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Molecular mapping and candidate gene analysis for fruit epidermal structure in cucumber

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Abstract

Firmness of skin is an important quality of processing cucumbers, which is a feature of the palisade epidermis in these types compared to flat epidermis in fresh cucumbers. This study was conducted to map the *Pe* (palisade epidermis) and analyse the candidate gene. Populations derived from the cross of two inbred lines, NCG122 with fruit flat epidermis and NCG121 with fruit palisade epidermis, were used to identify the inheritance of palisade epidermis and to map the gene involved in its development. The results showed that the palisade epidermis trait is controlled by a single gene, *Pe*, that is dominant over flat epidermis. Seven simple sequence repeat (SSR) and five insertion deletion (Indel) markers were identified to be linked to the *Pe* gene. It was mapped to cucumber chromosome 5 (Chr.5) between SSR14611 and Indelpe12 with genetic distances of 0.3 cM and 0.2 cM, respectively. The physical distance of the genomic region harbouring the gene was 227.5 kb with 26 predicted candidate genes. The accuracy of marker-assisted selection using the molecular markers, Indelpe12 and SSR14611, was 68% and 88%, respectively.

KEYWORDS
Cucumis sativus L., fruit epidermal structure, gene mapping, molecular marker, palisade epidermis

1 | INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the most important cultivated vegetable crops, ranking 4th in quantity of world vegetable production. It is estimated that China produces around three-fourths of the global total every year, and in 2015 that amounted to almost 50 million tonnes (Anon 2016). Cucumbers can be grouped into either processing or fresh market types according to the use of the fruit. In America and Europe, the main use is for processing cucumbers, whereas in Asia the main use is for fresh market types. Unlike fresh market cucumbers, an important quality of processing types is the firmness of the skin (Yu, Zheng, Kong, & Xiao, 2007), which is related to the structure of the cells in the epidermis. The epidermis in most plants is a single layer of cells that covers the leaves, flowers, roots, stems and fruits (Yang, Yue et al., 2014; Yang, Zhang et al., 2014). However, in cucumbers, the structure of the epidermal cells of the fruit varies according to genotype. Tkachenko (1935) analysed the epidermal structure and several other characters in cucumber and found two types of epidermal structure: one with cells perpendicular to the surface and the other with thin flat cells. Fanourakis and Simon (1987) reported that the epidermal cells of processing cucumbers are arranged perpendicular to the fruit surface (palisade type), while those of fresh market types consist of square or flat cells (flat type). Du, Li, Xu, and Chen (2012) confirmed the rectangular shape of the palisade cells in processing cucumbers by microscopic section.

An examination of the inheritance of the epidermis in processing cucumbers, and the identification of specific molecular markers linked to it could lead to improved quality. Fanourakis and Simon (1987) concluded that the expression of palisade or flat epidermal cells was determined by a single gene, and that the ratio of palisade:

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flat was 3:1 in an F_2 population, suggesting monogenic inheritance with dominance for palisade epidermal cells. The gene was named *Pe* (Fanourakis & Simon, 1987) and was mentioned in the gene list for cucumber (Wehner, 2005). So far there has been no report of the location of the *Pe* gene on the cucumber genome. In this study, we developed populations from the cross of inbred lines NCG121 with palisade epidermis, and NCG122 with flat epidermis. The main purpose of this work was to conduct chromosomal mapping and to identify candidate genes for *Pe* to facilitate marker-assisted selection (MAS) for processing cucumber and provides a foundation for fine mapping and gene cloning of *Pe*.

2 | MATERIALS AND METHODS

2.1 | Plant material

Two cucumber inbred lines, NCG122 and NCG121, were used as parental lines to develop segregating populations. NCG122 is a European fresh market cucumber with flat epidermal cells; NCG121 is an American processing cucumber with palisade epidermal cells. Two crosses were made between NCG122 (P_1) and NCG121 (P_2) to generate F_1 and reciprocal F_1 (F_1') populations. An F_2 population was generated by self-pollinating F_1, and F_1 was backcrossed with P_1 and P_2 to generate BC_1P_1 and BC_1P_2.

All experimental materials were grown in a greenhouse at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, P.R. China. Ten plants each of P_1, P_2, F_1 and F_1', and 240 plants of F_2, were planted in spring 2015 in a randomized complete block arrangement with three replicates, with distances between plants and lines of 25 and 55 cm, respectively.

2.2 | Phenotype confirmation

Fruits of P_1, P_2, F_1, F_1' and each plant of F_2, BC_1P_1 and BC_1P_2 were harvested at 15 days after blooming when the fruits were at marketable maturity. Fruits epidermal structures were observed cytologically in cross-section. Cross-sections (about 30 μm) of the epidermal cells of two fruits from each parental line were cut with a razor blade perpendicular to the long diameter and rated as either palisade or flat. Each section was observed at least twice with a Zeiss light microscope at 400×, and the experimental data were analysed using SAS 9.2 and Microsoft Excel 2013.

2.3 | Paraffin sections for histology

Fruit epidermal cells of P_1, P_2 and F_1 were dehydrated in a graded series of ethanol (70%, 85%, 95% and 100%), followed by a xylene/ethanol series (xylene/ethanol 1:3, 1:1, 3:1 and 100% xylene). Xylene was replaced gradually with paraffin (Paraplast Plus, Sigma, P3683) at 60°C for 2 days with four times replacement of paraffin. Sections of 10 μm were made using a HEISTION ERM3000 microtome, and stained with Fast Green. Each section was observed at least twice with a OLYMPUS light microscope at 400×.

2.4 | Construction of SSR linkage groups and preliminary chromosomal mapping for the *Pe* gene

Genomic DNA of P_1, P_2, F_1 and each plant of the F_2 population were prepared using the modified CTAB method described by He et al. (2013) and dissolved in water. The purity and concentration of DNA were measured using a UV spectrophotometer and diluted to 10 ng/μl. Samples of DNA from seven fruits with palisade epidermis and seven with flat epidermis were selected randomly from the F_2 population to construct two DNA bulks to use with the bulked segregation analysis (BSA) method of Michelmore, Paran, and Kesseli (1991).

A total of 2,112 pairs of SSR primers (Ren et al., 2009) were used to screen for polymorphisms in the two parental lines and the two bulks. The primers were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd., Shanghai, China. The PCR system for SSR markers was as reported previously (Zhang et al., 2013). The amplified products of the PCRs were separated on 6.0% non-denaturing polyacrylamide gels with 0.5× TBE buffer at a constant power of 150 V for 1–1½ hr, then stained with $AgNO_3$ (Bassam, Caetana-Anolles, & Gresshoff, 1991) and photographed with a digital camera.

A genetic linkage map was constructed with JoinMap 4.0 (Van Ooijen, 2006) using the phenotypic and molecular marker data obtained from the F_2 population. The map was constructed with a LOD threshold of 3.0. The recombination percentages were transformed to genetic map distances using Kosambi's mapping function (Kosambi, 1944). Comparison of this linkage group with that of Zhang et al. (2012) provided a putative position for the *Pe* gene on the cucumber chromosome.

2.5 | Development of new molecular markers and second mapping of the *Pe* gene

To target the putative position of the *Pe* gene, new primers were designed using the whole-genome sequence (Huang et al., 2009) and the re-sequence information of the parental lines. These new primers were used to screen for polymorphisms in the parents. Then, selected primers were used on the F_2 population to determine whether or not they were linked to the *Pe* gene. Using BLAST, sequence alignment was conducted between the genome sequences of the parents. 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). The software, Primer 3, was employed to design the Indel primers (http://frodo.wi.mit.edu/).

2.6 | Validity of the molecular markers linked to Pe for MAS breeding

In order to determine the accuracy of the molecular marker Indelpe12 and SSR14611 for MAS breeding, 16 accessions of cucumber germplasm with flat type fruit cell and eight accessions of

cucumber germplasm with palisade type fruit cell were used to investigate the validity of the flanking marker Indelpe12 and SSR14611 by performing MAS for the *Pe* locus.

2.7 | Sequence annotation and gene prediction in the genomic region harbouring the *Pe* gene

The sequences were aligned with the cucumber genome sequences (Huang et al., 2009) using BLASTN at an E-value cut-off of $1 \times e^{-20}$. Only matches with an identity of more than 95% were retained. Gene prediction and annotation were performed as in Zhao et al. (2015).

3 | RESULTS

3.1 | Genetic analysis of the fruit palisade epidermis trait in cucumber

The morphological phenotypes of the fruit epidermal cells in P_1, P_2, F_1, and $F_{1'}$ and each plant of F_2 were evaluated by light microscope. The parental lines, P_1 (NCG-122) and P_2 (NCG-121), had fruit palisade epidermis and fruit flat epidermis, respectively (Figure 1d, e). Both the F_1 and reciprocal F_1 had fruit palisade epidermis (Figure 1f). In the F_2 population, there were 168 plants with fruit palisade epidermis and 58 plants with fruit flat epidermis. Chi-square analysis ($X^2 = 0.0531 < 3.841$) indicated that the segregation ratio was 3:1. In the backcross of BC_1P_1, there were 37 plants with fruit palisade epidermis and 42 plants with fruit flat epidermis. Chi-square analysis ($X^2 = 0.3165 < 3.841$) indicated that the segregation ratio was 1:1. In the backcross of BC_1P_2, all plants had fruit palisade epidermis (Table 1).

3.2 | Preliminary chromosomal mapping for the *Pe* gene

From the 2,112 pairs of SSR primers, 282 (13.4%) showed polymorphisms. These were tested further on the bulk DNA samples prepared from plants with fruit palisade and fruit flat epidermis. Seven of these were used to analyse the DNA from 226 plants in the F_2

FIGURE 1 Phenotype pictures of the fruit and fruit epidermis microstructure of the parental lines and F_1. (a) Fruit of P_1 (NCG122); (b) Fruit of P_2 (NCG121); (c) Fruit of F_1 (NCG122 × NCG121); (d) Cytological structure of fruit flat epidermis in NCG122; (e) Cytological structure of fruit palisade epidermis in NCG121; (f) Cytological structure of fruit palisade epidermis in F_1

TABLE 1 Segregation ratios of plants with fruit palisade epidermis and fruit flat epidermis in NCG122 × NCG121 genetic populations

Populations	No. of total plants	No. of plants with fruit palisade epidermis	No. of plants with fruit flat epidermis	Expected ratio	Chi-square	Significance	*p*
P_1(NCG122)	30	0	30	–	–	–	–
P_2(NCG121)	30	30	0	–	–	–	–
F_1	30	30	0	–	–	–	–
BC_1P_1	79	37	42	1:1	0.3165	Not	.83
BC_1P_2	69	69	0	–	–	–	–
F_2	226	168	58	3:1	0.0531	Not	.61

population. Based on this data, a linkage group was constructed, using JoinMap 4.0, that included the seven SSR markers (Table 2). The total length of the linkage group was 62.9 cM with an average genetic distance of 8.99 cM. The *Pe* gene was mapped between two flanking SSR markers, SSR14611 and SSR11012, with genetic distances of 0.1 and 0.9 cM, respectively (Figure 2b), and was mapped putatively to the cucumber Chr.5.

3.3 | Second mapping of the *Pe* gene using new developed molecular markers

Using the re-sequencing information from NCG122 and NCG121, the sequence differences in the *Pe* mapping region were compared by BLAST analysis, and seven pairs of Indel markers were designed. Five of these showed polymorphisms among the parents and were used to analyse the F_2 mapping population. A new linkage group was constructed with seven SSR markers and five Indel markers (Table 2) that spanned a length of 63.4 cM. The *Pe* gene was located between Indelpe12 and SSR14611 on scaffold000006 with genetic distances of 0.2 cM and 0.3 cM, respectively (Figure 2b). Two recombination events were detected between Indelpe12 and *Pe*, as well as SSR14611 and *Pe*. The physical distance between the two flanking markers was 227.5 kb.

3.4 | Validity of the molecular markers linked to *Pe* for MAS breeding

In the F_2 population of NCG122 × NCG121, the flanking marker Indelpe12 and SSR14611 were tightly linked to *Pe* locus. A total of 24 accessions of cucumber germplasm with different type of fruit cell were used to testify the validity of the molecular marker Indelpe12 and SSR14611 (Table 3). According to the analysis of cucumber germplasm with different divers, the accuracy of Indelpe12 and SSR14611 was 68% and 88%, respectively. The accuracy of molecular marker Indelpe12 is too low to be used for MAS. Another molecular marker SSR14611 with high accuracy can be used for MAS. Therefore, the

TABLE 2 Sequence of SSR and Indel primers for the genetic linkage map of the fruit palisade epidermis gene in cucumber

Primer name	Forward primer (5′→3′)	Reverse primer (5′→3′)	Motif	Fragment size (bp)
SSR19836	TGCAGAAACATGAAAAGGGA	TGATTGAGTCCCATATGCCC	(AT)5	158
SSR15818	GGACATGTCAACTCCCCTGT	GCCTCTAGCCTGAAAGACCA	(AT)7	178
SSR11012	TCGTAATTTATGAAAATAGAACGGT	CGATTGCGCAAAATGTGTAT	(TA)9	146
SSR14611	ATTTGGCTTCCAGGTTGATG	GAATCGAAAACCGATGAGGA	(AT)5	242
SSR19172	CGAGGAGAAGGACTACGACG	CGTGTGGAGCTTTCTCACAA	(CAC)7	277
SSR23265	CCTCCCTCCATTTTCCACTA	TGAGCCAGTTGGGGTTTTAG	(AG)14	218
SSR21219	CCATTTCAATCCGCATAACC	CGCAATTGACGGCTATGTTA	(CTT)8	166
Indelpe06	CTTGCTACTTTGGTAGGTTT	ATGGAAACATGAGGGCTTT	Insertion (AAATTT CATATTAAACTA AATTGATTTTATGTC)	372/405
Indelpe07	GAATCACGTAAGCCAACCT	GCATCCCTCCTTGTATCTATC	Deletion (AGAAAAAAA)	216/207
Indelpe08	TCACCGACTTAAACGAGAT	TGACTATTGTCTTCCTCAAC	Deletion (TTTTGAA ACACATA)	445/431
Indelpe10	CAAAGGATTTCACTAAGGAGAG	CACACCAAGTGGCATATCA	Deletion (GGGTTGTC CATACAGAGAGATAA)	100/77
Indelpe12	GTCTTGAAGTCTAAGAGTGA	TAAACTTGCCTTGCCTTG	Deletion (TATTCGTAA CTTTTTAAAATGTTT GTTATAGAATCAAT TAACATTCTATAATTAAAGT)	270/212

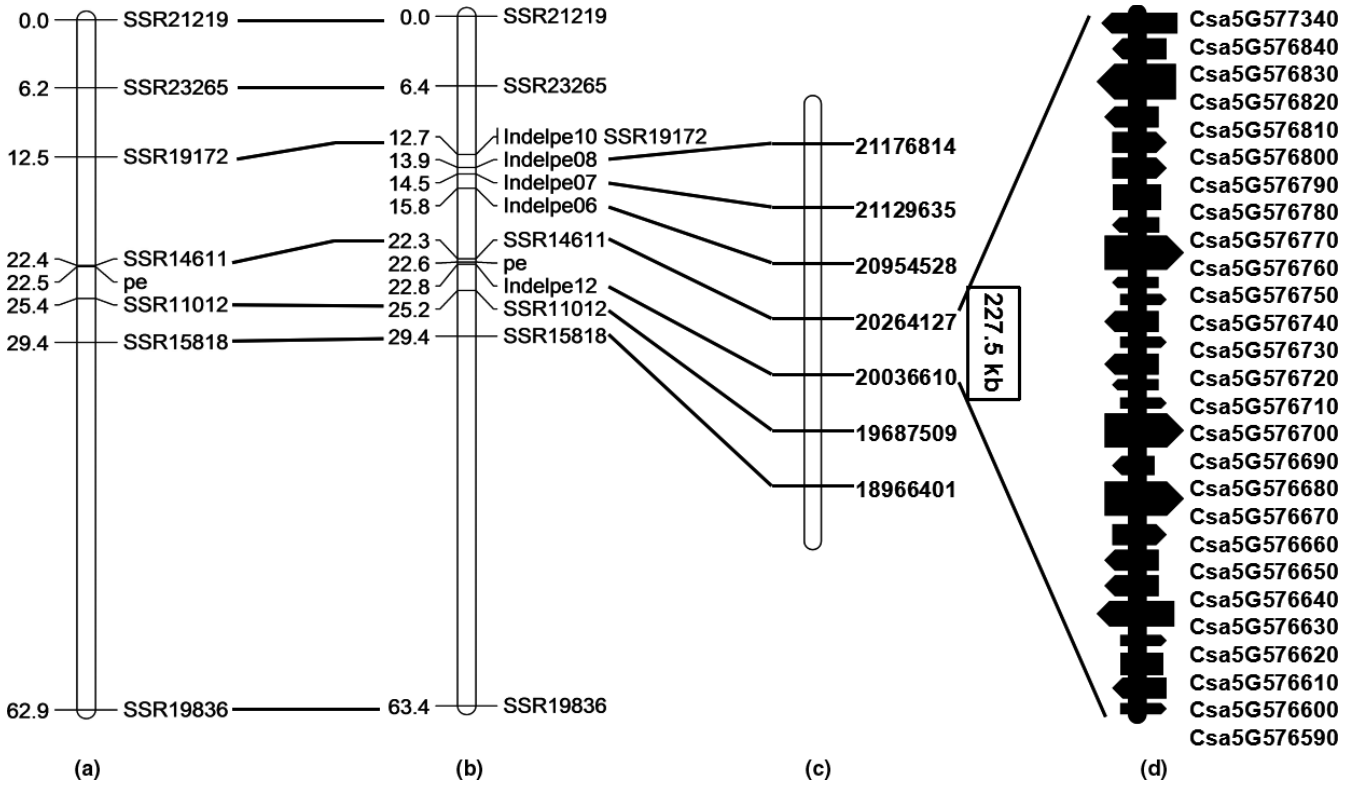

FIGURE 2 Genetic and physical maps of the fruit palisade epidermis (*Pe*) gene in cucumber. (a) SSR linkage map of the *Pe* gene for preliminary mapping; (b) genetic linkage of SSR and Indel markers of the *Pe* gene for second mapping; (c) physical map of partial markers; (d) predicted genes between the flanking markers in the Cucurbit genomics database

molecular marker SSR14611 can be used in the MAS of the palisade/flat type fruit cell trait in cucumber breeding.

3.5 | Annotation and gene prediction in the genomic region harbouring the *Pe* gene

The target genomic sequence obtained from the linkage map of the *Pe* gene was downloaded from the Cucurbit Genomics Database (http://www.icugi.org/) and showed 26 predicted genes and five unknown genes. Their relative location on the chromosome is shown in Figure 2c, and the functions of these annotated genes are shown in Table 4.

4 | DISCUSSION

In this study, the inheritance of fruit palisade epidermis in cucumber was analysed using populations covering six generations (P_1, P_2, F_1,

TABLE 3 Validity of the molecular marker Indelpe12 and SSR14611 was tested using 24 accessions of cucumbers

Germplasm	Type of fruit cell	Indelpe12	SSR14611	Germplasm	Type of fruit cell	Indelpe12	SSR14611
CG4	Flat type	a	a	CG57	Flat type	a	a
CG10	Flat type	a	a	CG63	Palisade type	b	a
CG30	Flat type	a	a	CG85	Flat type	b	a
CG33	Flat type	a	a	CG87	Palisade type	b	a
CG35	Flat type	b	a	CG89	Flat type	a	a
CG39	Flat type	a	a	CG91	Flat type	a	a
CG40	Flat type	a	a	CG99	Flat type	a	a
CG42	Palisade type	b	a	CG102	Flat type	a	a
CG43	Palisade type	b	a	CG105	Flat type	a	a
CG47	Palisade type	b	a	CG108	Flat type	a	a
CG50	Palisade type	b	a	CG109	Palisade type	b	h
CG55	Palisade type	b	a	CG116	Flat type	a	a

TABLE 4 Annotation of genes located in the *Pe* genetic mapping region

Predicted genes	Functions
Csa5M576590	Auxin efflux carrier; contains IPR004776 (Auxin efflux carrier)
Csa5M576600	Putative homeobox-leucine zipper protein; contains IPR003106 (Leucine zipper, homeobox-associated), IPR009057 (Homeodomain-like)
Csa5M576610	pH-response regulator protein palA/RIM20; contains IPR004328 (BRO1 domain), IPR025304 (ALIX V-shaped domain)
Csa5M576620	ATP synthase subunit beta; contains IPR005722 (ATPase, F_1 complex, beta subunit), IPR020971 (ATP synthase, F_1 beta subunit), IPR027417 (P-loop containing nucleoside triphosphate hydrolase)
Csa5M576630	Histone chaperone asf1; contains IPR006818 (Histone chaperone, ASF1-like)
Csa5M576640	Acyl-ACP thioesterase; contains IPR002864 (Acyl-ACP thioesterase)
Csa5M576650	Phosphatidylserine synthase, putative; contains IPR004277 (Phosphatidyl serine synthase)
Csa5M576660	Fructose-bisphosphate aldolase; contains IPR000741 (Fructose-bisphosphate aldolase, class-I), IPR013785 (Aldolase-type TIM barrel)
Csa5M576670	2-oxoglutarate and iron-dependent oxygenase domain-containing protein 2; contains IPR005123 (Oxoglutarate/iron-dependent dioxygenase)
Csa5M576680	Unknown protein
Csa5M576690	Unknown protein
Csa5M576700	Zinc finger protein, putative; contains IPR000571 (Zinc finger, CCCH-type)
Csa5M576710	Unknown protein
Csa5M576720	Putative two-component response regulator family protein; contains IPR009057 (Homeodomain-like)
Csa5M576730	Unknown protein
Csa5M576740	Cytochrome b6/f complex subunit V; contains IPR003683 (Cytochrome b6/f complex, subunit 5), IPR007802 (Cytochrome b6/f complex subunit VI)
Csa5M576750	Alpha/beta hydrolase fold; contains IPR016969 (Uncharacterized conserved protein UCP031088, alpha/beta hydrolase, At1g15070)
Csa5M576760	Receptor protein kinase-like protein; contains IPR001611 (Leucine-rich repeat), IPR011009 (Protein kinase-like domain), IPR013210 (Leucine-rich repeat-containing N-terminal, type 2)
Csa5M576770	Nucleotidyl transferase domain-containing protein; contains IPR014729 (Rossmann-like alpha/beta/alpha sandwich fold)
Csa5M576780	Fibre protein Fb34; contains IPR009606 (Protein of unknown function DUF1218)
Csa5M576790	AT4g03420/F9H3_4; contains IPR008507 (Protein of unknown function DUF789)
Csa5M576800	Mitochondrial import inner membrane translocase subunit tim44, putative; contains IPR007379 (Tim44-like domain)
Csa5M576810	Mitochondrial import inner membrane translocase subunit tim44, putative
Csa5M576820	Unknown protein
Csa5M576830	Mitochondrial carrier protein, putative; contains IPR002067 (Mitochondrial carrier protein), IPR023395 (Mitochondrial carrier domain)
Csa5M576840	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein; contains IPR003406 (Glycosyl transferase, family 14)
Csa5M577340	Chaperone protein dnaJ; contains IPR001623 (DnaJ domain)

F_2, BC_1P_1 and BC_1P_2). The results showed a segregation ratio of 3:1 for palisade epidermis:flat:epidermis in F_2 population indicating that the trait for palisade epidermis is controlled by a single dominant gene, *Pe*. And the segregation ratio in BC_1P_1 and BC_1P_2 population also confirmed this conclusion. The fruit palisade epidermis trait was dominant over the fruit flat epidermis trait. This result is consistent with the former study by Fanourakis and Simon (1987).

Using a series of SSR and Indel markers, the *Pe* gene was putatively mapped to Chr.5. Two molecular markers, Indelpe12 and SSR14611, were found to be tightly linked to the *Pe* locus with distances of 0.1 cM and 0.3 cM, respectively. These markers confirmed the location of the *Pe* gene on Chr.5, and that it is located in a region of 227.7 kb on scaffold 000006. Comparison of this region with the data for cucumber on the Cucurbit Genomics Database showed 26 predicted genes and five unknown genes as shown in Table 3.

In plant, Auxin promotes cell growth and elongation. The content and distribution of auxin in the cells affect the formation of plant cells. The distribution of auxin in different tissues and cells is a complex process, which is mainly affected by the following genes: auxin influx carrier protein, auxin signalling F-BOX, transport inhibitor response protein, auxin-responsive protein IAA, auxin-induced protein AUX, auxin response factor, indole-3-acetic acid-amido synthetase GH3, indole-3-acetic acid-induced protein ARG, auxin-induced protein 15A (Teale, Paponov, & Palme, 2006). Based on the results of cucumber genome annotation, two genes, *Csa5M576590* and *Csa5M576630*, attract our attention. The annotation of

Csa5M576590 was Auxin efflux carrier PIN1, PIN1 mediate cellular auxin efflux by limiting the rate in Arabidopsis (Petrasek et al., 2006). The annotation of *Csa5M576630* is Histone chaperone asf1. In Arabidopsis, asf1 play an important role in cell division and early cell differentiation (Sun, Zhou, Zhang, Yanlei, & Huang, 2002). These two genes are genes associated with cell growth and division in Arabidopsis. But the function of these two genes in cucumbers is lacking study. The relationship between these two genes and epidermal cell development also requires further study.

In the last few years, molecular marker technology had been widely used in crop breeding research. MAS has been proved an efficient way to accelerate the breeding of plants. In this study, *Pe* was mapped to a 227.5 kb region (containing 26 candidate genes) of scaffold000006, which lays a solid foundation for the cloning of *Pe*. In the process of developing new molecular markers, we analysed seven Indel markers and obtained five polymorphic markers that are linked to the *Pe* locus. Based on the identification of 24 different cucumber germplasm, the molecular marker SSR14611 can be used for MAS. This finding will facilitate the breeding of processing cucumber in the near future.

The publication of the complete sequence of the Chinese long cultivar (9930) provided an invaluable resource for biological research and gene mapping. The genetic mapping of several cucumber genes was finished. These genes included fruit dull gene (*D*) (Yang et al., 2013), heavy netting of mature cucumber gene (*H*) (Miao et al., 2011), uniform colour gene (*u*) (Yang, Yue et al., 2014; Yang, Zhang et al., 2014), fruit tumour gene (*Tu*) (Yang, Yue et al., 2014; Yang, Zhang et al., 2014; Zhang et al., 2010), black spine (*B*) (Li, Wen, & Weng, 2013), yellow fruit (*yf*) (Lu et al., 2015), white rind gene (*w*) (Liu et al., 2016), fruit bitter (Zhang et al., 2013), glabrous *(Gl)* (Cui et al., 2016; Zhao et al., 2015), fruit spine density (Zhang, Liu et al., 2016; Zhang, Wang et al., 2016). However, chromosomal mapping of palisade epidermis has not been reported so far.

This study is the first report for gene mapping of the palisade epidermis in cucumber. It will help people to understand the mechanisms for structure formation in the epidermis, and to facilitate the breeding of processing cucumbers.

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