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Molecular Mapping and Candidate Gene Analysis for Numerous Spines on the Fruit of Cucumber

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Abstract

Number of spines on the fruit is an important quality trait in cucumber. The inheritance and identification of molecular markers for fruit spine density gene can provide a basis for breeding and lay the foundation for gene cloning. Cucumber inbred lines NCG-122 with numerous spines and NCG-121 with few spines were used for genetic analysis and gene mapping in this study. Genetic analysis showed that the numerous spines trait in NCG-122 was qualitative, and a single recessive nuclear gene (*ns*) controlled this trait. The few spines trait was dominant over the numerous spines trait. In the preliminary genetic mapping of the *ns* gene, 8 SSR markers were found to be linked to *ns*, which mapped to chromosome 2 (Chr.2) of cucumber. The closest flanking markers SSR22338 and SSR11596 were linked to the *ns* gene, with genetic distances of 10.2 and 1.7cM, respectively. One-hundred and thirty pairs of new SSR primers and 28 pairs of Indel primers were developed based on sequence information in the preliminary mapping region of *ns*. Fifteen SSR markers and 2 Indel markers were identified to be linked to the *ns* gene after analysis on the F_2 mapping population using the new molecular markers. The 2 closest flanking markers, SSRns-127 and SSR04219, were 0.7 and 2.4 cM from *ns*, respectively. The physical distance between SSRns-127 and SSR04219 was 266.1 kb, containing 27 predicted genes. Csa2G285390 was speculated as the probable candidate gene for numerous spines. The accuracy of the closest linked marker to the *ns* gene, SSRns-127, for MAS breeding was 95.0%.

Subject areas: Genomics and gene mapping
Key words: *Cucumis sativus* L., gene prediction, inheritance, marker-assisted selection, molecular marker

Cucumber is one of the world's most produced vegetables, and fruit quality is a major focus in breeding programs. Fruit quality, including the density of the fruit spines, has an important impact on the marketability of cucumber. In China, northern Chinese type cucumber with many fruit spines is very popular, whereas American processing type cucumber with a few spines is mainly cultivated in

the United States. The fruit of European greenhouse cucumber has a smooth surface with almost no spines. The clarification of the inheritance and identification of molecular markers for the fruit spine density gene will provide a theoretical basis for breeding of fruit quality and lay the foundation for fine mapping and gene cloning.

It was reported that several genes, such as *s*, *s-2*, *s-3*, *ss*, and *ns*, were related to the formation of cucumber fruit spines (Strong 1931; Tkachenko 1935; Caruth 1975; Fanourakis 1984; Fanourakis and Simon 1987). The *ns* gene conferring numerous spines was first reported by Fanourakis (1984). It was recessive to the *Ns* gene conferring few spines. The *ns* gene was linked with *ss* conferring small spines (Fanourakis and Simon 1987). In the F_2 segregation population of WI2757×TMG-1, the ratio of plants with numerous spines to few spines fruit was 3:1. It was concluded that the numerous spines trait in WI2757 was also governed by the recessive gene, *ns* (Wal et al. 1997). Walters (2001) reported that *ns* was linked with several genes, such as *D* (dull fruit skin), *de* (determinate habit), *F* (female), *Tu* (tuberculate fruit), and *u* (uniform immature fruit color). However, there are few reports of gene mapping and molecular markers linked to numerous spines in cucumber. Miao (2011) identified one major quantitative trait locus (QTL) for the density of fruit spines, *Fsd6.1*, on chromosome 6 (Chr.6) using recombinant inbred lines (RIL). The flanking linked simple sequence repeat (SSR) markers were SSR14652 and SSR20680 within a genetic distance of 4.7 cM. So far, there has been no research to locate the *ns* gene on the cucumber genome, and more research is needed to identify molecular markers tightly linked to the gene for use in molecular marker assisted selection (MAS).

In this study, inbred lines NCG-122 with numerous spines and NCG-121 with few spines were used to construct a genetic population for inheritance analysis and chromosomal mapping of *ns*. The results will provide the foundation for fine mapping, gene cloning, and MAS breeding of cucumber spines.

Materials and methods

Experimental Materials

Cucumber inbred lines NCG-122 and NCG-121 (see Figure 1) were used as the parents for the F_1, reciprocal F_1 (F_1'), and F_2 populations. There were 182 plants in the F_2 population. The 2 inbred lines were preserved by T. C. Wehner. There are numerous spines on the fruit surface of NCG-122 (Figure 1a), and few spines on the fruit of NCG-121 (Figure 1b). NCG122 and NCG121 belong to European greenhouse type and American processing type cucumber, respectively. About 2112 pairs of SSR markers were used to map the *ns* gene (Ren et al. 2009).

Experiment Design

All experiments were conducted in the plastic tunnel of the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, during 2012 and 2013. Ten plants each of P_1, P_2, F_1, F_1', were planted in spring 2013 in a randomized complete block with 3 replications. A total of 182 plants of F_2 were also planted. The distances between plants and lines were 25 and 55 cm, respectively.

Trait Investigation and Data Statistics

The number of fruit spines (numerous or few) 10 days after blooming was determined for at least 2 fruits for each plant. Fruit spines were assessed by 2 investigators. Phenotypes were categorized as having either numerous or few spines by visual observation. The segregation ratio was analyzed using SAS 9.2 and Microsoft Excel 2003 software.

Construction of SSR Linkage Groups and Preliminary Chromosomal Mapping for the *ns* Gene

The modified CTAB method (He et al. 2013) was used for the isolation of genomic DNA from each of the 10 plants used for P_1, P_2, F_1, F_1', and for each plant of the F_2 population. The SSR reaction system was described in Zhang et al. (2014). For codominant markers, JoinMap 4.0 (Van Ooijen 2006) was used. The linkage map was constructed by screening SSR markers that showed polymorphisms between paternal and maternal parents, and then using the bulked segregation analysis (BSA) method (Michelmore et al. 1991) to obtain DNA from 7 plants with numerous spines or few spines in the F_2 generation to form the gene pool for primer screening. Finally, we used primers that had been screened for polymorphisms to analyze the genotypes of each plant in the F_2 population, and construct the linkage map using JoinMap 4.0.

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

Development of New Molecular Markers and Second Mapping of the *ns* Gene

In the preliminary mapping region containing the *ns* gene, new SSR primers were designed using the full sequence information of the

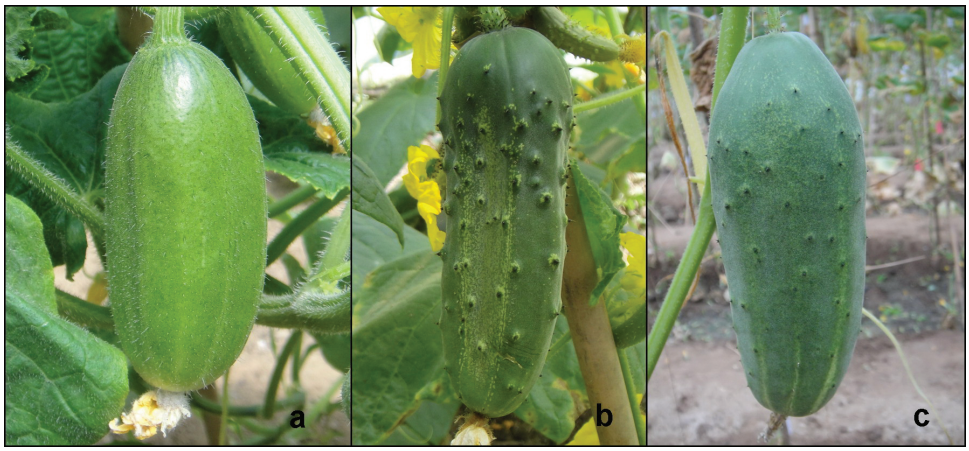

Figure 1. Phenotype pictures of the parental lines and F_1. (**a**) P_1 (NCG-122 fruit with numerous spines); (**b**) P_2 (NCG-121 fruit with few spines); (**c**) F_1 (fruit with few spines).

cucumber genome (Huang et al. 2009). The new primers were first used to screen for polymorphisms in the parents. Selected primers were then used in the F_2 population to determine whether or not they were linked to the *ns* gene.

Using BLAST, sequence alignment was conducted between the genome sequence of 115 core accessions having either numerous spines or few spines genotypes. Insertion and deletion (Indel) markers were designed based on the insertion and deletion points and used in the Indel reaction system described by Zhang et al (2011).

Validation of the Closest Marker Linked to the *ns* Gene for MAS Breeding

An F_2 population including 60 plants was used to validate the closest marker linked with the *ns* gene to determine the accuracy of the markers for MAS breeding. The F_2 population was derived from the cross of Coolgreen×NCG-127. The fruits of Coolgreen and NCG-127 have numerous spines and few spines, respectively. There were 13 plants having numerous spines and 47 plants having few spines in the F_2 population (Table 2).

Sequence Annotation and Gene Prediction in the Genomic Region Containing the *ns* Gene

The sequences were aligned with the cucumber genome sequences (Huang et al. 2009) using BLASTN at an E-value cutoff of 1×10^{-20}. Only matches with an identity of more than 95% were retained. Gene prediction and annotation was performed as in Zhang et al. (2013b).

Data Availability

In fulfillment of data archiving guidelines (Baker 2013), we have deposited the primary data underlying these analyses as follows:

1. Sampling locations, morphological data and microsatellite genotypes: Dryad.
2. DNA sequences: Genbank accessions F234391-F234402; NCBI SRA: SRX0110215.
3. Final DNA sequence assembly uploaded as Online Supplementary Information.

Results

Inheritance of the Numerous Spines Fruit Trait in Cucumber

The parental lines, NCG-122 and NCG-121, had numerous spines and few spines, respectively (Figure 1a and b). The F_1 and reciprocal F_1 of NCG-122×NCG-121 both had few spines (Figure 1c). In the F_2 population, there were 136 plants with numerous spines and 46 plants with few spines. Chi-square analysis ($X^2 = 0.007 < 3.841$) indicated that the segregation ratio was 3:1. Therefore, the expression of the numerous spines trait in NCG-122 is consistent with control by a single recessive nuclear gene, *ns*. The few spines trait was dominant over the numerous spines trait.

Construction of SSR Linkage Groups and Preliminary Chromosomal Mapping for the *ns* Gene

Of the 2112 pairs of SSR markers tested, 282 (13.4%) showed distinct polymorphisms between the parental lines P_1 (NCG-122) and P_2 (NCG-121). These markers were further used to look for polymorphisms in the bulked genomic DNA from plants with numerous spines or few spines. Eight markers (2.8%) were chosen to analyze the DNA from 182 plants in the F_2 population. The resulting data was used to construct a linkage group using JoinMap 4.0 (LOD = 10). The total length of the linkage group was 25.9 cM, and the average genetic distance was 3.2 cM. The *ns* gene was located between SSR22338 and SSR11596 with a genetic distance of 10.2 and 1.7 cM, respectively (Figure 2b). Combined with the integrated genetic map of cucumber reported by Zhang (2012) (Figure 2a), the present linkage group had 7 common markers on cucumber Chr. 2, so the *ns* gene was mapped putatively to the cucumber Chr.2.

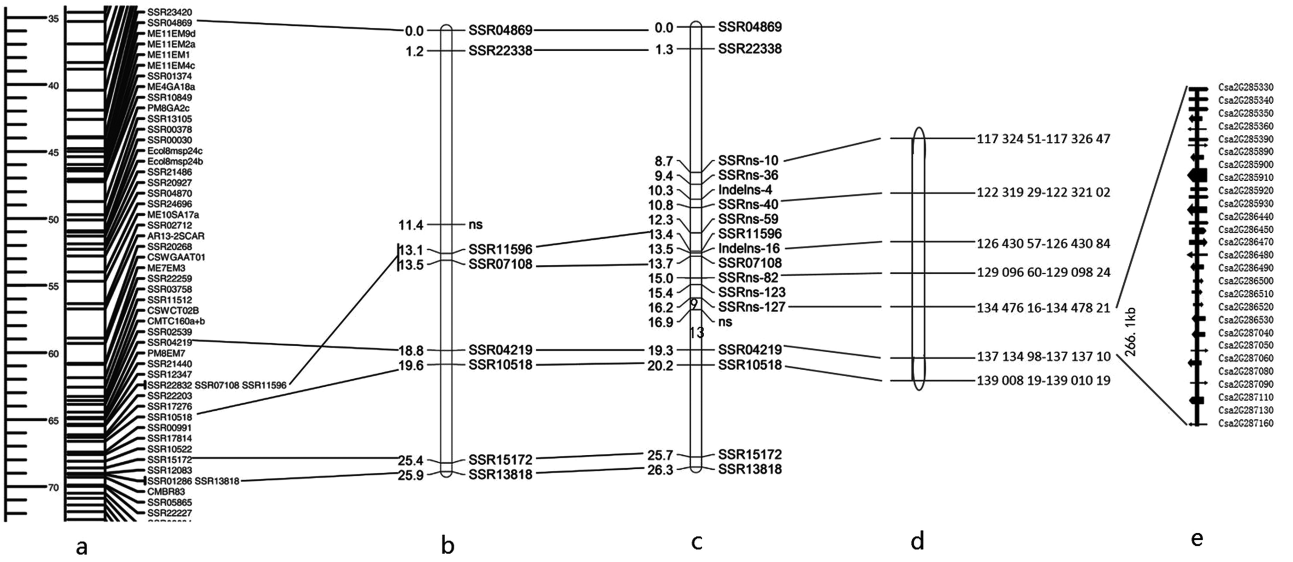

Figure 2. Molecular marker linkage and chromosomal mapping of the numerous spines gene in cucumber. (**a**) Chromosome 2 of cucumber; (**b**) SSR linkage of the numerous spines gene for preliminary mapping; (**c**) linkage of molecular markers to the *ns* gene for second mapping; (**d**) physical map of partial molecular markers; (**e**) predicted genes among the flanking markers. (The number on the bars shows number of recombinant plants between *ns* and flanking markers.)

Second Mapping of the *ns* Gene Using New Molecular Markers

Based on the genome sequence at the preliminary mapping region of the *ns* gene, 128 pairs of new SSR primers were designed, of which 10 pairs had polymorphisms among the parents. Using resequencing information, the sequence differences in the *ns* mapping region were compared by BLAST analysis, and 28 pairs of Indel markers were designed, 3 of which showed polymorphisms among the parents. The 13 pairs of SSR primers or Indel markers that were selected were used to analyze the F_2 mapping population. Then a new linkage group was constructed with 15 SSR primers, 2 Indel markers, and a total length of 26.3 cM (see Table 1 for names and sequences of the primers). Among these, the markers most closely linked to the *ns* gene were SSRns-127 and SSR04219, with genetic distances of 0.7 and 2.4 cM, respectively (Figure 2c).

Validation of the Closest Linked Marker for MAS Breeding

The accuracy of the SSR marker SSRns-127 was tested using 60 plants of the F_2 population of Coolgreen×NCG-127 (Table 2). Plants 8, 13, 15, 16, 17, 21, 24, 44, 46, 48, 54, 55, and 60 had numerous spines. The remaining 47 plants had few spines. For the marker SSRns-127, there were 12 plants (6, 8, 13, 15, 16, 21, 24, 44, 46, 48, 55, and 60) with the same parental allele as NCG-122 (numerous spines). The remaining 48 plants had the same parental allele as NCG-121(few spines), or with both alleles. The result was identical to the phenotype investigation, except for plants 6, 17, and 54 (Table 2). The fruit of 6 had few spines, but the same allele as NCG-122. The fruits of 17 and 54 had numerous spines, but had both alleles. Thus, the accuracy rate for SSRns-127 was 95%.

Table 1. Sequence of SSR and Indel primers for the genetic linkage map of the numerous spines gene in cucumber

Primer name	Forward primer	Reverse primer	Motif	Fragment size
SSR04869	CCAACACCACCCTTCGTTTA	CGGAACCGTTCGTCTTCTT	(TC)27	202
SSR22338	GGTGGATGAAGAGGGGAAAT	TACTCCTTCCTTCGCCCTTT	(AGA)16	159
SSRns-10	TGAATGCATCTCTGTTGTGGA	GGCCATCCTCAAGTTCTCAA	(TCAC)5(CT)10	197
SSRns-36	TGATCACTTGCTTGGTGTTACC	GGCGTTTAGGCCCATAATTT	(AT)10	152
Indelns-4	AGAAAAGACGTTTGTTGGAG	ACCCTTATCGAGTCTTCAAA	Insertion (ACATG)	223/228
SSRns-40	GCCATATTAAGAAAAACCAATTCAC	GCTCTGCTTGATTTCTTCTGC	(AAG)9	172
SSRns-59	AAGCAAAATGGGAATCCAAG	CCCTCCCTCCTTTGCTTAAT	(AAAG)5	240
SSR11596	TCACATAGGCTTGCTCCAAA	TCAAACACCGCGAAAGAGAT	(TA)18	150
Indelns-16	ATGACAAATGATCTGGTGGT	CAATATCCCGTTGTTGTCTT	Deletion (TTTCA)	228/223
SSR07108	TAAGCAATTCCAGGAGAGGG	GTTCTTTGATGGGTGCCTGT	(GA)19	210
SSRns-82	AGATCCTAAAAGGGGATCTTGA	TCCAGAGGTTTTTCCTTTTCTC	(TA)9	155
SSRns-123	CGAAGGTGAAGGCAAAGAAG	TTTAGGGTTTCCAGCCGATA	(AC)9	198
SSRns-127	AGTGACAAAAAGACTAACTCAACAAA	TTGGGTATATAGATTGTCACTACTCCT	(TTA)7	206
SSR04219	GAGACATTGTGGGCATTTGA	CTCATTTTCATCCAAAGGGC	(AT)17	218
SSR10518	TCTAATTCGCTCCGGATGAT	TTGCAGCGAACAATCCTGTA	(ATTA)5(A)30	211
SSR15172	GGTGTGGGTTATTTTGGCAC	GAAGAAATCAAAGAGGGGGC	(CTCTTT)7	168
SSR13818	TTGTTAGTTCATTTGAGGTGTCAAG	TCCATATTAACTCTCTCAGGCTAACA	(TA)32	189

Table 2. Validity of the SSRns-127 marker tightly linked to the numerous spines gene was tested using an F_2 population with 60 plants derived from the cross of Coolgreen×NCG-127

Material code	Fruit spines Phenotype	SSRns-127	Material code	Phenotype	SSRns-127	Material code	Phenotype	SSRns-127
Coolgreen	Numerous	b	F_2-19	Few	h	F_2-40	Few	h
NCG127	Few	a	F_2-20	Few	h	F_2-41	Few	a
F_1	Few	h	F_2-21	Numerous	b	F_2-42	Few	a
F_2-1	Few	h	F_2-22	Few	h	F_2-43	Few	h
F_2-2	Few	a	F_2-23	Few	h	F_2-44	Numerous	b
F_2-3	Few	h	F_2-24	Numerous	b	F_2-45	Few	h
F_2-4	Few	a	F_2-25	Few	a	F_2-46	Numerous	b
F_2-5	Few	h	F_2-26	Few	a	F_2-47	Few	h
F_2-6	Few	b	F_2-27	Few	h	F_2-48	Numerous	b
F_2-7	Few	a	F_2-28	Few	h	F_2-49	Few	a
F_2-8	Numerous	b	F_2-29	Few	a	F_2-50	Few	h
F_2-9	Few	h	F_2-30	Few	h	F_2-51	Few	h
F_2-10	Few	h	F_2-31	Few	h	F_2-52	Few	h
F_2-11	Few	a	F_2-32	Few	h	F_2-53	Few	h
F_2-12	Few	h	F_2-33	Few	h	F_2-54	Numerous	h
F_2-13	Numerous	b	F_2-34	Few	h	F_2-55	Numerous	b
F_2-14	Few	a	F_2-35	Few	a	F_2-56	Few	a
F_2-15	Numerous	b	F_2-36	Few	a	F_2-57	Few	a
F_2-16	Numerous	b	F_2-37	Few	a	F_2-58	Few	h
F_2-17	Numerous	h	F_2-38	Few	h	F_2-59	Few	a
F_2-18	Few	a	F_2-39	Few	h	F_2-60	Numerous	b

Annotation and Gene Prediction in the Genomic Region Harboring the *ns* Gene

The physical distance between SSRns-127 and SSR04219, 2 flanking markers tightly linked to the *ns* gene, was about 266.1 kb (Figure 2d). A total of 27 predicted candidate genes are located in the region, and their distribution on the chromosome is shown in Figure 2. The functions of these annotated genes are shown in Table 3. According to previous studies (Larkin et al. 1993, 1994, 2003), the MYB-bHLH-WD40 complex is involved in epidermal outgrowth, or trichome, development in Arabidopsis and tobacco. Of the 27 annotated genes, Csa2G285390 belongs to the WD40 class, and may be the most probable candidate gene for fruit spine density.

Discussion

Inheritance of Numerous Spines Trait

In this study, cucumber line NCG-122 with numerous spines was crossed with line NCG-121 with few spines, and the segregation ratio of numerous to few was found to fit a 3:1 ratio. This result suggested that the numerous spines trait in NCG-122 was controlled by the single recessive gene, *ns*, which provided a basis for the further study of the *ns* gene at the molecular level.

Development of Molecular Markers and Gene Mapping for the *ns* Gene in Cucumber

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

In this study, SSR markers were used to map the *ns* gene on Chr. 2 within a region of 11.9 cM in the first mapping. Based on this preliminary mapping information, new SSR primers and Indel markers were used to shorten the region from 11.9 to 3.1 cM, with a physical distance of about 266.1 kb. These results provided the foundation for fine mapping and molecular cloning of the *ns* gene. The marker position in the linkage group was consistent with previous map (Zhang et al. 2010). By comparing the QTL for fruit spine density (*Fsd6.1*) identified by Miao et al (2011), we concluded the *ns* gene was different from *Fsd6.1*. They were located on different chromosomes. The reason for this may be that different parental lines were used in the 2 studies, with differences in allelic segregation at these 2 loci.

Table 3. Annotation of genes located in the *ns* genetic mapping region

Predicted genes	Functions	Predicted genes	Functions
Csa2G285330	Glycoside hydrolase family 43; Glycosyl hydrolase family 43 5-bladed beta-propellor domain	Csa2G286490	Protein kinase catalytic domain; Serine/threonine-protein kinase domain; kinase ATP binding site
Csa2G285340	Transcription factor GRAS	Csa2G286500	Zinc finger LIM-type; Ubiquitin interacting motif
Csa2G285350	Ribosomal protein L26/L24P eukaryotic/archaeal; KOW; Translation protein SH3-like	Csa2G286510	Protein kinase catalytic domain; Serine-threonine/tyrosine-protein kinase
Csa2G285360	Porin eukaryotic type	Csa2G286520	Pentatricopeptide repeat
Csa2G285390	WD40 repeat; WD40 repeat-like-containing domain	Csa2G286530	Pentatricopeptide repeat
Csa2G285890	Helix-loop-helix DNA-binding domain	Csa2G287040	Ankyrin repeat; Ankyrin repeat-containing domain
Csa2G285900	Lipase class 3	Csa2G287050	Proteinase inhibitor I13 potato inhibitor I
Csa2G285910	RNA recognition motif domain; Nucleotide-binding alpha-beta plait	Csa2G287060	mRNA splicing factor thioredoxin-like U5 snRNP; Thioredoxin fold; Thioredoxin-like fold
Csa2G285920	Peptidase M24 structural domain; Peptidase M24A methionine aminopeptidase subfamily 1	Csa2G287080	Allergen V5/Tpx-1-related; CAP domain
Csa2G285930	Transcriptional factor B3; Restriction endonuclease type II EcoRII N-terminal	Csa2G287090	Domain of unknown function DUF547
Csa2G286440	Calcium-binding EF-hand; EF-hand-like domain	Csa2G287110	POX; Homeodomain-like; Homeodomain-related
Csa2G286450	Calcium-binding EF-hand; EF-hand-like domain	Csa2G287130	Zinc finger C2H2-type
Csa2G286470	Pentatricopeptide repeat; Tetratricopeptide-like helical	Csa2G287160	Transcriptional factor B3; Restriction endonuclease type II EcoRII N-terminal
Csa2G286480	Multicopper oxidase type 1; Multicopper oxidase type 2; Multicopper oxidase type 3		

Application of Molecular Markers Tightly Linked to the *ns* Gene for MAS Breeding

In recent years, DNA molecular marker technology has become widely used in crop breeding. Using DNA markers that are closely linked to the quality traits under investigation, MAS can improve breeding efficiency and accelerate the breeding process. Therefore, it was important to identify molecular markers that are tightly linked to the *ns* gene for breeding new cultivars using MAS to meet different market demands in the world.

In this study, SSRns-127 was identified as tightly linked to the *ns* gene, with a genetic distance of 0.7 cM. With an F_2 population of 60 cucumber plants, the accuracy of the 2 markers for MAS were achieved 95.0%. This study identified linked markers, rather than the gene itself, which has some limitations in breeding, for example transgenic modification of the specific gene. Further research will involve the development of gene markers that are co-segregating with the *ns* gene.

Prediction of Candidate Genes for the *ns* Gene

Trichome is a special structure of most plant epidermis. In Arabidopsis, the MYB-bHLH-WD40 complex was involved in trichome development (Larkin et al. 1993, 1994, 2003). As described above, the *ns* gene was within a region of 13447.6–13713.7kb on cucumber Chr. 2, and 27 predicted candidate genes were identified from the genomic map of cucumber. In the 27 annotated genes, Csa2G285390 belonged to WD40 with the function of a repeat-like-containing domain and was speculated as the most probable candidate gene. Further research is needed in this area.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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Data Availability

Data deposited at Dryad: http://dx.doi.org/10.5061/dryad.2vj5h

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