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ORIGINAL ARTICLE

Fine genetic mapping of target leaf spot resistance gene *cca-3* in cucumber, *Cucumis sativus* L.

Changlong Wen[1,2] · Aijun Mao[1,2] · Congjuan Dong[1,2] · Huyu Liu[1,2] · Shuancang Yu[1,2] · Yang-Dong Guo[3] · Yiqun Weng[4] · Yong Xu[1,2]

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Abstract
Key message **The cucumber target leaf spot resistance gene *cca-3* was fine mapped in a 79-kb region harboring a CC-NB-ARC type R gene that may be responsible for the hypersensitive responses to infection of the target leaf spot pathogen in cucumber.**

Abstract The target leaf spot (TLS) is one of the most important foliar diseases in cucumber (*Cucumis sativus* L.). In this study, we conducted fine genetic mapping of a simply inherited recessive resistance gene, *cca-3* against TLS with 193 $F_{2:3}$ families and 890 F_2 plants derived from the resistant cucumber inbred line D31 and the susceptible line D5. Initial mapping with microsatellite markers and bulked segregant analysis placed *cca-3* in a 2.5-Mbp region of cucumber chromosome 6. The D5 and D31 lines were re-sequenced at 10× genome coverage to explore new markers in the target region. Genetic mapping in the large F_2 population delimited the *cca-3* locus in a 79-kb region with flanking markers Indel16874230 and Indel16953846. Additional fine mapping and gene annotation in this region revealed that a CC-NB-ARC type resistance gene analog, *Csa6M375730*, seems to be the candidate gene for *cca-3*. One single nucleotide polymorphism (SNP) was found in the NB-ARC domain of this candidate gene sequence between D31 and D5 that may lead to amino acid change, thus altering the function of the conserved NB-ARC motif. This SNP was validated in the segregating population as well as 24 independent cucumber lines. There was significantly higher level of *cca-3* expression in the leaves of D5 (susceptible) than in D31 (resistant), and the expression level was positively correlated with the areas of necrotic spots on leaves after inoculation. It seems the *cca-3* resistance gene was able to induce hypersensitive responses to the infection by TLS pathogen.

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C. Wen, A. Mao, and C. Dong contributed equally to this work.

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✉ Changlong Wen
wenchanglong@nercv.org

Yang-Dong Guo
yaguo@cau.edu.cn

1 National Engineering Research Center for Vegetables, Beijing Vegetable Research Center (BVRC), Beijing Academy of Agricultural and Forestry Sciences, Beijing 100097, China

2 Beijing Key Laboratory of Vegetable Germplasms Improvement, Beijing 100097, China

3 College of Agriculture and Biotechnology, China Agricultural University, Beijing 100193, China

4 USDA-ARS, Vegetable Crops Research Unit, Horticulture Department, University of Wisconsin, Madison, WI 53706, USA

Introduction

Target leaf spot (TLS), caused by the phytopathogenic fungus *Corynespora cassiicola* (Berk. and Curt.) has a wide range of hosts over 530 plant species (Li et al. 2012) including important crops such as cucumber (Dixon et al. 2009; Teramoto et al. 2011; Yang et al. 2012a), rubber (*Hevea brasiliensis*) (Qi et al. 2009), tomato (*Solanum lycopersicum*) (Oka et al. 2006), kiwifruit (*Actinidia chinensis*) (Yuan et al. 2014), balsam pear (*Momordica charantia* var. *abbreviate*) (Li et al. 2010), and scarlet sage

(*Salvia splendens* Ker-Gawl.) (Furukawa et al. 2008). This disease has been widely reported in cucumber in many countries including Japan (Miyamoto et al. 2009, 2010), the United States (Ishii et al. 2007), Korea (Kwon et al. 2003), Sri Lanka (Watudura et al. 2003), and China (Yang et al. 2012a; Li et al. 2012). TLS is one of the most important foliar diseases in both field and greenhouse cucumbers (Dixon et al. 2009; Teramoto et al. 2011), which infects leaves of seedling and adult plants. Under severe infection, the cucumber yield could be reduced by 20–70 % (Yang et al. 2012a).

In cucumber, symptoms of TLS generally occur on the foliage; the infection often appears as light brown spots which grow larger and turn necrotic in the center surrounded by dark brown margins. Diameters of the individual lesions vary 4–10 mm, but in many cases the lesions coalesce and produce large irregular necrotic areas with subsequent drying and shedding of leaves (Blazquez 1967). Race differentiation of *C. cassiicola* in cucumber is unclear, but in *Hevea* rubber, Qi et al. (2009) recognized two physiological races (1 and 2) based on the different reactions to rubber clones. Oliveira et al. (2007) investigated the pathogenicity of 15 *C. cassiicola* isolates from several hosts; inoculation tested on 12 different plant species revealed that the isolates from cucumber infecting other hosts showed the widest host range.

The inheritance of TLS resistance in cucumber has been investigated in several studies. Abul-Hayja et al. (1978) found that a single dominant gene, *Cca*, in Royal Sluis 72502 cucumber line for resistance to TLS. Using an F_2 population derived from Q5 (resistant) × P57-1 (susceptible), Wang et al. (2010) identified a molecular marker CSFR33 that was linked with the susceptible *cca-1* locus from Q5. Fu et al. (2012) further validated this marker by testing in 30 diverse germplasm lines and reported an accuracy rate of 93 %. Yang et al. (2012a) studied the inheritance of TLS resistance with populations derived from PI 183967 (resistant parent, wild cucumber *C. sativus* var *hardwickii*) and Xintaimici (susceptible parent), and revealed that a single recessive gene *cca-2* controls TLS resistance in PI 183967. This gene was mapped in cucumber chromosome 6 and microsatellite (SSR) markers linked with this resistance gene were also identified. Based on linked markers, both *cca-1* and *cca-2* are located in chromosome 6, but their allelic relationship is unknown. The genetic and physical distances of linked markers from the two studies are still far away from the target gene. In addition, these markers are not breeder friendly and not amendable for high-throughput genotyping. Therefore, the objective of the present study was to conduct fine mapping of TLS resistance and develop markers that can be used in high-throughput genotyping. We investigated the inheritance of TLS resistance in D31 cucumber lines using F_1, F_2 and $F_{2:3}$ populations derived from D5 (susceptible inbred line) and D31 (resistant inbred line). Through genetic mapping in a large F_2 population, we were able to delimit the *cca-3* locus carried by D31 into a 79-kb genomic DNA region that contained a resistance gene analog (RGA).

Materials and methods

Plant materials

Two cucumber inbred lines, D5 and D31 were used to develop segregating populations, which were self-pollinated progeny of two introduction lines ZN26 and 5SB, respectively. D31 was resistant to target leaf spot. The other parent D5 was susceptible to TLS. A single F_1 plant of D5 × D31 was self-pollinated to produce F_2 population, each plant of which was self-pollinated to generate F_3 family. Two populations of D5 × D31 mating were used for linkage analysis and fine mapping of the TLS resistance gene with molecular markers, which contained 193 $F_{2:3}$ families and 890 F_2 plants.

Preparation of *C. cassiicola* inoculum

TLS-infected cucumber leaves were collected from the greenhouse of the Beijing Vegetable Research Center (BVRC). Lesions on leaves were cut out, surface sterilized and plated onto the potato dextrose agar (PDA) medium in Petri dishes and incubated at 25 °C. After 2 days, emerging mycelia were sub-cultured onto fresh PDA plates. The identity of *C. cassiicola* was verified by microscopic examination of the shape and size of its conidia (Fig. 1) which was performed by the Plant Disease Diagnostics Laboratory of BVRC. Cultures used for inoculation was from a single spore to ensure genetic uniformity. Pure isolates were grown on PDA plates for 10 days at 28 °C in the dark. Spores were re-suspended in distilled water and filtered through three layers of muslin cloth. The concentration of the spore suspension was adjusted to 3–4 × 10^4 spores/ml which was determined by counting six fields for each sample in a standard Hawksley haemocytometer.

Plant inoculation and disease assessment

In 2012 and 2013, inoculation tests were conducted on F_2 plants, $F_{2:3}$ families (30 plants per family) and two parental lines and their F_1 in the greenhouses (around 25 °C with 16 h light and 8 h dark). Phenotypic data from F_3 families were used as reference in constructing the resistant and susceptible bulks with corresponding F_2 plants. The second true leaves of 3-week-old seedlings were sprayed with spore suspensions. Flats with seedings were kept at 28 °C

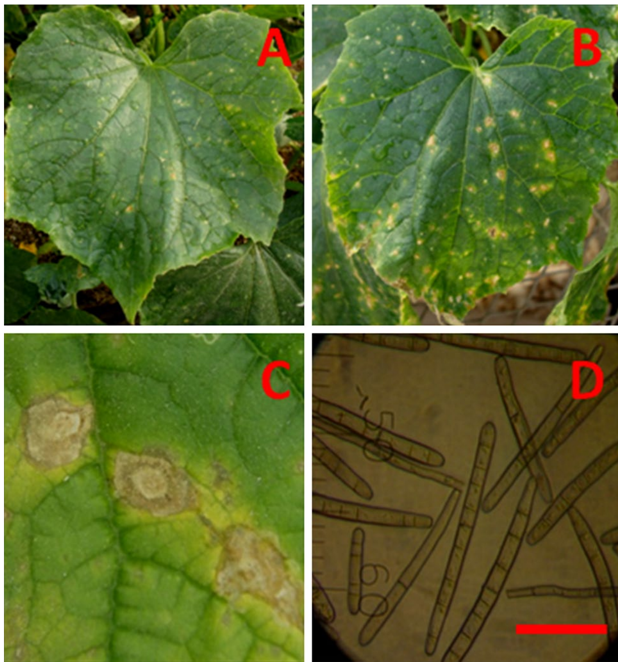

Fig. 1 Typical symptoms of TLS on D31 (**a**) and D5 (**b**, **c**) parental lines and the morphology of *C. cassiicola* pathogen (**d**, *bar* 5 μm). Ten days after inoculation, a few small lesions could be observed on leaves of resistant D31, whereas there were many lesions and typical necrotic spots on susceptible D5 (**b**, **c**)

for 24 h in a dew chamber with a 100 % relative humidity, and then maintained at 25 °C for 5 days in a phytotron. Disease development on each leaf was recorded 10–15 days after inoculation. The disease rating scale was as follows: 0 = no apparent symptoms, 1 = < 5 %, 2 = 5–25 %, 3 = 26–50, 4 = 51–75 %, and 5 = >75 % of leaf area diseased. Plants with disease rating scales 0, 1 and 2 were classified as resistant (*R*); those rated with scale 3, 4 and 5 were treated as susceptible (*S*). The genotypes at the TLS resistance gene locus of F_2 plants (A, B or H) were inferred from segregation to TLS inoculation in corresponding F_3 families.

Genetic mapping strategy and marker analysis

We first located the TLS resistance locus in a chromosomal region using SSR markers and the BSA (bulked segregant analysis) method. A total of 202 SSRs from published maps by Yang et al. (2012a, b) and Cavagnaro et al. (2010) were used to screen parental lines D31 and D5. All polymorphic markers were applied to the resistant and susceptible DNA bulks. Each bulk was composed of equal amount of DNAs of 30 F_2 plants based on phenotypic results of F_3 population.

The next-generation sequencing was used for marker development and fine mapping of TLS resistance gene. D5 and D31 genomes were re-sequenced at 10× genome coverage using Illumina Hi-Seq2000. Raw reads from the two parental genomes were assembled with the SOAP program (http://soap.genomics.org.cn/) (Li et al. 2008). Single nucleotide polymorphism (SNP) and insertion/deletion (Indel) variations between D5 and D31 in the initially mapped 2.5 Mbp region were identified by sequence alignment.

Young leaves of individual cucumber plants were harvested, flash frozen in liquid nitrogen, and stored at −80 °C. Genomic DNA was extracted with the CTAB method (Murray and Thompson 1980). For SSR analysis, all PCR reactions were performed in a PTC0200 Thermal cycler (Bio-Rad, Mexico) in 10 μl volumes containing 1 μl of template DNA (25 ng μl^{-1}), 1 μl 10× PCR buffer, 200 μmol L^{-1} dNTPs, 0.5 μmol L^{-1} each of forward and reverse primer, 0.25 U *Taq* DNA polymerase (Biotechnology, Beijing, China). A "touch-down" PCR program was employed for all primer sets: 95 °C for 5 min, 8 cycles of 94 °C, 30 s denaturation; 60 °C (−1 °C cycle), 1 min annealing and 72 °C, 1 min extension; followed by 32 cycles of 94 °C, 30 s denaturation; 53 °C, 30 s annealing and 72 °CC, 1 min extension; and 72 °C, 5 min.

We employed the KASPar platform (LGC Genomics, UK) to conduct SNP genotyping in segregating populations. The "touch-down" PCR program for KASPar SNP genotyping was: 95 °C for 15 min, 10 cycles of 94 °C, 20 s denaturation; 61 °C (−0.6 °C/cycle) 60 s annealing; followed by 26 cycles of 94 °C, 20 s denaturation; 55 °C, 60 s annealing.

Linkage analysis of the TLS resistance locus with molecular markers in $F_{2:3}$ and F_2 mapping populations was performed with JOINMAP 4.0 (Stam 1993) using a minimum LOD threshold of 4.0 and the Kosambi mapping function (Kosambi 1944). The genotype of different population plants was described as A (recessive, homozygous resistance), H (heterozygous susceptible) and B (dominant, homozygous susceptible).

DNA sequence analysis and gene prediction for TLS resistance candidate gene

The potential candidate genes of the TLS resistance locus in D5 and D31 were sequenced with the Sanger method. Sequencing primers were designed based on the 9930 V2.0 CDS sequence (Huang et al. 2009) using Primer 3.0 with expected amplicon size from 800 to 1000 bp in the predicted gene region. Neighboring primer pairs were designed to amplify PCR products with at least 79 bp overlap with its preceding fragment. Gene annotation was performed with the program FGENESH (http://sunl.softberry.com/) and function prediction was conducted with BLASTx at the NCBI (National Center for Biotechnology Information) website (http://blast.ncbi.nlm.nih.gov).

Allelic diversity of the candidate gene region in diverse cucumber lines

To identify of resistance phenotype and the genotype at the TLS candidate resistance gene locus, 24 cucumber lines were genotyped at the non-synonymous SNP site within the candidate gene. Each line was phenotyped for responses to TLS pathogen inoculation (30 plants per line), and at the same time subjected to SNP genotyping with the KASP platform.

Quantitative reverse-transcription PCR (qRT-PCR) analysis of TLS candidate resistance gene

Quantitative RT-PCR analysis of the TLS candidate resistance gene was conducted following Li et al. (2013). Primers of the candidate gene were designed with the Primer 3.0 (http://primer3.ut.ee/); the sequences of the two primers were:

cca3-qRT-PCR-F:
GGAGAATAAGTCATTTGGTAGAAACTCCC,
and cca3-qRT-PCR-R:
TGGACATACGTGATATCAGTTTGCAAC.

Root and leaf tissues from both parental lines were flash frozen in liquid nitrogen, and total RNA was isolated with RNeasy Plant Mini Kit (Qiagen, Germany) following manufacturer's instructions. The first-strand cDNA synthesis was performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, USA). All qRT-PCR experiments were performed with three biological replications and three technical replicates. The cucumber ubiquitin extension protein gene was used as the reference gene (Wan et al. 2010), and qRT-PCR system was performed using the SYBR Green PCR master mix (Applied Biosystems Inc., USA) in LightCycler®480 Real-Time PCR detection system (Roche, Switzerland).

Correlation of TLS lesion size with candidate gene expression level

In our preliminary observation, we found some link between TLS candidate gene expression and the lesion size on the infected leaves. We systematically investigated correlation between the two factors in host plant–TLS pathogen interactions. Nine cucumber inbred lines, five resistant and four susceptible, were inoculated with TLS isolate. Ten days after inoculation, the amount of necrotic spots with lesion size $\geq$0.5 mm on one leaf of each line was recorded. Quantitative RT-PCR analysis of the TLS candidate resistance gene was conducted with all nine cucumber lines. Data from ten plants of each line were obtained for correlation analysis with candidate gene expression data. The Gene-E program was used to generate a heat-map (Ferreira 2001).

Results

Inheritance of TLS resistance in D31 cucumber

Responses of plants to *C. cassiicola* inoculation in different populations and parents in 2012 and 2013 are summarized in Table 1. The results confirmed that D31 was resistant and D5 was susceptible to TLS inoculation (Fig. 1), and the F_1 was similar to D5 suggesting the recessive nature of TLS resistance in D31. In 2012, among 90 $F_{2:3}$ families tested, 24 were homozygous resistant, 44 heterozygous susceptible, and 22 were homozygous susceptible, which was consistent with 1:2:1 segregation ratio ($\chi^2 = 0.333$, $P = 0.847$) (Table 1). The 2013 test with $F_{2:3}$ families and F_2 plants

Table 1 Segregation to TLS inoculation in F_2 and $F_{2:3}$ populations derived from D31 × D5 cross

Years	Materials	Segregation				Expected	χ^2	$\chi^2_{0.05}$
		# Plants tested	*R*	*H*	*S*			
2012	F_1	30	–	–	30	–	–	–
	$F_{2:3}$	90	24	44	22	1:2:1	0.333	5.991
	F_2	90	24	–	66	1:3	0.133	3.841
2013	D5 (P1)	30	–	–	30	–	–	–
	D31 (P2)	30	30	–	–	–	–	–
	F_1	30	–	–	30	–	–	–
	$F_{2:3}$	193	51	95	47	1:2:1	0.212	5.991
	F_2	890	220	–	670	1:3	0.037	3.841
	D5 (P1)	24	–	–	24	–	–	–
	D31 (P2)	30	30	–	–	–	–	–

R homozygous resistant, *S* homozygous susceptible, *H* heterozygous susceptible

Table 2 Information of SSR and Indel markers used for framework TLS resistance gene mapping in this study

Type	Marker names	Position in Chr 6 (9930 V2.0)	Forward primer (5′–3′)	Reverse primer (5′–3′)
SSR	SSR14652	15,195,088–15,195,217	CCAATTGAATGCACGAACAA	TCATTACATTTCCCCCATTTT
SSR	SSR00842	15,254,488–15,254,692	CCTCCGCCTTTCTTTCTTTT	CGCCCAAATTGAACGAATAA
Indel	Indel16624801	16,624,737–16,624,907	GGTAACATTTTGCTACATTTGCC	GTTGAAGGGTATGATTGCAAGTAC
Indel	Indel16874230	16,874,179–16,874,348	CTTTGGATTCCCAGCACGTATAG	GCAATTTACCATAAAAGGCAGTGAC
Indel	Indel16923460	16,923,433–16,923,586	AGACGATGATGTATCACTCTGTAATCG	AGAGGAACCATGAAGAAGAAGAA-GAAG
Indel	Indel16938038	16,937,643–16,937,832	CACATAATTAGATAAAAGATTACGTTTGA-TAACC	GGAAACTGGGTGGAGAATATTGTCT-TAC
Indel	Indel16953846	16,953,760–16,953,910	AAAGAAAGAACGACAATTTTCACA	CAAATTCGGTAATCTAAACTTCCA
Indel	Indel16955630	16,955,503–16,955,702	AATTAGACAAGAAGTAACCTTGAAATG	TCATGTGCCTATTCTTAAAACCATC
Indel	Indel17042567	17,042,434–17,042,671	GGTACACAATCGTTTAGATTTGAC	GCGTTAGAATGTTGGATAGCC
Indel	Indel17050110	17,050,022–17,050,191	AACTGTAAAATGGGTGTCAACGAAG	GTTGGGTACGCACTGTGTTAATTTT
Indel	Indel17115616	17,115,514–17,115,697	AATTCCTTGTGGTGGTTGTGACTAT	GTGCTACAAAGACTTAGTGAATGATG
Indel	Indel17115721	17,115,665–17,115,822	AAGATGTTGATGGATATCCTAGGTCAC	CATCAAACATAGTCACAACCACCAC
Indel	Indel17129263	17,129,181–17,129,369	AGAAGACACCTTCCTTCATCAATAACG	CCTTCACAACGATTCTTCAACAAGT
Indel	Indel17132990	17,132,921–17,133,116	CAATTTGATTTCTAATGCCTTTTTCTAC	GATACAAGCTAACAAGTTG-CAAAAACA
Indel	Indel17156286	17,156,190–17,156,455	CATTGCCACCAAACCAACTACG	CCATGTTTACGTGTTGGGCG
SSR	UW083827	17,169,463–17,169,597	TTTTGTCCAAAGCACATAAATGA	AAACATCTCTCTTCCACTCTCCA
Indel	Indel17213546	17,213,497–17,213,656	GGCAGCACATTCATTGGACTC	CAACAGTTCAAGAAACCGGGTG
SSR	UW083726	17,324,928–17,325,067	CAAGCCCAGACACTCACAGA	CATATCCACATCTGCAGAAAACA
SSR	SSR15067	13,578,213–13,578,213	AACCACTCCCACTTGCCTAA	AAAAATATTCAAACCCAATTTTTGA
SSR	SSR01179	17,574,097–17,574,242	ACCAAAATGCCAACCACTTC	CGTCCCTCACACCTTCTTTC
SSR	SSR03932	19,924,338–19,924,510	CTTTTTGGGGACCCTTCATT	CACGAATGCTGCTCTAACCA
SSR	SSR06424	20,676,218–20,676,384	GCTCCCTCAATTGGGTAACA	AACCGAAATTCATATGTGGAAAA
SSR	SSR04637	9,966,467–9,966,610	ATCTGGTACCGCTGTTTTGC	GTGTTTGATGTACGCGGTTG
SSR	CS41	15,109,733–15,109,902	TGGTGGTGGGACTCCACTAT	GTGGAGGAAGCAGGAGATGA
SSR	UW085138	15,629,908–15,630,110	TGGTGATTAGTAGAGGGTCAAATTC	TGTCCTTTCCTTCTACTTCTTGC
SSR	UW043074	17,465,180–17,465,302	ATTTCTTTTGAGGCCCCATT	TTTTCACAATGGTAGCTTTCG
SSR	SSR14239	10,910,748–10,910,896	GGAGCCGAGAACATCAAAAG	AGAGATCCTCTCACGCCTTG
SSR	SSR13884	11,069,339–11,069,635	GGATAATCCTGATTCCTGTGG	TGTGCAACTGAAAACGAAGC
SSR	SSR00259	10,732,342–10,732,566	TCCACGTAGACATTGTGAGGTC	CGAGTGTAGCTCAATTAATATGGTG
SSR	SSR06500	10,260,783–10,260,952	TGACAAAACACTACCCACCG	AGCAAATTCAACCGTTCATGT
SSR	SSR05348	19,590,934–19,591,065	ACTGCTGGAAAAAGCTGGTG	AGCCTATTTCCCCGCTCTTA
SSR	SSR21885	21,186,690–21,186,990	AAGATTCAGGAGGAGGGGAA	AGTTCCACAAGGCACAGGTC
SSR	SSR13941	19,971,364–19,971,552	ATCAAAGAAGGAAGAGGGGC	AAAGCATGGAGAGTAACATCCAA

were also in agreement with the fact that the target leaf spot resistance in D31 is controlled by a single recessive gene, which was consistent with previous studies (Wang et al. 2010; Yang 2012). Since the allelic relationships among the three recessively inherited TLS resistance genes (cca-1 by Wang et al. 2010; *cca-2* by Yang 2012; present study) was not known, we hereby designated the resistant gene in D31 *cca-3*.

Genetic mapping of *cca-3* locus

Among 202 SSR markers screened, 19 (9.2 %) were polymorphic between the two parental line D5 and D31. When the 19 were applied to the resistant and susceptible bulks, four markers from cucumber chromosome 6, SSR14652, UW083827, UW083726 and SSR00842, were polymorphic. Detailed information of SSR and other Indel markers used in framework genetic mapping is presented in Table 2. Linkage analysis with 193 $F_{2:3}$ families of those markers identified SSR14652 and UW083827 flanking the *cca-3* locus (Fig. 2) suggesting *cca-3* was located in chromosome 6. In the 9930 draft genome assembly (V2.0), the physical distance between the two markers was approximately 2.5 Mb.

To identify new markers in the 2.5 Mbp region, the D5 and D31 parental lines were re-sequenced with Illumina at

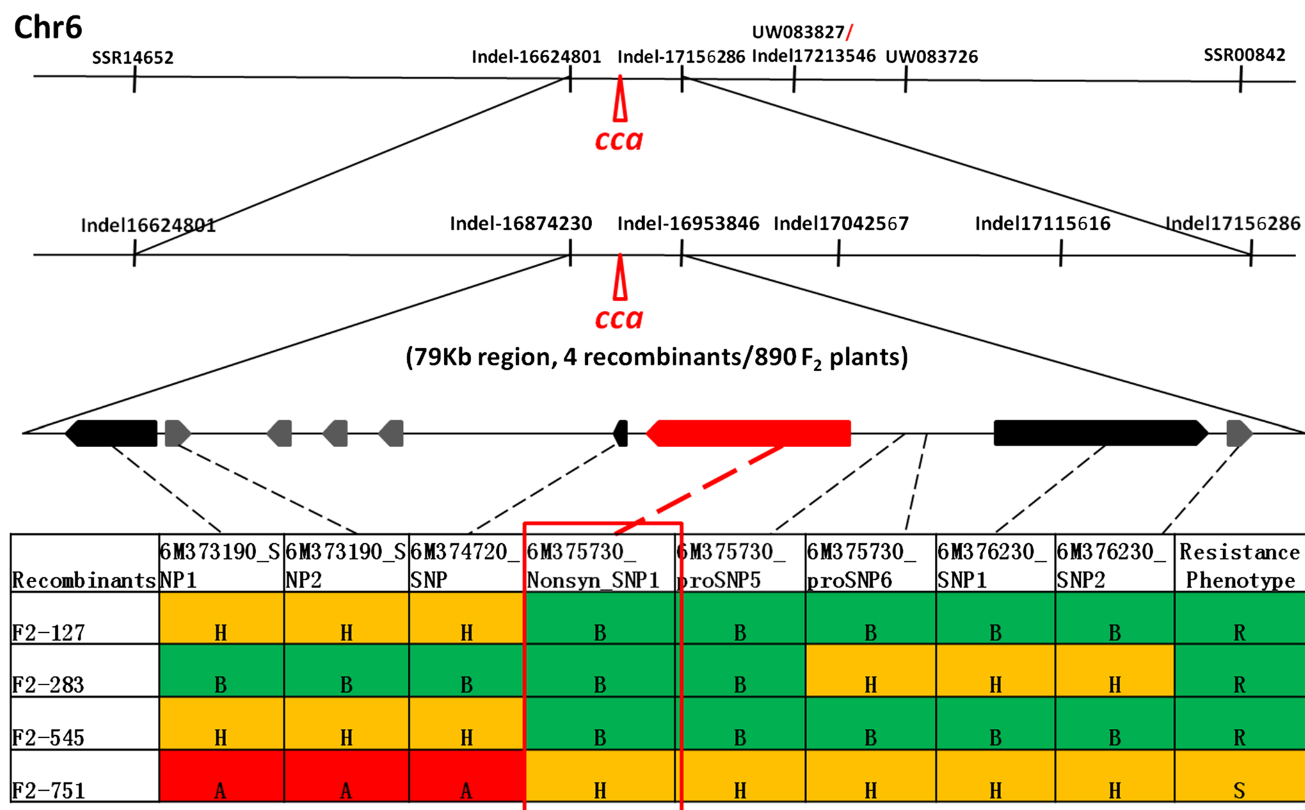

Fig. 2 Fine mapping of TLS-resistant gene *cca-3* in cucumber. The *cca-3* gene was mapped in chromosome 6 with two flanking markers Indel16874230 and Indel16953846, in which nice genes were predicted. Four of the nine genes harbor SNP mutations between D5 and D31. Eight SNP markers were used to screen 890 F_2 plants and four recombinants were identified, which were self-pollinated to generate F_3 families for phenotyping and inferring F_2 genotype at *cca-3* locus. Finally, of the 2 SNP markers co-segregating with the *cca-3* locus, the non-synonymous mutation at 1318 bp location (C-T) seems to be causal SNP. Genotype designations *A* D5; *B* D31, *H* F1 (heterozygous)

10× genome coverage. Alignment of the reads with 9930 reference genome identified many SNP and Indel variations between D5 and D31, from which 21 Indel markers were selected and tested for polymorphism between D31 and D5. Seven Indel markers were mapped using the $F_{2:3}$ population. Information of the seven markers is provided in Table 2. At this time, two Indel markers, Indel16624801 and Indel17156286, were the closest flanking markers for *cca-3* (Fig. 2); the physical distance between them was approximately 530 kb in the 9930 V2.0 draft genome sequence. Next, 19 new Indel markers were developed in the 530-kb region, and seven were polymorphic (Table 2). These markers were applied to both the 193 $F_{2:3}$ and 890 F_2 plants for fine mapping. Four recombinants were identified among 890 F_2 plants, which were used for high-resolution genetic mapping with the new polymorphic Indel markers. Lastly, two flanking Indel markers, Indel16874230 and Inde16953846 were identified, which were 0.25 cM apart genetically, and 79 kb physically in the 9930 draft genome between them (Fig. 2).

Annotation, gene prediction in 79-kb *cca-3* gene region and validation of candidate gene

We manually annotated the 79-kb region delimited by Indel16874230 and Indel16953846. Nine genes were predicted in this region by the FGENESH program, including *Csa6M373190.1*, *Csa6M373200.1*, *Csa6M374700.1*, *Csa6M374710.1*, *Csa6M374720.1*, *Csa6M375720.1*, *Csa6M375730.1*, *Csa6M376230.1*, and *Csa6M376240.1*. Information about the position and predicted functions of these genes is presented in Table 3. Among them, there were two RGA2-like genes, *Csa6M375720.1* and *Csa6M375730.1* encoding disease resistance proteins that seem to be good candidates of the *cca-3* locus.

Nine SNPs were detected in 4 out of the 9 predicted genes between the two parental lines. To further fine map the *cca-3* gene, 8 KASPar type SNP markers were designed (Table 4) and tested in the four recombinants of D5 × D31 F_2 population identified early (Fig. 2). This successfully narrowed down the *cca-3* locus into a region of

Table 3 Predicted genes in the 79-kb region of cucumber chromosome 6 harboring TLS resistance gene *cca-3*

Gene no.	Predicted genes	CDS start position in Chr 6	Predicted gene function	*E* value
1	Csa6M373190.1	16,886,921	Transcription factor BIM2-like	0.0
2	Csa6M373200.1	16,891,553	Transcription factor BIM2-like	2e−68
3	Csa6M374700.1	16,905,411	Conserved hypothetical protein	0.13
4	Csa6M374710.1	16,908,684	Hypothetical protein	2e-79
5	Csa6M374720.1	16,912,208	Hypothetical protein	1e-56
6	Csa6M375720.1	16,932,329	Disease resistance protein RGA2-like	3e-86
7	Csa6M375730.1	16,934,210	Disease resistance protein RGA2-like	0.0
8	Csa6M376230.1	16,946,354	Tetratricopeptide repeat protein 33-like	0.0
9	Csa6M376240.1	16,951,863	Chalcone synthase (CHS2) gene	2e-05

Table 4 Information of KASPar SNP markers used for fine mapping of TLS resistance in this study

Type	Marker names	SNP position	D5 allele-1 (5′–3′)	D31 allele-2 (5′–3′)	Reverse primer (5′–3′)
SNP	6M373190_SNP1	16,886,901	ATAATCTGCACCAC-GAGTCCC	GATAATCTGCACCAC-GAGTCCT	CTAATTCACATTAGAAA-CAGTAATCGCACAA
SNP	6M373190_SNP2	16,887,197	GTGAACAAGGACTAAGAT-GCAACAT	GTGAACAAGGACTAAGAT-GCAACAA	CTTGTTTATCCT-TAACATTTGGGTGT-CAAAA
SNP	6M374720_SNP	16,912,569	GTATTTGTTTTTGTGTAA-GGCTCTGG	TGTATTTGTTTTTGTGTAA-GGCTCTGT	CGGCTCTGTTTTCTGAG-TAATTAGATGAA
SNP	6M375730_NonsynSNP1	16,936,147	TGCATTATTCTCCAAAGAT-TATCACC	GTGCATTATTCTCCAAA-GATTATCACT	CCTTGTGCCATCCATT-GTTTTATCAAATCA
SNP	6M375730_proSNP5	16,939,578	ACTGAAGTTGAGGTTAT-GATAGCATTA	CTGAAGTTGAGGTTATGA-TAGCATTG	CTCCTCCTCAAGCAAGT-CATCAATTG
SNP	6M375730_proSNP6	16,939,708	GATTCCAAACCATTGTTG-TATATCAAAG	GATTCCAAACCATTGTTG-TATATCAAAC	TATTAAAACAATACAGTT-GCAGTTACATCAAAACA
SNP	6M376230_SNP1	16,948,053	AAGCACATGGCCTTGGAG-TATAATT	GCACATGGCCTTGGAG-TATAATC	GATATTTTCCTCAAGCTTT-GACATTTAGATGT
SNP	6M376230_SNP2	16,948,559	CATCATGGGCTGAGGTA-CAACTA	ATCATGGGCTGAGGTA-CAACTC	GGCGCATCAGAGATATTG-GTATTGTATT

27 kb that only contained the two RGA genes. We cloned these two RGA genes from D5 to D31, and found that *Csa6M375720.1* only had 298 bp, which seemed to be a truncated, non-functional gene (supplemental Figs. S1, S2). Therefore, the other R-type gene, *Csa6M375730.1* was the most possible candidate gene for *cca-3*.

The whole length of *Csa6M375730.1* gene was 3255 bp in the two parental lines with high similarity with typical R genes. FGENESH predicted one exon (coding region, CDS) and no intron in this RGA, but the gene structure was different from the CDS in 9930 and Gy14 (supplemental Fig. S3). The candidate gene had several SNPs in the CDS, but only the one at 1318 bp location was non-synonymous (C-T, Fig. S1) resulting in an amino acid substitution from H (histidine) in the resistant line D31 to Y (tyrosine) in the susceptible line D5 (Fig. S2).

Information on the deduced protein and domains in *Csa6P375720.1* and *Csa6P375730.1* is presented in Fig. 3. The non-synonymous SNP site was localized in the NB-ARC domain suggesting the amino acid substitution may alter the function of *cca-3* candidate gene. A marker 6M375730_NonsynSNP1 from this non-synonymous SNP at 1318 bp (Table 4) was used in KASPar assay to screen plants in the D5 × D31 populations and 24 additional cucumber lines (Table 5). It turned out that this marker was co-segregating with the phenotypes in the D5 × D31 populations. This marker was also consistent with the TLS inoculation responses in all 24 lines except the cucumber line Zh12 (Supplemental Fig. S4).

Expression analysis of *cca-3* candidate gene

We carried out qRT-PCR analysis for the *cca-3* candidate gene *Csa6M375730.1* in leaf and root tissues of the two parental lines, and the results are presented in Fig. 4. We found that its expression in the roots was low and there was no significant difference between D5 and D31. In contrast, the expression of *cca-3* candidate gene expression in leaves of the susceptible parent D5 was 2.58 times higher than that in the resistant parent D31 (Fig. 4).

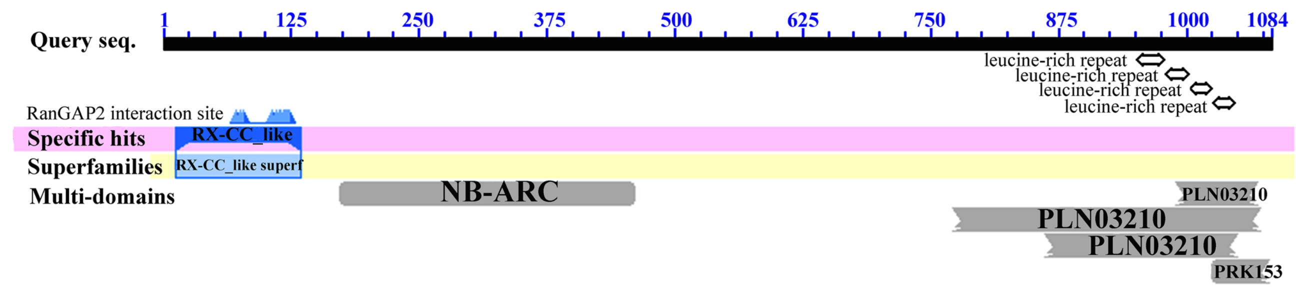

Fig. 3 Conserved motifs of *Csa6M375720* and *Csa6M375730* candidate gene proteins. In **a**, the predicted protein of Csa6M375720 contains 65 amino acids with no conserved R type motif; in **b**, the predicted protein of Csa6M375730 has 1084 amino acids and harbors the RX-CC_like, NB-ARC, PLN03210, and four leucine-rich repeat motifs, which are typical domains in R genes. Thus, Csa6M375730 seems to a possible candidate gene of *cca-3*

Table 5 Relationship between target leaf spot resistance phenotype and genotype based on 6M375730_NonsynSNP[b] in independent 24 cucumber lines

Code	Germplasm name	Source	Ecotype	Phenotype[a] (S or R)	6M375730_ NonsynSNP[b]	6M375730_ SNP1 genotype	6M375730_ SNP5 genotype
1	D22	China	East Asian	S	C:C	C:C	C:C
2	D8	China	East Asian	R	T:T	T:T	T:T
3	D33	China	East Asian	R	T:T	T:T	T:T
4	CK3 (F1 hybriD)	China	East Asian	S	C:C	C:T	C:T
5	Zh12	China	East Asian	S	T:T	T:T	T:T
6	Zh1	China	East Asian	S	C:C	C:C	C:C
7	D1	China	East Asian	S	C:C	C:C	C:C
8	BJ109 (F1 hybriD)	China	East Asian	S	C:T	C:T	C:T
9	D11	China	East Asian	R	T:T	T:T	T:T
10	Zh15	China	East Asian	S	C:C	C:C	C:C
11	Zh2	China	East Asian	S	C:C	C:C	C:C
12	CK2 (F1 hybriD)	China	East Asian	S	C:T	C:T	C:T
13	BJ207	China	East Asian	S	C:C	C:C	C:T
14	D23	China	East Asian	S	C:C	C:C	C:C
15	D26	China	East Asian	S	C:C	C:C	C:T
16	D25	China	East Asian	S	C:C	C:C	C:C
17	D12	China	East Asian	S	C:C	C:C	C:C
18	D32	China	East Asian	S	C:C	C:C	C:C
19	Zh13 (F1 hybriD)	China	East Asian	S	C:T	C:T	C:T
20	JY2	China	East Asian	S	C:C	C:C	C:C
21	WI2757	USA	Eurasian	R	T:T	T:T	T:T
22	Z298	USA	Eurasian	S	C:C	C:C	C:C
23	PI 330628	USA	India	R	T:T	T:T	T:T
24	JY35	China	East Asian	S	C:C	C:C	C:C

[a] Reactions to inoculation tests: *R* resistant, *S* susceptible

[b] Marker genotype designation: *C:C* homozygous genotype which is the same as the susceptible line D5, *T:T* homozygous genotype which is the same as resistant line D31, *C:T* heterozygous genotype that was the same as the susceptible F_1 hybrid of D5 × D31

In our preliminary observations, we found a correlation between TLS lesion size and the expression level of the candidate gene. Nine cucumber lines including four susceptible lines (JY35, D5, JY2 and Zh1) and five resistant lines (D31, WI2757, D33, D11 and D8), were used to validate this observation. Lesion areas caused by TLS infection on the leaves of the nine lines were measured 10 days after inoculation. The expression level of this candidate gene in

all nine lines was also measured, and the results are shown in Fig. 5. The result suggested that the higher the *cca-3* candidate gene expression, the more necrotic spots in the susceptible lines (Fig. 5).

Discussion

Inheritance analysis and molecular mapping of TLS resistance in cucumber

Previous studies have indicated that host resistance in cucumber to target leaf spot pathogen can be either dominant (*Cca*) (Abul-Hayja et al. 1978) or recessive (*cca-1* in Wang et al. 2010; *cca-2* in Yang 2012). In the present study, we found that the TLS resistance in the cucumber line D31 was also conferred by a single recessive gene, *cca-3*, which was probably originated from the US germplasm line WI2757 (Peterson et al. 1982), was shown to be controlled by a simply inherited recessive gene, (Table 1). Wang et al. (2010) identified an EST-SSR marker CSFR33 linked with TLS resistance gene *cca-1* in a cucumber line Q5. Yang (2012) conducted genetic mapping of a recessively inherited TLS resistance gene *cca-2* in the wild cucumber *C. sativus* var. *hardwickii* and identified two flanking SSR markers SSR10954 and SSR16890. We delimited the *cca-3* locus in a 79-kb region on chromosome 6 (Fig. 2). Based on marker sequence information, the marker CSFR33 linked with *cca-1* was located at approximately 15.11 Mbp of the chromosome 6 pseudomolecule in the 9930 draft genome (Version 2.0); the physical positions of SSR10954 and SSR16890 were located at 9.45 and 14.85 Mbp in chromosome 6, respectively. In our study, the co-segregation SNP marker with *cca-3* was located at 16.94 Mbp in chromosome 6 (Fig. 2). Physically, the three genes seem to be different. It is not known if the strains used or inoculation methods may have contributed to the discrepancies, or they are truly different loci. Since the markers for *cca-1* and *cca-2* were not closely linked, it is still possible that the three genes are allelic. As such, the allelic relationships among the three genes merit further investigation.

Candidate gene for the *cca-3* locus

In the 79-kb region harboring *cca-3*, 9 genes were predicted including two RGA genes *Csa6M375720.1* and *Csa6M375730.1*. Since *Csa6M375720.1* was only 298 bp in length with no typical domain or motif of R genes, it was presumed as a pseudogene (Fig. 3) leaving *Csa6M375730.1* as the only possible candidate gene for *cca-3*. The whole length of *Csa6M375730.1* gene was 3255 bp with typical CC-domain, NB-ARC and LRR domain. A marker designed from the non-synonymous

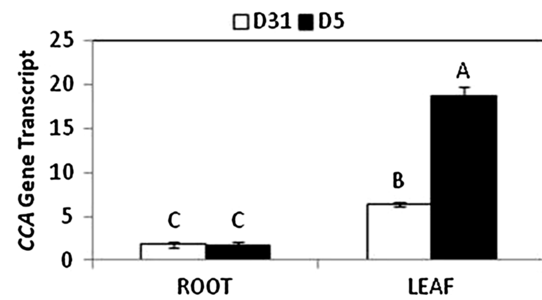

Fig. 4 Expression of the *cca-3* candidate gene (*Csa6M375730.1*) in roots (*left*) and leaves of D5 and D31 revealed with qRT-PCR. There is no significant difference in the root between the two *lines*, whereas the susceptible line D5 shows significant higher expression in the leaves than the resistant D31. *Error bars* represent the standard error. *Bars marked by the same letter* are not significantly different according to Duncan's test ($P \leq 0.05$). Values are the average of three independent experiments

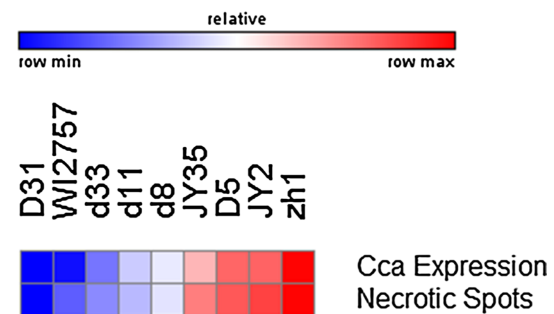

Fig. 5 Positive correlation of *cca-3* candidate gene expression level and the degree of damage caused by TLS infection in terms of lesion sizes. Candidate gene expression and lesion size were measured 10 days after inoculation from nine *cucumber lines*. The heat-map was drawn with Gene-E program. The *color bar* above map showed the relative intensity of gene expression or amount of necrotic spots. *Blue color boxes* indicate low expression or less necrotic spots; *red color boxes* represents high expression or more necrotic spots. Each value was the average of three independent observations

SNP within the NB-ARC domain of *Csa6M375730.1* was co-segregating with phenotypes within the F_2 population, and this SNP genotype was also consistent with TLS inoculation responses among all but one (Zh12) of 24 independent cucumber lines examined (Table 5). These lines of evidence suggested that *Csa6M375730.1* may be the most possible candidate gene for *cca-3*. For Zh12, it may be necessary to exam the whole sequence of *Csa6M375730.1* to see if there are other genetic variations within this gene to be responsible for the inconsistent result. It is also worthy to investigate the allelic variations at *cca-3* locus in other TLS resistance resources such as Q5 and PI 183967 reported previously.

Most plant disease resistance genes (R genes) belong to the NB-LRR (nucleotide-binding site, leucine-rich repeat)

type, which contains a C-terminal LRR domain, a varying N-terminal effecter domain, and a central NB domain. The C-terminal module contains ARC sub-domains, and the N-terminal domain frequently contains either Toll/Interleukin-1 receptor (TIR) or coiled-coil (CC) motifs (Meyers et al. 2003; Pan et al. 2000). NB-LRR types RGAs often exist in plant genomes in clusters. In cucumber, there are approximately 70 NB-LRR RGAs (Huang et al. 2009; Wan et al. 2013; Yang et al. 2013), and some disease resistances have been associated with this type of R genes, for example, the scab resistance gene *Ccu* (Kang et al. 2013; Zhou et al. 2015). Most NB-LRR type R genes are dominant. In the present study, TLS resistance gene *cca-3* was recessively inherited (Table 1). In addition, the expression level of the *cca-3* candidate gene, *Csa6M375730.1*, was higher in the leaves of the susceptible line D5 than in the resistant D31 line (Fig. 4). The expression level was also positively correlated with the number and size of necrotic spots (diameter > 0.5 mm) on leaves after inoculation (Fig. 5). It is possible that expression of *cca-3* gene may induce HR and subsequent cascades leading to systemic acquired host resistance in the resistant line. However, these phenomena may merit further investigations.

High-throughput sequencing and genotyping for fine genetic mapping

Since the release of the cucumber draft genomes (Huang et al. 2009; Yang et al. 2012b) and the use of NGS technologies, rapid progress is being made in molecular mapping and gene cloning in cucumber (Qi et al. 2013). High-throughput re-sequencing has been employed in cucumber fine genetic mapping (for example, Amano et al. 2013; Li et al. 2013; Ecke et al. 2015; Kang et al. 2011; Zhang et al. 2013; Zhang et al. 2014; Yang et al. 2014). More recently, other high-throughput technologies derived from next-generation sequencing have also been developed, such as genotyping by sequencing (GBS), restriction-site-associated DNA sequencing (RAD), the specific length amplified fragment sequencing (SLAF) were reported in cucumber genetic research, and several important genes or quantitative trait loci (QTLs) were fine mapped or cloned (e.g., Pang et al. 2013; Yang et al. 2014; Wei et al. 2014; Xu et al. 2015). In this study, we re-sequenced the D5 and D31 genomes with next-generation sequencing technology, and identified numerous Indel and SNP variations between the two lines. We took advantage of the high-throughput genotyping technology based on LGC Genomics KASPar system. Both Indel and SNP markers could be used in genotyping with this system with very high throughput (Pariasca-Tanaka et al. 2015). NGS combined with high-throughput genotyping allowed us to fine map the *cca-3* locus in a relatively small region in very short time suggesting the power of these new technologies in expediting traditional plant breeding.

In summary, in the present study, the combined use of next-generation sequencing and high-throughput SNP genotyping allowed us to quickly locate the *cca-3* locus into the small genomic DNA region and identify a candidate gene for TLS resistance. This lays a good foundation for further functional studies for molecular mechanisms of host resistance against the TLS pathogen in cucumber. The co-segregated SNP marker we designed amendable for high-throughput genotyping will be useful in marker-assisted breeding for TLS resistance in cucumber.

Author contribution statement CW, AM and YX designed the experiments. CW and CD performed marker development and genetic mapping. AM, CW and YG participated in physical mapping. HL and SY conducted gene expression analysis. CD, CW and YW wrote the paper. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that the study compiles with the current laws of the countries in which the experiments were performed.

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