2 Amplifications of the three SCAR markers C_SC24 (**a**), C_SC933 (**b**), and C_SC69 (**c**) in two parents, two bulks, and the 20 individual plants that comprising the two bulks. *PT* tuberculate fruit parent S52, *PN* non-tuberculate fruit parent S06, *BT* tuberculate fruit bulk, *BN* non-tuberculate fruit bulk. *Asterisk* indicates the recombinant

one side of the *Tu*/*tu* locus and other four-ones (C_SC933, M25OD3, M18EM6, and SSR04323) on the other side. The two flanking markers closest to the *Tu*/*tu* locus are the co-dominant SSR marker SSR16203 and the SCAR marker C_SC933, at a genetic distance of 1.4 and 5.9 cM, respectively.

A RILs population of S94 × S06 (224 individuals) from our laboratory was used to map the *Tu*/*tu* locus to a certain linkage group of the published cucumber genetic map (Yuan et al. 2008). Two SCAR markers (C_SC24 and C_SC69) are polymorphic between the two parents (S94 and S06). Data from phenotype survey, the two new SCAR markers and the other molecular markers that exist in the genetic map were combined for linkage analysis using the MAPMAKER/EXP3.0 program. The results show that the *Tu*/*tu* locus maps between the SCAR marker C_SC69 and the SSR marker CS15 in the distal end of linkage group 6 (LG6) of the S94 × S06 reference map and is linked to the *D*, *u*, and *ss* (Fig. 3a).

SSR markers flanking the *Tu*/*tu* locus in the study were used as anchor loci to map the *Tu*/*tu* locus on one of cucumber chromosomes (Fig. 3b, c). The six SSR markers (SSR03943, SSR01498, SSR07100, SSR03529, SSR16203, and SSR04323) linked to the *Tu*/*tu* locus all were found on chromosome 5 (Chr.5) of the published integrated genetic and cytogenetic map of cucumber genome of a RIL population from the cross Gy14 × PI 183967 (Ren et al. 2009). Thus, we demonstrated the presence of the *Tu*/*tu* gene in the region between the two SSR markers, SSR16203 and SSR04323, on the cucumber Chr.5 (Fig. 3c, black block showed).

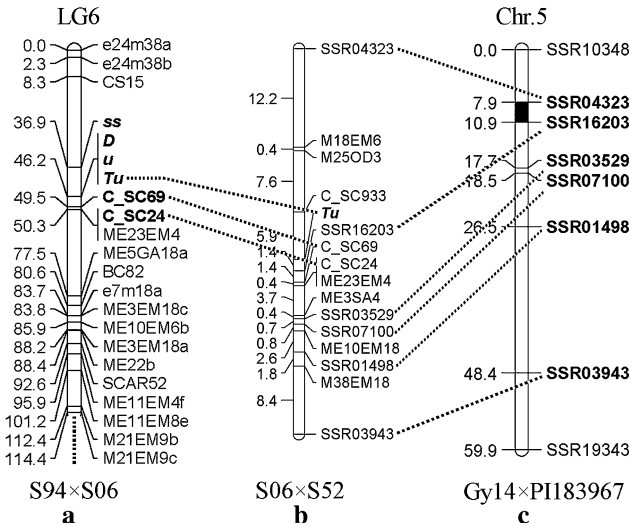

Fig. 3 **a** Mapping of the *Tu* gene to LG 6 of the published cucumber linkage map of a RIL population from the cross S94 × S06 (Yuan et al. 2008). **b** A local linkage map of the region surrounding the *Tu* gene. **c** Six SSR markers linked to the *Tu*/*tu* locus anchored the *Tu*/*tu* gene on Chr.5 of the integrated genetic and cytogenetic map of a RIL population from the cross Gy14 × PI 183967 (Ren et al. 2009). Distances are shown in centimorgans. *Dotted lines* indicate the common markers. *Black block* indicates that the *Tu*/*tu* gene located in the region between the two SSR markers SSR16203 and SSR04323 on Chr.5

Testing in 62 cucumber lines using the SCAR markers C_SC24 and C_SC69

The C_SC24 and C_SC69 markers were tightly linked to the *Tu*/*tu* locus in the F_2 population of S06 × S52. We used 28 warty fruit (*TuTu*) and 34 non-warty fruit (*tutu*) cucumber lines to investigate the validity of the two SCAR markers in a MAS for the *Tu*/*tu* locus. Out of the 62 cucumber lines of diverse origins (Table 1), C_SC24 and C_SC69 could correctly predict the fruit phenotype of 55 and 58, respectively. These results showed that both of the SCAR markers could be used in the assisted selection of the warty fruit/smooth fruit trait in cucumber breeding. For the C_SC24 marker, although the smooth fruit-associated band was incorrectly amplified in 6 of 28 warty fruit lines, the warty fruit-associated band was generated incorrectly in only one of 34 non-warty fruit lines (Table 1); thus, the C_SC24 marker tends to predict the non-warty fruit

phenotype well. For the C_SC69 marker, on the contrary, all mismatches presented in 34 non-warty fruit lines (Table 1), of which four lines amplified the warty fruit-associated band; thus, the C_SC69 marker can correctly predict the warty fruit phenotype.

Discussion

Warty fruit is the strumae of cucumber fruit surface and one of the highly valuable external quality traits. Previous reports on the warty fruit trait have been limited to study on the genetic law (Strong 1931; Poole 1944; Andeweg 1956; Fanourakis 1984; Pierce and Wehner 1990; Walters et al. 2001), to date, regarding the molecular basis of the warty fruit trait, very little information was reported. For many organisms, map-based or positional cloning is the primary strategy for identifying and characterizing genes with unknown biochemical products (He et al. 2008). In the present study, we reported the molecular mapping of the *Tu*/*tu* gene. We identified nine SRAP and six SSR markers linked to the *Tu*/*tu* gene using the method of BSA and located the gene on cucumber Chr.5, between the two SSR markers SSR16203 and SSR04323. The works represent a prerequisite step for positional cloning of the *Tu* gene.

As a relatively new molecular marker technique, SRAP has been broadly applied to study genetic diversity (Ferriol et al. 2003) and gene mapping (Rahman et al. 2007; Li and Quiros 2001) as well as to aid in linkage map construction (Yuan et al. 2008). Normally, the genetic background of the cucumber is rather narrow (Horejsi and Staub 1999). In comparison to other random markers, the SRAP marker has higher polymorphism in the cucumber (Yuan et al. 2008; Li et al. 2008). Out of 736 SRAP primer combinations were tested in our study, 348 pairs were polymorphic (47.3%) between the two parents. The high polymorphism enabled us to obtain nine SRAP markers linked to the *Tu*/*tu* locus.

SRAP marker is less convenient for large-scale MAS in plant breeding. Consequently, the three SRAP markers tightly linked to the *Tu*/*tu* locus were developed to SCAR markers, following the lead of several researchers who converted non-special amplified markers into special amplified markers, such as SCAR from SRAP, RAPD, and AFLP (Tian et al. 2005; Rahman et al. 2007; Li et al. 2008). However, for SRAP marker ME6EM9, the direct conversion into a SCAR marker proved difficult, so the additional flanking region needs to be sequenced. Recently, Adaptor ligation-based PCR-mediated walking (Mibus and Tatlioglu 2004), inverse PCR methods (Knopf and Trebitsh 2006) and BAC library-mediated walking strategy (Li et al. 2008) have been confirmed as being efficient in isolating flanking sequence in cucumber. In our study, we obtained approximate 1.0 kb sequences using BAC library strategy, and find the polymorphism of the primary dominant SRAP marker located in 5′ end of the forward primer ME6. Based on the different nucleotides of the two parental plants, we developed a dominant SCAR marker.

With six linked SSR markers, *Tu*/*tu* was anchored in the region between SSR markers SSR16203 and SSR04323 on cucumber Chr.5 (Fig. 3c, black block showed). The region located at distal end of Chr.5, which demonstrated the previous hypothesis (Fanourakis 1984). It is difficult to obtain the markers tightly linked to the genes located on chromosome centromere and telomere area; thus, this mapping result of *Tu*/*tu* on chromosome might explain why we always could not get closer markers linked to *Tu*/*tu* locus in one side by screening lots of molecular markers, where the nearest marker was C_SC933 (5.9 cM). In fact, in order to find new markers that more closely linked to *Tu*/*tu* locus, we also analyzed the markers between SSR16203 and SSR04323 on Chr.5. On the Ren's map (Ren et al. 2009), we found that 38 SSR markers besides SSR04323 shared at 7.9 cM locus and six SSR markers besides SSR16203 shared at 10.9 cM locus, and there was no other marker locus between the two loci; therefore, we chose all six markers of 10.9 cM locus and ten markers of 7.9 cM locus that were near 10.9 cM locus to analyze the linkage relationship with the *Tu*/*tu* locus in F_2 population (S06 × S52). The result revealed that of these markers, one marker was located at the same locus with SSR16203, one was weakly linked to *Tu*/*tu* (more than 15 cM), and the rest markers could not generate the polymorphic bands in two parents (S06 and S52). We analyzed that it might be due to the different mapping parents, mapping populations, and calculation methods. Based on the above analysis, we did not obtain any new markers more closely linked to *Tu*/*tu* locus; thus, new markers still need to be developed by the BAC library and the sequence of cucumber genome.

In the work, the *Tu*/*tu* locus, with the two SCAR markers (C_SC24 and C_SC69), were together mapped to linkage group 6 (LG6) of the published genetic map (Yuan et al. 2008) (Fig. 3a), and the *Tu*/*tu* locus was located on the cucumber Chr.5 of the updated linkage map (Ren et al. 2009) (Fig. 3c), indicating that LG6 is equivalent to Chr.5. On the LG6, *Tu* was linked with *ss* at a genetic distance of 9.3 cM and co-segregated with *D* and *u* (Fig. 3a). Previous studies (Fanourakis and Simon 1987) also showed that the four morphological markers (*ss*, *Tu*, *D*, and *u*) were linked with each other. Due to the linkage relationship between the four traits, *D*, *u*, and *ss* also were mapped on the Chr.5 of cucumber. Therefore, studies on the *Tu* gene will help in understanding the roles of *D*, *u*, and *ss*.

Markers that are tightly linked (<5 cM) to the agronomic traits can be used for MAS programmes (Tanksley 1983). In this study, two SCAR markers linked tightly to

the *Tu*/*tu* locus, C_SC69 (2.8 cM) and C_SC24 (3.2 cM), can be used in the assisted selection of the warty/smooth fruit trait in cucumber breeding by evaluating the validity of 62 cucumber lines of diverse origins (Table 1). The ability of the C_SC69 marker to predict the warty fruit trait (94%) was higher than that of the C_SC24 marker (89%). C_SC24 is a co-dominant marker and distinguishes the homozygous from heterozygous (Fig. 2a); although the C_SC24 marker has a lower ability to predict the warty fruit trait than C_SC69, it can supply more genetic information. The efficiency for MAS of the warty fruit trait would be increased greatly if using C_SC69 and C_SC24 together with C_SC933, the linked marker on another side of *Tu*/*tu*. In addition, we also analyzed the validity of SSR marker SSR16203 (1.4 cM). The result revealed that the allelic variation for SSR16203 markers was observed in warty fruit and smooth fruit germplasm. Allelic variation in microsatellites has been shown to be the common occurrence in crop plants (Udupa et al. 1999; Lakshmi Padmaja et al. 2005). It is presumed that the variation in the number of repeating units in microsatellite causes the length variations in diverse germplasm.

This study found the cytological mechanism of fruit tumor formation. In plants, many types of tumors that are similar to the fruit tumor of cucumber arise from an increase in cell number caused by cell division (Fig. 1e); examples include the callus induced in plant tissue culture, genetic tumors in tumor radish lines and tobacco lines, and nodules on legume roots, etc. Previous research has shown that the formation of these types of tumors involves a change in the concentration of an endogenous hormone, in particular, cytokinin and auxin (Ichikawa and Syono 1991; Lohar et al. 2004; Matveeva et al. 2004; Il'ina et al. 2006). Matveeva et al. (2004) studied the genetic tumor of the crop-root in tumor radish lines and showed that changes in the concentration of the endogenous cytokinin induce tumor initiation. Oldroyd (2007) reported that the nodule organogenesis on legume roots involved the production of the hormone cytokinin. Fruit tumors are different from other types of tumors in phenotype and sites of formation, but they are all strumae of the specific sites that arise by an increase in cell number. Therefore, based on previous data on the tumor trait, we presume that the formation of the fruit tumor on the cucumber would involve a plant hormone cytokinin. Localized increase in cytokinin may activate cortical cell division and lead to formation of the nodule primordium (Oldroyd 2007). In the study, we also found that the fruit tumor organogenesis was derived from the division of several layers of cells that lie near the fruit spine-base cell by the cytological observations of the entire development of fruit tumor in cucumber (data not shown), so we further presume that localized increase in cytokinin of several layers of cells that lie near the fruit spine-base cell would initiate tumor cell division and eventually lead to formation of the cucumber fruit tumor. In the present study, the result of the cytological observation indicated the putative mechanism of fruit tumor formation; however, further studies will be required to isolate the *Tu* gene for truly elucidating its mechanism.

This work is the first report of the development of markers that are tightly linked to the *Tu*/*tu* gene and mapping of *Tu*/*tu* on cucumber chromosome. This information will facilitate further map-based cloning of the *Tu* gene. At present, we have enlarged the population of S06 × S52 to approximate 1,500 individuals. Next, large population, BAC library from our laboratory and complete draft sequences of the cucumber genome (Cucumber Genome DataBase, http://cucumber.genomics.org.cn/cucumber/cucumber/index.jsp) will be used for the physical mapping of *Tu*/*tu* and the isolation of candidate gene.

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