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ORIGINAL PAPER

Fine mapping of pepper trichome locus 1 controlling trichome formation in *Capsicum annuum* L. CM334

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Abstract Trichomes are present on nearly all land plants and protect plants against insect herbivores, drought and UV radiation. The trichome-bearing phenotype is conferred by the dominant allele of the pepper trichome locus 1 (*Ptl1*) in *Capsicum annuum*, Mexican 'Criollo de Morelos-334' (CM334). A genetic analysis using simple sequence repeats from pepper cDNA identified the HpmsE031 marker as tightly linked to *Ptl1* in 653 individuals of an F_2 population derived from a cross between CM334 and Chilsungcho varieties. A bacterial artificial chromosome (BAC) library from CM334 covering 12× of the genome was screened using the HpmsE031 SSR marker as a probe and three BAC clones were identified. The *Ptl1* region was covered by one 80 kb BAC clone, TT1B7. Fluorescence in situ hybridization (FISH) confirmed that TT1B7 localized to pepper chromosome 10. One co-dominant marker, Tco, and one dominant marker, Tsca, were successfully developed from the TT1B7 BAC sequence. Tco mapped 0.33 cM up from *Ptl1* and Tsca mapped 0.75 cM down from *Ptl1*. Analysis of the BAC sequence predicts the presence of 14 open reading frames including 60S ribosomal protein L21-like protein (*Solanum demissum*), protein kinase 2 (*Nicotiana tabacum*), hypothetical proteins, and unnamed protein products. These results will provide not only useful information for map-based cloning of *Ptl1* in *Capsicum* but also the starting points for analysis of R-gene cluster inked with *Ptl1*.

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Introduction

Trichomes are small hairs of epidermal origin on the surface of various plant organs and protect plants against damage by UV radiation, drought, herbivores and pathogens (Espigares and Peco 1995; Hagley et al. 1980; Valverde et al. 2001). In *Arabidopsis*, trichomes are well characterized both morphologically and genetically due to their simple single cell structure (Hülskamp 2000; Schellmann et al. 2002; Schnittger and Hülskamp 2002; Szymanski et al. 2000; Walker et al. 2000; Wasteneys 2000). There are approximately 40–60 trichome-related genes in *Arabidopsis* of which a major gene, *Glabrous 1*(*GL1*), suppresses the initiation of trichomes, resulting in nearly complete loss of leaf trichomes. In the whole plant, the complete loss of trichomes is caused by loss-of-function both in *GL1* and in *TRANSPARENT TESTA GLABRA1* (*TTG1*) genes, which negatively regulate trichome initiation (Hülskamp et al. 1994; Walker et al. 1999). Additionally, weakly semi-dominant mutations in *TRIPTYCHON* (*TRY*) also result in a partial loss of trichome initiation by negative regulation and give rise to limited trichome clustering (Hülskamp et al. 1994; Schnittger and Hulskamp 2002; Szymanski et al. 2000). The *try* mutant also displays elevated branch number and DNA content in trichomes. Even though the *caprice* (*cpc*) mutation in *Arabidopsis* does not affect the trichome phenotype, trichome initiation was suppressed by overexpression of *CPC* (Wada et al. 1997).

Although the presence of trichomes is not in itself a typical trait in most plants except for cotton, many researchers have demonstrated that trichomes are tightly associated with resistance to herbivorous insects and plant pathogens (Ashraf et al. 1999; Cardoso 2008). Trichome-bearing plants exhibit more resistance against whiteflies and aphids than glabrous plants, such as tomato and potato (Gentile and Stoner 1968; Goffreda and Mutschler 1989). A recent report demonstrated a close relationship between trichomes and resistance against *Phytophthora capsici* in pepper (Egea-Gilabert et al. 2008). In their study all the resistant pepper plants had trichomes on their stems. The R-gene cluster on pepper chromosome P10 is predicted to include resistance loci for pepper mottle virus, potato virus Y (Caranta et al. 1997; Grube et al. 2000a), and tomato spotted wilt virus (Jahn et al. 2000). Genetic and QTL analysis in our laboratory (manuscript in preparation) showed that the presence or absence of trichomes on the pepper main stem was controlled by two loci (*Ptel*1 and *Ptel*2), which are located on a region of pepper chromosome 10 corresponding to the R-gene cluster for potyvirus resistance (Grube et al. 2000a). Although the direct role of trichomes in disease resistance of pepper is to be elucidated, previous reports in other members of Solanaceae have suggested the possible link of trichomes with resistance to both biotic and abiotic stresses. In this study, we developed BAC-derived markers to perform fine mapping of *Ptl*1 toward map-based cloning of the *Ptl1* and characterization of the R-gene cluster region on pepper chromosome P10.

Materials and methods

Plant materials and phenotyping trichomes on the main stem

Two landrace cultivars of *Capsicum annuum*, Mexican 'Criollo de Morelos-334' (CM334) and Korean 'Chilsungcho' were obtained from Dr. Palloix (INRA-Avignon, France) and Dr. Kim (Kyungpook National University, Korea), respectively. Each cultivar was self-pollinated to obtain next generation seeds, and one seed per each cultivar was grown and used for production of F_1 hybrid seeds, with a trichome-bearing CM334 used as the maternal plant and a glabrous Chilsungcho used as the pollen donor. In a previous study, we generated a first mapping population of 100 F_2 progeny to construct a SNU5 linkage map and subjected the data to QTL analysis for various traits including pepper trichomes (Min et al. 2008). The pepper trichome locus 1 (*Ptl1*) is considered to be a common locus boosting the density of trichomes in a qualitative manner on the main stem and calyx organs. For fine mapping of *Ptl1*, a new F_2 mapping population consisting of 653 individuals was produced by self pollination of a different F_1 seed from the same parents. Each of the F_2 seeds was grown in a greenhouse. Forty-day-old plants were monitored for the presence of trichomes on the surface of the third node of the main stem by visual observation (Fig. 1).

Genomic DNA extraction

Leaf samples were collected from parent plants and their F_2 progeny at 4–6 weeks after germination was also collected and stored at −80°C prior to use. Genomic DNA was isolated by the cetyl trimethyl ammonium bromide (CTAB) protocol (Murray and Thompson 1980; Shi et al. 2008).

BAC library screening and analysis

The CM334 BAC library with 12× genome coverage (Yoo et al. 2003) was screened with the PCR fragment of HpmsE031 SSR marker as a probe. The SSR marker was previously shown to co-segregate with *Ptl1* in the first mapping population of 100 F_2 individuals. For Southern hybridization, the amplicon was labeled by the random hexamer method with [α-^{32}P] dCTP (Feinberg and Vogelstein 1983)

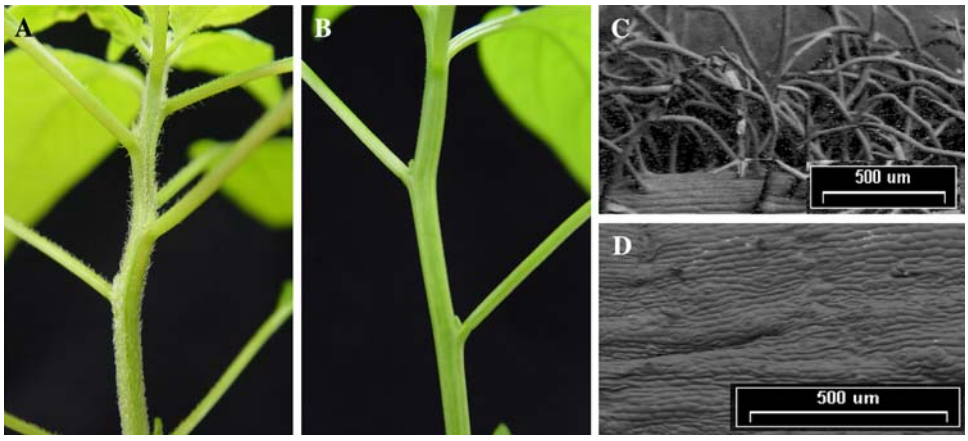

Fig. 1 Main stem trichome phenotypes of *Capsicum annuum* cultivars CM334 (**a** and **c**) and Chilsungcho (**b** and **d**). Photographs **a** and **b** are magnified images. **c** and **d** were taken by a scanning electron microscope

at 37°C for 1–2 h and denatured by addition of a 0.4 N NaOH solution to a final concentration of 0.2 N. After prehybridization of BAC membranes at 65°C for 1 h, the labeled probes were added to the hybridization solution (6× SSC, 0.5% SDS, 5× Denhardt's reagent) and allowed to hybridize for 16–20 h. The membrane was washed twice for 30 min in a washing solution containing 0.1% SDS/2× SSC and 0.1% SDS/1× SSC, respectively. The final membranes were placed in plastic sheets and exposed to Agfa X-ray films for 1–2 days.

To determine the size of BAC clones, positive BAC DNA were digested with *Not*I and fractionated by pulsed-field gel electrophoresis using a CHEF DRIII (Bio-Rad, Hercules, CA, USA) on 1% agarose gel for 12 h at 11°C with an initial pulse time of 10 s, at a 120° angle and 6 Vcm^{-1}.

BAC sequencing and sequence analysis

The BAC clones were fully sequenced by the National Instrumentation Center for Environmental Management (NICEM) at Seoul National University, Korea. The BAC contig was assembled (Macrogen, Korea) using a fingerprinting contig program (FPC; Luo et al. 2003). Repeat sequences were filtered by RepeatMasker (http://www.repeatmasker.org) and JDotter (http://athena.bioc.uvic.ca). The gene coding regions were predicted by FGENESH (http://linux1.softberry.com) or GENESCAN (http://genes.mit.edu) and BALSTX (E value $< 3 \times 10^{-15}$) to distinguish between exon and intron regions of fully sequenced BAC clones.

Fluorescence in situ hybridization

Fluorescence in situ hybridization was carried out as described by Kwon and Kim (2009) with minor modifications. The hybridization mixture contained 50% formamide (w/v), 10% dextran sulfate (w/v), 5 ng/μl of sheared salmon sperm DNA and probe DNA labeled with biotin 11-dUTP or digoxygenin 11-dUTP (Roche) in 2× SSC. After overnight hybridization, the slides were washed twice in 2× SSC for 5 min, once in 50% formamide/2× SSC for 10 min, twice in 2× SSC and once in 4× SSC/0.2% Tween 20 at 42°C. To detect the probes, fluorescein avidin DCS (Vector Laboratories, USA) and anti-avidin-D (Vector Laboratories, USA) or rhodamine anti-digoxygenin (Roche, Germany) and Texas red anti-sheep IgG (Vector Laboratories, USA) were added on slides for 1 h at 37°C in 1% bovine serum albumin or goat serum. After counterstaining with DAPI (1 mg/ml), slides were examined with a fluorescence microscope containing a Cool SNAP CCD camera (Delta Vision, USA). All images were pseudo-colored and improved for optimal brightness and contrast using Adobe Photoshop. We measured chromosome length for karyotype analysis using a LaserPix S/W (Bio-Rad, Germany).

SSR and PCR for marker development

The PCR reactions for the HpmsE031 SSR marker were performed in a 25-μl volume containing 10–25 ng of genomic DNA as template, 10 pmol/μl of each primer, 1 unit of *Taq* polymerase (Takara, Japan) and 10× buffer solution. Forward and reverse primers were labeled with r-[^{32}P] or r-[^{33}P] ATP using T4 polynucleotide kinase. Amplification was performed in a thermocycler (BioRad, USA) according to the following parameters: initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final extension at 72°C for 10 min. Radioisotope-labeled PCR products were separated on a 6% acrylamide gel in 1× TBE buffer. After electrophoresis, the gel was dried at 80°C on a Whatman 3 MM paper in a gel dryer (Bio-Rad, USA) for 2 h and exposed to X-ray film for 2–3 days.

For PCR-based markers from BAC-derived sequences, primers were designed using Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The PCR mixture composed of 3 μl of DNA, 2 μl of 10 × PCR buffer (TaKaRa), 0.3 μl of 10 mM dNTP mixture (TaKaRa), 0.2 μl of 5 U/μl Taq polymerase (Roche, USA), 1 μl of 10 pmol/μl each primer and 14.5 μl of distilled water. PCR was carried out on a PCR machine (PTC-200, MJ research, USA) with the following parameters: initial denaturation at 94°C for 3 min, 29 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were separated on a 1% agarose gel in 0.5× TAE containing ethidium bromide (EtBr) and visualized under UV light.

Linkage analysis

Linkage analysis was performed using the Kosambi (1944) function in Mapmaker 3.0b (Lander et al. 1987) at a logarithm of odds (LOD) value of 8.0 and 20 cM of maximum distance.

Results

Trichome phenotypic analyses

CM334 were found to have high trichome density on the surface of stem (Fig. 1a, c). However, there were no trichome in any part of Chilsungcho and surface was smooth. CM334 trichome on stem was a simple trichome, which had no branch, no glands and no prickle (Fig. 1b, d). F_1 has trichome on surface of stem and calyx.

Further confirmation of co-segregation of HpmsE031 with *Ptl1*

In the first mapping population of 100 F_2 individuals from a cross between CM334 and Chilsungcho, the *r*-[^{32}P] labeled SSR marker, HpmsE031, co-segregated with *Ptl1*, the locus controlling trichome density on the surface of the pepper main stem. Based on this earlier result, this marker was applied to further screen 653 F_2 individuals from a different F_1 hybrid seed from the above same parent plants, and co-segregation of the marker with the *Ptl1* locus was again seen without any recombination events (Fig. 2).

BAC library screening

HpmsE031 PCR bands were rechecked by changing the labeling of the primer pairs from *r*-[^{32}P] to *r*-[^{33}P]. This experiment demonstrated that the original band obtained by *r*-[^{32}P] labeling consisted of three types of polymorphic bands (Fig. 2). Sequence comparison of four SSR bands revealed that the number of the TC repeat motif was different, a discrepancy reflecting the polymorphism of HpmsE031 in CM334 and Chilsungcho.

The band specific to CM334 was extracted and used as probe for BAC library screening. The 12× BAC libraries (Yoo et al. 2003) were screened and 3 out of 8 positive BAC clones were finally selected after checking the existence of probe sequence by PCR analysis with the HpmsE031 primer pair and certification of the TC repeats specific to CM334 in the PCR product (Fig. 3a, c). The average insert size of these clones, named TT1B5, TT1B7 and TT1B9 BAC, was about 80 to 150 kb in pulsed-field gel electrophoresis (Fig. 3b). DNA fingerprinting with five restriction enzymes (*Eco*RI, *Bam*HI, *Xba*I, *Xho*I and *Hae*III) resulted in a contig of TT1B5 and TT1B7 in which almost all sequence of TT1B5 was included in TT1B7. Finally, we selected TT1B7 BAC clone for complete sequencing.

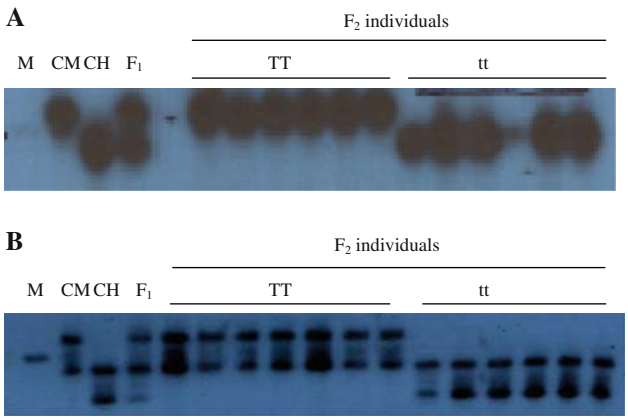

Fig. 2 Segregation of the co-dominant SSR marker HpmsE031 in *Capsicum annuum* CM334 (CM), *C. annuum* Chilsungcho (CH) and F_1 and F_2 hybrid offspring. *M* 330 bp DNA ladder, *TT* trichome-bearing and *tt* glabrous. **a** Amplification results of the SSR marker HpmsE031 labeled with ^{33}P. **b** Amplification results of the SSR marker HpmsE031 labeled with ^{32}P

FISH of TT1B7 for chromosome location

FISH was used to localize TT1B7 on a pepper chromosome. A BAC clone (TG420B1) positive for the TG420 RFLP marker located on the long arm of pepper chromosome 10 was used as positive control. High-resolution pachytene FISH demonstrated that TT1B7 (red) and TG420B1 (green) localized on three or four metaphase chromosomes of CM334 at low stringency washing, whereas a strong signal for the TT1B7 was only detected on one chromosome at high stringency washing condition (Fig. 4). This result indicated that TT1B7 localized on pepper chromosome 10 near TG420.

Fine mapping and size of the *Ptl1* region

TT1B7 (GU108230) was completely sequenced for fine mapping of the *Ptl1* region. The total length of the sequence is approximately 80 kb, including the HpmsE031 sequence at base pair 21,368. Analysis of the BAC sequence predicts the presence of 14 open reading frames including 60S ribosomal protein L21-like protein (*Solanum demissum*), protein kinase 2 (*Nicotiana tabacum*), hypothetical proteins and unnamed protein products. In total, 13 primer pairs designed from the sequence were tested for polymorphisms between the two parents. No polymorphisms were found in any of the PCR products generated using these 13 primer pairs. To find polymorphic sites, all PCR products were sequenced and compared to those of TT1B7. The results demonstrated that the sequences of the PCR bands amplified in CM334 were well conserved, whereas in Chilsungcho, some single point mutations were found. Based on the sequence discrepancies, PCR primer pairs were designed for a dominant marker (Tsca) and a co-dominant marker (Tco) (Fig. 5). Both Tco and Tsca were tightly linked at 0.3 cM and 0.7 cM flanking *Ptl1* on chromosome 10, respectively. The physical locations of three markers on the TT1B7 sequence start from base pair 32,355 for Tco, 21,167 for HpmsE031 and 8,500 for Tsca (Table 1, Fig. 6).

Discussion

In *Arabidopsis thaliana* and cotton develop through a transcriptional regulatory network that differs from the regulating trichome formation in Solanaceous family (Serna and

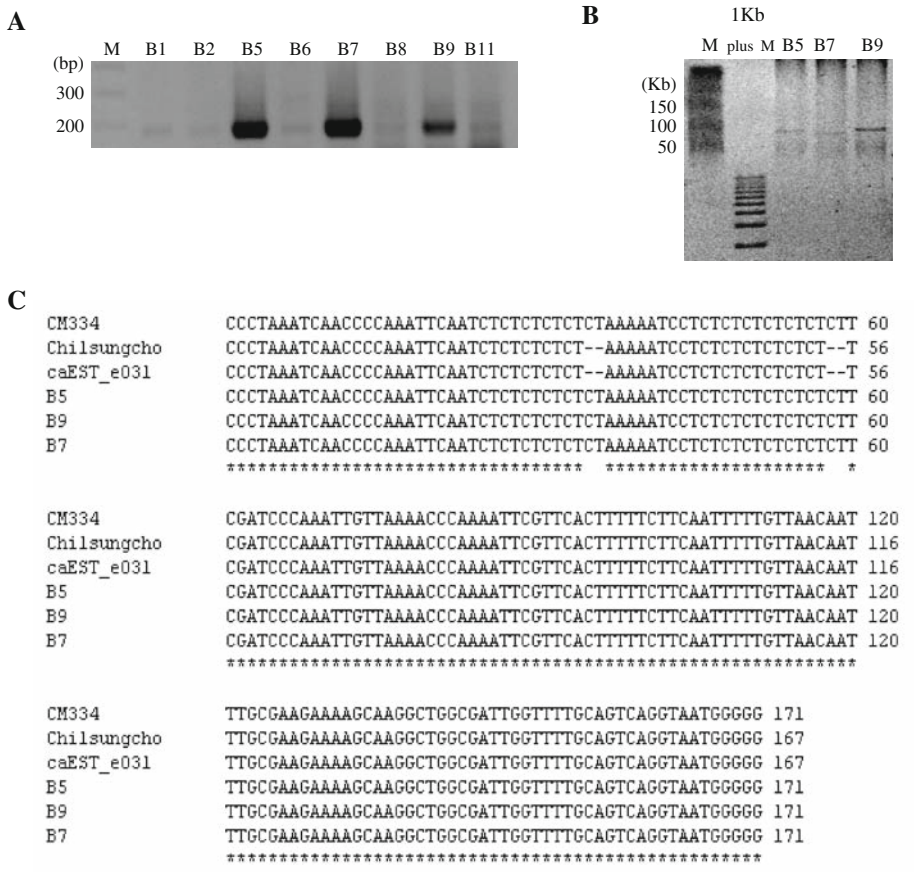

Fig. 3 Analysis of positive BAC clones identified with an [r-^{33}P]-labeled HpmsE031 probe. **a** PCR amplification with HpmsE031 primers. **b** Pulsed-field gel electrophoresis of three BAC clones. The inserts were digested with *Not*I, separated on 1% agarose gel and visualized by ethidium bromide staining. Lambda concatamer size marker. The insert size of each BAC clone was calculated to range from 80 to 100 kb. **c** Nucleotide sequence alignment of simple sequence repeat sequences amplified with HpmsE031 primer pair. caEST_031 (pepper EST), B5 (TT1B5 BAC clone), B7 (TT1B7 BAC clone) and B9 (TT1B9 BAC clone)

Martin 2006). The overexpression of an R-like bHLH factor from maize, which confers excess trichomes on leaves and stems of Arabidopsis (Lloyd et al. 1992) had no effect in trichome formation in tobacco (Lloyd et al. 1992; Mooney et al. 1995; Payne et al. 1999), petunia (Quattrocchio et al. 1993; Spelt et al. 2000) or tomato (Mooney et al. 1995). We tried to develop markers in pepper by PCR with 15 primers derived from five major gene-related trichome development of *Arabidopsis*, CPC, GL1, GL2, GL3 and TTG1. However, there was no polymorphism between CM334 and Chilsungcho and the PCR product showed multi-band pattern (data not shown).

The region of *Ptl1* on pepper chromosome 10 was further narrowed with BAC sequence derived markers developed in this study. Symmetric comparison of the SNU5 map (Lee et al. 2009) to other linkage maps from inter- and intra-specific pepper populations indicated that the *Ptl1* region may be near a hot spot zone containing an R-gene cluster for potyvirus resistance (Grube et al. 2000b). In addition, the hot spot zone has been known to contain genes for disease resistance against other pathogens, such as pepper mottle virus (Caranta et al. 1997; Grube et al. 2000a), potato virus Y (Caranta et al. 1997) and tomato spotted wilt virus (Jahn et al. 2000). The comprehensiveness of disease resistance genes in this zone is probably why many researchers have concentrated on this region (Grube et al. 2000b).

Using a normal mapping strategy, *Ptl1* was fine mapped between the Tco and Tsca markers from the sequence of the TT1B7 BAC clone probed with HpmsE031. For most of chromosome 2 in tomato, the pair/centimorgan relationship is <200 kb/cM. Some hot spots were detected between 120 and 140 cM, and some cold spots between 72 and 73 cM (Koo et al. 2008). The pepper genome is three times larger than that of tomato (Arumuganathan and Earle 1991). Assuming that 1 cM in the 2,800 Mb pepper genome would theoretically correspond to a physical distance of 200 to 400 Kb, the interval between Tco and Tsca markers were 1.08 cM, which would be 23,855 bp in physical distance. In addition, we used the TG420 marker to determine the position of the HpmsE031 marker on chromosome 10 (Jahn et al. 2000). The marker TG420 has a genetic map length of 15.5 cM from HpmsE031, whereas BAC clone TG420B1 flanking the same position with HpmsE031 (Fig. 4). These observations imply that great care must be taken during fine mapping of hot spot zones in pepper, because over evaluation of map distances in this region will probably preclude fine mapping.

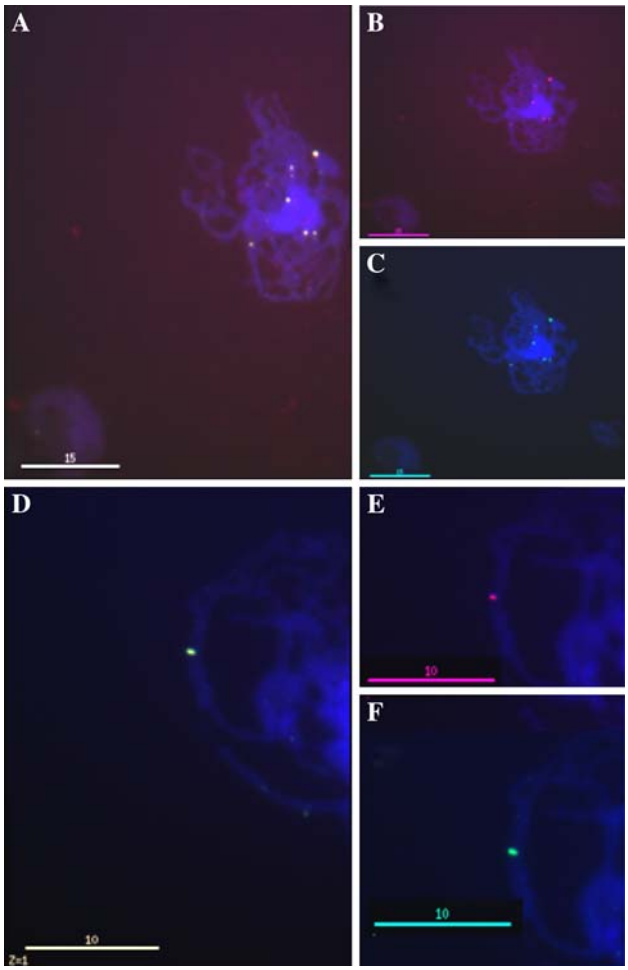

Fig. 4 Fluorescence in situ hybridization analysis for chromosome localization of BAC TT1B7. TG420B1 was used as a landmark for the R-gene cluster region on pepper chromosome 10. Hybridization was followed by low (**a–c**) or high (**d–f**) stringency washing. An intense signal was seen with high stringency washing. **d** Hybridization of TT1B7 BAC clone (*red*) was clearly seen. **f** TG420B1 BAC clone (*green*) co-localized with TT1B7 BAC clone on chromosome 10

Only a few markers have been successfully developed in the R-gene cluster for potyvirus resistance on pepper chromosome 10 (Grube et al. 2000a), even though we have searched for polymorphisms using several marker technologies, such as the conventional amplified fragment length polymorphism (AFLP) and the newly developed reverse random amplified polymorphism and WRKY sequence-based markers during the past decade (Lee et al. 2009). A total of 28 primers from the TT1B7 BAC clone sequence were tested for polymorphisms. Sequence comparisons showed single base pair polymorphisms between CM334 and Chilsungcho in PCR products from eight primer combinations which could not be transformed into molecular markers due to lack of availability of restriction enzyme sites (data not shown). In addition, BSA-RAPD using 200 primers failed to detect polymorphisms between the two parents (data not shown).

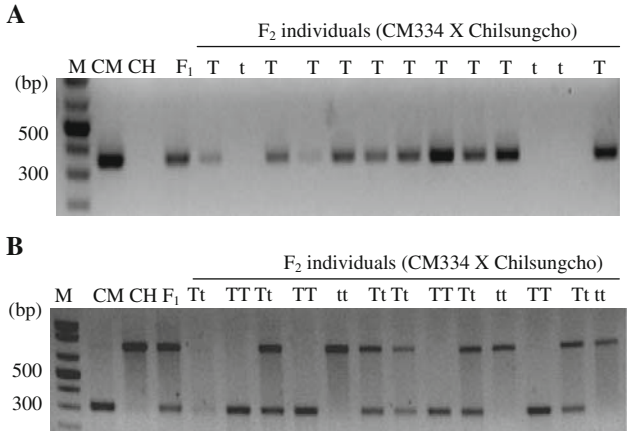

Fig. 5 Segregation of Tsca and Tco markers developed from a TT1B7 BAC clone in CM334 (CM), Chilsungcho (CH) and their F_1 and F_2 hybrid offspring with trichome bearing (TT), glabrous (tt) and hetero (Tt) genotypes. As much as 13 F_2 plants were randomly selected

These results indicate that there is a high degree of sequence conservation between CM334 and Chilsungcho pepper plants. On the other hand, FISH analysis with TT1B7 BAC clone show that signals were distributed on several pepper chromosomes at low stringency washing conditions, while just one signal was detected at high-resolution stringency conditions. Multiple signals on CM334 chromosomes indicate that some sequences homologous to TT1B7 may be present on some chromosomes due to a few repeat sequences in the BAC clone.

Isolation of several genes by map base cloning has been successfully used in various plant species (Desloire et al. 2003; Koizuka et al. 2003; Komori et al. 2004). There are several essential steps of cloning method, including detailed mapping of the target gene region, constructing a large insert BAC library, screening the library with the closest linked markers for chromosome walking and contig assembly and identifying the target gene from the selected fragments by transformation. In this study, we present a localized linkage map covering the Ptl1 gene region and a BAC library from one parent, CM334. With our present findings, we can sequence the full BAC and develop the new SCAR and CAPS markers that link closely with the *Ptl1* gene. Sequence analysis demonstrated that this sequence interval included a type of protein kinase gene which could be the *Ptl* gene. Further studies on loss- and gain-of-function of the predicted genes in the TT1B7 BAC sequences will provide the direct evidence on the role of *Ptl1* in pepper trichome formation. Furthermore, the fine map of pepper *Ptl1* locus obtained in this study could provide the starting point not only for unveiling the R-gene cluster linked with *Ptl1* but also for understanding the regulatory networks of trichome formation in Solanaceous plants

Table 1 Primers used in this study for markers which were polymorphic between CM334 and Chilsungcho

Marker	Primer sequence (5′→3′)	Tm (°C)	Product size (bp)
HpmsE031-F	CCCTAAATCAACCCCAAATT	50	167
HpmsE031-R	CCCCCATTACCTGACTGCAA	56	
Tsca-F	AAACGCCATCATTCGTTTTC	55	356
Tsca-R	CATGAAAGTTGACCCGAACA	54	
Tco-1F	TCATTTTCCATACCTATACACC	47	300
Tco-1-1R	TCAGTCAAACTAGACAGCAGATCC	54	
Tco-1-2F	AATGTGTCATATAGGCTGATTGA	50	
Tco-1-2R	AGATCATAGGATCATTAACAGTTG	48	

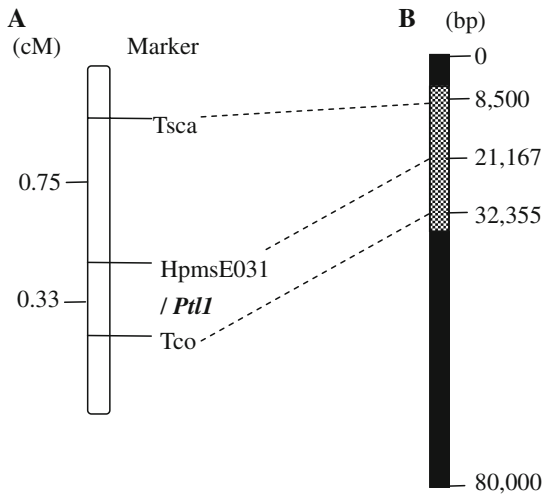

Fig. 6 Fine mapping of the *Ptl1 (I)*. A genetic linkage map of the *Ptl1 (I)* locus which controls trichome density in pepper was generated using a recombinant population from a cross between CM334 and Chilsungcho. Genetic distance in centimorgan (cM) was calculated using the Kosambi function (Kosambi 1944). Numbers on the left indicate genetic distances between markers (cM). **a** Genetic map constructed using 653 individuals of an F_2 population of CM334 × Chilsungcho. The F_2 population was tested using PCR-based markers near the HpmsE031 and the Ptl1 (I) morphological marker derived from the BAC clone. **b** Physical map of TT1B7 BAC clone. The *Ptl1 (I)* gene is located between Tsca and Tco, a segment about 21 kb in length

which is considered as significantly different from that of the well-known Arabidopsis and cotton.

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