

## Research Paper

# *Cscs* encoding chorismate synthase is a candidate gene for leaf variegation mutation in cucumber

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Variegation is a frequently observed genetic phenomenon in landscaping. In this study, an ethyl methanesulfonate induced variegated leaf (*Csvl*) mutant in cucumber (*Cucumis sativus* L.) was identified. The *Csvl* mutant displayed green-yellow-white variegation phenotype throughout the whole growth cycle, while the leaf of wild type plants was normal green. The photosynthetic pigment contents and photosynthetic parameters of *Csvl* was significantly lower than wild type. The cytology observation results showed that the mesophyll cells of *Csvl* mutant contained defective chloroplasts. Genetic analysis indicated that variegated leaf phenotype was monogenic recessive inheritance. MutMap and genotyping results revealed that *Csa6G405290* (*Cscs*), encoding chorismate synthase, was the candidate gene for variegated leaf mutant in cucumber. The expression level of *Cscs* was similar between wild type and variegated leaf mutant leaves. Transcriptome profile analysis of leaves of *Csvl* mutant identified 183 candidate genes involved in variegated leaf development in cucumber, including genes that encode heat shock protein, zinc finger protein. *Cscs* may regulate variegated leaf in cucumber by interacting with these genes. In a word, these results revealed that *Cscs* might regulate the variegated leaf phenotype in cucumber.

**Key Words:** chorismate synthase, cucumber, leaf variegation, MutMap, RNA-seq.

## Introduction

Leaf is a significant organ for photosynthesis, respiration, and nutrient transformation in plants, the change in leaf color is determined by genotype and growth environment (Esteban *et al.* 2008, Sakamoto *et al.* 2009). Leaf variegation, forming different colored sectors in the same leaf, is a frequently observed genetic phenomenon in landscaping (Yu *et al.* 2007). In nature, the variegated plants usually occur in tropical and subtropical forests (Cui and Guan 2013). Some adaptive advantages of variegated leaves caused by the genotype can play a role in protecting themselves so that they can persist in the local populations. For example, the brightly colored patches can be alert to predators and attract insects to pollinate (Chen *et al.* 2017b, Sheue *et al.* 2012). Some previous studies have been conducted on chemical reagent induced leaf variegation

mutants. At present, extensive studies have been conducted on the molecular mechanism of leaf variegation. The Arabidopsis *var2* mutant phenotype was attributed to a mutation in the subunit of the filamentation temperature-sensitive (FtsH) complex located in the thylakoid, which may participate in chloroplast development and biogenesis (Chen *et al.* 2000). Complete loss of the FtsH complex caused the chloroplast to develop stagnant, while slight loss led to plant leaf variegation (Putarjunan *et al.* 2013). Recent studies on rice stripe mutants showed that *stripe1-2* (*stl-2*) encoded a small subunit of ribonucleotide reductase 1 (RNRS1), and the mutation of Val-171 residue in *stl-2* impacted the attachment of RNRS1 to iron, leading to defective chloroplast development (Chen *et al.* 2015). The tomato *gh* gene encoded a plastid terminal oxidase, which was involved in carotenoid biosynthesis as a phytoene desaturase cofactor, and its deficiency led to photobleaching in leaves under high-light, mainly due to the ability to synthesize colored carotenoids in *gh* mutant (Shahbazi *et al.* 2007).

Cucumber (*Cucumis sativus* L.) is one of the important horticultural crop worldwide, whose genome was sequenced successfully (Huang *et al.* 2009). However, the narrow hereditary basis of cucumber has seriously restricted its

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molecular breeding progress. In order to increase the genetic resources of cucumbers, several research groups have constructed cucumber mutant libraries, and some important mutation traits have been obtained, such as dwarf plant, fruit peel color and fruit thorn and tumor (Boualem *et al.* 2014, Fraenkel *et al.* 2012, Wang *et al.* 2014, Xue *et al.* 2016). Researchers have performed cucumber gene function analysis by genomic information and techniques combined with large-scale construction of cucumber mutant library, such as super compact (SCP) mutant *C257* (Wang *et al.* 2017), virescent yellow leaf mutant *vyl* (Song *et al.* 2018), male sterile cucumber mutant (Han *et al.* 2018), male flower mutant *406a* (Chen *et al.* 2016), light green peel mutant *lgp* (Zhou *et al.* 2015) and tendril-less mutant *td-1* (Chen *et al.* 2017a). Spontaneous mutants of cucumber were also used to identify the glabrous genes *gl-2*, *csg11/Mict* (Li *et al.* 2015), *csg13* (Cui *et al.* 2016, Pan *et al.* 2015), *Tril* (Wang *et al.* 2016a, Zhao *et al.* 2015), white peel gene *w* (Liu *et al.* 2016), short hypocotyl1 gene *SH1* (Bo *et al.* 2016) and *CsDET2* involving in BR biosynthesis (Hou *et al.* 2017) respectively. The screening of these mutagenic and spontaneous mutants and the identification of target genes promote the molecular breeding in cucumber.

Currently, MutMap method has been successfully used to precisely locate genetic loci that cause mutations from different species. And it greatly reduced the number of genetic crosses and the number of mutant progeny populations (Takagi *et al.* 2015). The MutMap method was first applied to the re-sequencing of seven mutants of the rice cultivar 'Hitomebore' crossing with their parents. The SNP molecular markers were then used to quickly locate the gene that caused the target phenotype (Abe *et al.* 2012). Abe *et al.* (2012) mapped the trait related mutation regions by analyzing the differences in the SNP frequency of the offspring DNA pool and wild-type parent, and quickly located four rice semi-dwarf, two light green leaves, and one male sterility related candidate mutation sites (Abe *et al.* 2012). Besides, MutMap technology was applied to the identification of salt tolerance gene in rice (Takagi *et al.* 2015) and root development gene *CTR1* in Arabidopsis (Tabata *et al.* 2013). MutMap method has also successfully identified some important genes in cucumber, such as *gl2* encoding a C-type lectin receptor-like tyrosine protein kinase (Xu *et al.* 2015), *Ycf54* gene controlling the cucumber light green leaf and fruit (Lun *et al.* 2016), plant dwarf gene *CsaVFB1* (Lin *et al.* 2016), light green peel gene *CsaARC5* (Zhou *et al.* 2015) and yellow green peel gene *CsMYB36* (Hao *et al.* 2018).

In our previous study, we isolated an ethyl methanesulfonate (EMS) induced variegated leaf (*Csvl*) mutant in cucumber (*Cucumis sativus* L.) (Xue *et al.* 2016). The wild type leaves showed normal green, while the leaves of the *Csvl* mutant displayed variegation. *Csvl* mutant had lower photosynthetic pigment content and net photosynthetic rate than wild type plants. The morphology of chloroplasts was defective in *Csvl* mutant. *Csa6G405290* (*Cscs*), encoding

chorismate synthase, was identified to be the candidate gene for variegated leaf mutant in cucumber by conducting MutMap and kompetitive allele specific PCR (KASP) genotyping analysis. The expression level of *Cscs* was similar between wild type and *Csvl* mutant. Transcriptome sequencing also identified some potential target genes which might interact with *Cscs* to regulated the variegated leaf phenotype in cucumber. Over all, the results obtained in the present study are beneficial to further dissect the molecular mechanism leaf variegation formation in cucumber.

## Materials and Methods

### Plant materials and growth condition

In our previous study, we isolated some ethyl methanesulfonate (EMS) induced important mutants in cucumber, such as few spines mutant, fruit color mutant, short fruits mutant, round leaf mutant, dwarf mutant, no flower mutant, variegated leaf mutant, long fruits mutant and leaf crimple mutant (Xue *et al.* 2016). In the present study, the *Csvl* mutant was investigated. The F<sub>2</sub> population was crossed between wild type and *Csvl* mutant. The *Csvl* mutant, wild type plants, and F<sub>2</sub> population were used for phenotypic characterization, casual gene identification and genotyping. All plants were grown in the greenhouse of Xiangyang farm of Northeast Agricultural University, Harbin, China.

### Phenotype identification of *Csvl* mutant in cucumber

The color of the cotyledons and leaves were observed since the seeds emergency. Other traits observation and comparison between *Csvl* and wild type plants were performed at 60 d after sowing. Plant height was measured by using a tape. Leaf area of the top 7<sup>th</sup> node leaf was measured using a leaf area meter. Five plants of each type were measured, each measurement was repeated three times, and *t* test was conducted for statistical analysis.

### Transmission electron microscopy analysis

To investigate morphology of chloroplast, the leaves of the top 4<sup>th</sup> node of wild type and *Csvl* plants grown for 50 d were sampled. The yellow, white, light green, normal green leaf tissues were treated for transmission electron microscopy analysis. In short, five different buffers were used in sequence, namely 2.5% glutaraldehyde, 0.1 M phosphate, 1% osmate acid, a series of ethyl alcohol, and a variety of acetone. The prepared samples were cut and stained with uranyl acetate and lead citrate. Finally, transmission electron microscopy (H-765, Hitachi Ltd., Japan) was used for observation. Three replicates were performed for each sample.

### Photosynthetic pigment content quantification

Photosynthetic pigment was extracted from leaves of 5-week-old wild type and *Csvl* mutant plants. 0.2 g fresh leaf sample was collected from a fully expanded leaf (variegated or green) of the top 3<sup>rd</sup> or 4<sup>th</sup> node, and then steeped in

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80% acetone for 48 h. The quantification of pigments was conducted using a microplate spectrophotometer (Epoch, Bio Tek Instruments Inc., USA) according to the method reported previously (Garcia and Nicolas 1998, Wellburn 1994). The photosynthetic pigment content of 10 plants of each type was measured for a total of three measurements. It's worth noting that all measurement operations should be carried out in the dark to avoid photosynthetic pigment degradation.

### Photosynthetic parameter determination

Photosynthetic rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), and intercellular CO<sub>2</sub> molar fraction (Ci) of fully expanded true leaf from wild type and *Csv1* mutant plants were determined using portable photosynthesis system (LI-6400XT, LI-COR Inc., USA). A total of 10 measurements were taken for each type of leaf on sunny day 9:00–11:00.

### Genetic analysis

The formation of variegation in F<sub>2</sub> population were observed after the obvious period of variegation phenotype. The number of plants in the wild type and the variegation leaf phenotype was counted and subjected to  $\chi^2$  test.

### RNA extraction

Total RNA of root, stem, leaf, tendril, female flower and male flower in wild type plants and leaf in *Csv1* were extracted using TRIzol reagent (Invitrogen, USA), Dried RNA samples were dissolved in H<sub>2</sub>O treated with diethylpyrocarbonate. UV-spectrophotometer was used to measure RNA concentration and purity. Its quality was detected by 1% agarose gel electrophoresis. Reverse transcription was carried out using the ReverTra Ace qPCR RT Kit (TOYOBO) in accordance with the manufacturer's instruction.

### Whole genome re-sequencing, SNP calling and filtering

DNA of leaves of wild type and F<sub>2</sub> population plants were extracted according to CTAB method (Murray and Thompson 1980) respectively. The mutant DNA bulk from 17 F<sub>2</sub> plants with variegation phenotype and DNA of one wild type were re-sequenced respectively as a commercial service at Annoroad Genomics (Beijing).

MutMap technique (Takagi *et al.* 2015, Xu *et al.* 2015) was used to identify candidate genes of *Csv1* mutant. The mixed pool data of mutant bulk and the data of wild type were compared with the reference genome ([ftp://www.icugi.org/pub/genome/cucumber/Chinese\\_long/v2/](ftp://www.icugi.org/pub/genome/cucumber/Chinese_long/v2/)) (Huang *et al.* 2009) to obtain the single nucleotide polymorphism (SNP) data set after the sequencing was completed. Then, the SNPs identified in both wild type and mutant DNA pool were removed, SNPs unique to mutant DNA pool were obtained. The SNP-index is calculated by dividing the frequency of the mutation sequence at this locus by the total depth of the locus (Li *et al.* 2009).

### KASP genotyping

KASP (Saxena *et al.* 2014, Semagn *et al.* 2014), was applied for SNP genotyping. The DNA samples from the variegation phenotype and normal leaf color phenotype plants in F<sub>2</sub> population were selected. KASP Primer mix containing two different, allele specific, competing forward primers with unique tail sequences and one reverse primer and universal KASP Master mix containing the commonly used FRET cassette fluorescent primers, ROX internal reference dyes, Klear Taq DNA polymerase, dNTP and MgCl<sub>2</sub> were added into the sample DNA for PCR amplification. Following completion of the KASP PCR, the test results were read and the SNP site genotype data of the sample was analyzed.

### Quantitative reverse transcription PCR (qRT-PCR) analysis

To identify the expression of candidate gene in different tissues, gene-specific primers were designed on-line (<https://www.genscript.com/tools/real-time-pcr-tagman-primer-design-tool>) based on candidate gene CDS sequence information (**Supplemental Table 1**). The *CsEF1 $\alpha$*  gene was amplified as the reference gene. The Taq SYBR<sup>®</sup> Green qPCR Premix (Yugong Biolabs, Inc., CN) and iCycler iQTM5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) were used to perform the qRT-PCR. The program used in this assay was as follows: 96°C for 1 min, 95°C for 15 s, 56°C for 15 s, 72°C for 45 s, 45 cycles. Four technical replicates were set for each sample and Tukey's test and *t* test were conducted for statistical analysis.

### Bioinformatics analysis

The protein sequences of the candidate gene and its homologues were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>). The structure of the gene and the full length of DNA were searched in the Cucurbit Genomics Database (<http://cucurbitgenomics.org/organism/2>). Multiple sequence alignment analysis was performed using DNAMAN version 8.0. Prediction of subcellular localization of eukaryotic proteins was conducted by TargetP on line (<http://www.cbs.dtu.dk/services/TargetP>).

### RNA-seq analysis

To further probe the mechanism of variegated leaf development, the differentially expressed genes from *Csv1* and wild type were screened by RNA-seq. Leaves of wild type and *Csv1* plants, with length equal to 3 cm, were harvested at 53 d after sowing for RNA extraction. At least 20  $\mu$ g of total RNA ( $\geq 600$  ng/ $\mu$ L) was prepared for Solexa sequencing. The Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used to evaluate total RNA quantitation. A total quantity of 1  $\mu$ g RNA per sample with RIN values above 7 was used for library preparation. Then constructed libraries were tested to qualitative analysis and sequenced using Illumina HiSeq instrument in line with manufacturer's instructions (Illumina, San Diego, CA, USA).

Genome website (<http://www.icugi.org/cgi-bin/ICuGI/index.cgi>) was consulted for the reference genome, and



gene model annotation files. Hisat2 (v2.0.14) was used for indexing of the reference genome and clean data was employed for comparison (Kim *et al.* 2015). The read numbers mapped to each gene were calculated using Integrative Genomics Viewer. Gene expression levels were calculated using software Htseq (v0.6.1). Analysis of the overall expression of the transcriptome using the standard RPKM method (Reads per kilobase per million mapped reads) to calculate the expression levels of genes (Mortazavi *et al.* 2008).

Analysis of differential expression in wild type and *Csvl* was conducted using the DEGSeq Bioconductor package (Robinson and Oshlack 2010). The *p* values were adjusted using the Benjamini and Hochberg method. Corrected *p* value of 0.05 was set as the threshold for significantly differential expression.

### Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differential expression genes

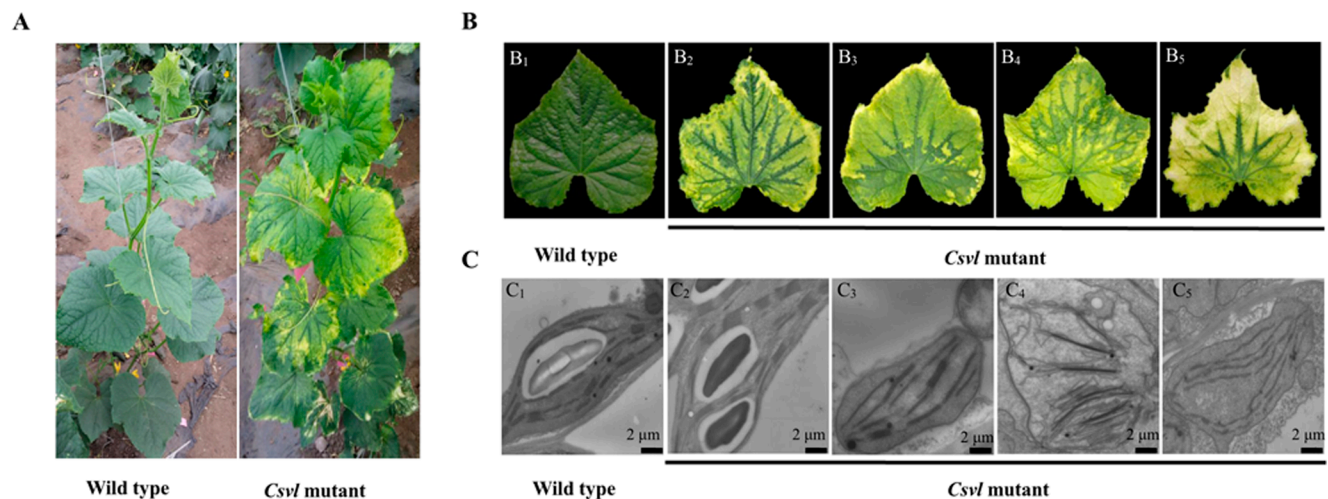
The gene length bias was corrected by GO enrichment analysis implementing the GO-Term Finder. The GO terms with corrected *p* values of less than 0.05 were considered significantly enriched after correction by Bonferroni. KEGG is the main public database on pathway (Kanehisa *et al.* 2008). Pathway significance enrichment can identify the major biochemical metabolic and signal transduction pathways that differentially expressed genes are involved. After the data correction, statistical enrichment of differential expression genes in the KEGG pathways were tested (Mao *et al.* 2005).

## Results

### Phenotypic characterization of a cucumber variegated leaf (*Csvl*) mutant

The variegated leaf *Csvl* mutant in cucumber was identified in our previous study (Xue *et al.* 2016). Phenotype identification results showed that the color of wild type leaves of each node appeared normal green throughout the growth cycle, while the color of each leaf of *Csvl* plants appeared variegation when the leaves grew to 3 cm during 2–3 true leaves stage (Fig. 1A). The typical character of leaf variegation was the formation of yellow or white and green sectors in the same leaf. Yellow sectors were mainly observed in the central region of the leaves, randomly, or near from leaf margins, and the white sectors were mainly at the leaf margins (Fig. 1B<sub>2</sub>–B<sub>5</sub>). The green sector of the leaves of *Csvl* plants showed normal green similar to those of the wild type (Fig. 1B<sub>1</sub>–B<sub>5</sub>). Furthermore, the plant height and leaf area of *Csvl* mutant plants were significantly reduced compared to those of wild type plants (Supplemental Fig. 1). The plant height of wild type was  $191 \pm 19.67$  cm, which was significantly higher than *Csvl*, whose height was  $146 \pm 6.52$  cm (Supplemental Fig. 1A). The leaf area of *Csvl* was  $174.92 \pm 17.37$  cm<sup>2</sup>, which was significantly less than that of wild type ( $442.67 \pm 69.92$  cm<sup>2</sup>) (Supplemental Fig. 1B). In addition, the fertility, leaf angle, and leaf shape of the *Csvl* mutant were not significantly different from wild type plants (data not shown) (*p* < 0.05).

It is speculated that the formation of variegated leaf may be related to the development of chloroplasts. Therefore the chloroplast morphology of wild type and mutant was observed and compared. The result showed that the chloroplast of wild type leaf included well-organized thylakoids and starch grain. The mesophyll cell of the *Csvl* mutant



**Fig. 1.** Comparison of the phenotype of wild type and variegated leaf mutant *Csvl* in cucumber. A: Wild type and variegated leaf mutant *Csvl* plants grown for 60 d. B: Wild type leaf (B<sub>1</sub>) and the main variegated leaf phenotype in *Csvl* mutant (B<sub>2</sub>, B<sub>3</sub>, B<sub>4</sub> and B<sub>5</sub>). C: Observation of chloroplast ultrastructure of wild type leaf and mutant with different color sectors. (C<sub>1</sub>) Wild type. (C<sub>2</sub>) Green sector in *Csvl* mutant. (C<sub>3</sub>) Light green sector in *Csvl* mutant. (C<sub>4</sub>) Yellow sector in *Csvl* mutant. (C<sub>5</sub>) White sector in *Csvl* mutant. Magnification times: 25000 ×.

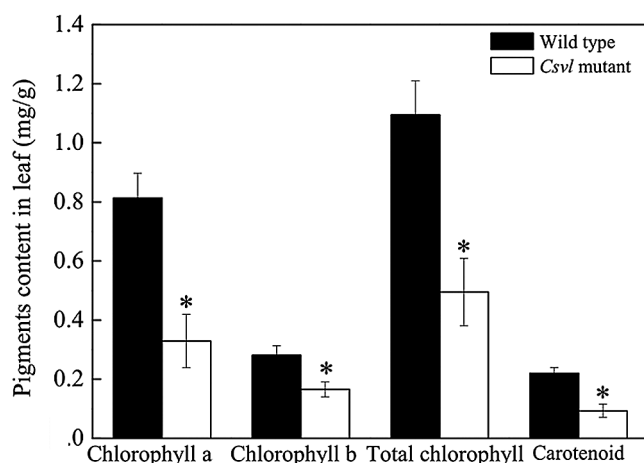
*Cscs* involves in the formation of leaf variegation in cucumber

contained a visible chloroplast but no structural feature of normal chloroplast, including the loose grana lamellae, unclear membrane structure and irregular shape (Fig. 1C<sub>1</sub>–C<sub>5</sub>). The thylakoid packed into grana and starch grain were present in mesophyll cell in the green part of the *Csvl* mutant (Fig. 1C<sub>2</sub>). In contrast, plastid hypoplasia in light green mesophyll cell had no starch grain and less grana lamellae (Fig. 1C<sub>3</sub>). The plastids of yellow and white leaves were hypoplastic, lacked starch grains and contained unstacked and loose thylakoids (Fig. 1C<sub>4</sub>, 1C<sub>5</sub>).

### Comparison of photosynthetic pigment content and photosynthetic parameter in wild type and *Csvl* mutant

Abnormal chloroplast morphology may affect chlorophyll synthesis, so the contents of photosynthetic pigments in *Csvl* mutant and wild type were determined. The result showed that the content of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid from mutant was significantly decreased by 59.54%, 41.14%, 54.80% and 57.71% than in wild type (Fig. 2). The analysis of photosynthetic pigment content showed that chloroplast did not completely lose the ability to synthesize chlorophyll, but the content of photosynthetic pigment decreased.

The leaf color is closely related to photosynthesis, and the photosynthetic rate in the leaf variegation mutant may be decreased. Therefore, the photosynthetic parameters in *Csvl* mutant and wild type were measured at 50 d after planting. The result showed that photosynthetic rate of reduction in *Csvl* mutant was 59.05% compared to in wild type (Supplemental Table 2). The stomatal conductance, intercellular CO<sub>2</sub> concentration and transpiration rate of mutant significantly increased by 47.73%, 11.59% and 28.01% than that of wild type respectively (Supplemental Table 2).



**Fig. 2.** Contents of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid in *Csvl* mutant and wild type leaves. ‘\*’ indicate the significant difference of target between wild type and *Csvl* mutant with  $p$  value < 0.05. Data were presented as means  $\pm$  SD of five replicates.  $t$  test was conducted for statistical analysis.

### Identification of candidate genes for *Csvl* mutant

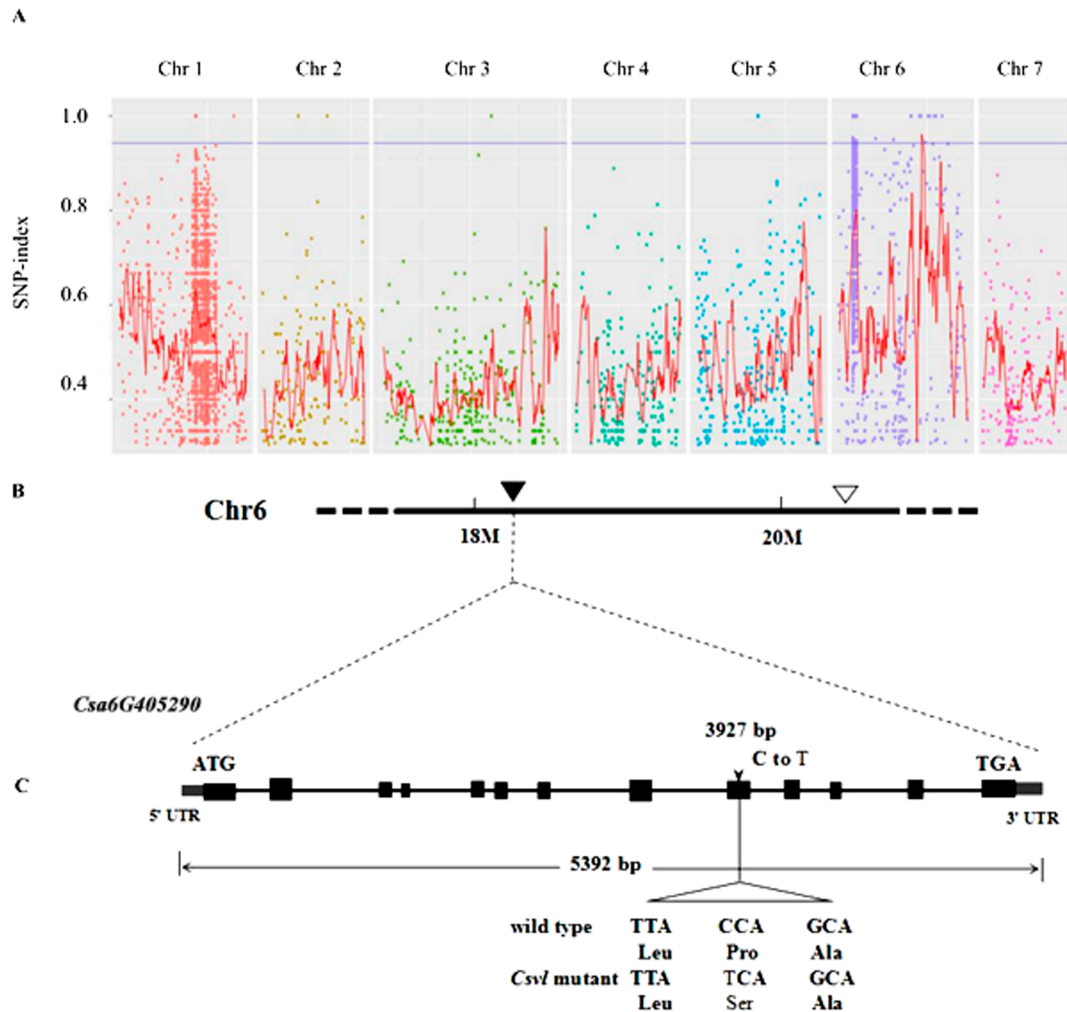
In order to identify the genetic characteristics of the variegated leaf phenotype in cucumber, *Csvl* was crossed with wild type. The F<sub>1</sub> plants had the same phenotype as the wild type with no variegated leaves. However, the significant phenotypic segregation was occurred in the F<sub>2</sub> population. The result of  $\chi^2$  test ( $\chi^2 = 0.59$ ,  $p > 0.05$ ) showed that the ratio of wild phenotype and variegation phenotype plants in F<sub>2</sub> population was 3:1 (WT:*Csvl* = 65:17), which revealed that the phenotype of *Csvl* mutant was regulated by a single recessive gene.

MutMap technique was then used to identify the candidate gene of the *Csvl* mutant. The DNA bulk from 17 F<sub>2</sub> plants showing leaf variegation phenotype (mutant pool) and DNA of one wild type was re-sequenced respectively. A total of 6408 pool-specific SNPs were detected in *Csvl* mutant (Supplemental Table 3), and the distribution map of SNP site for all seven chromosomes of cucumber was plotted by calculating the SNP-index (Fig. 3A). Candidate SNPs were selected in accordance with; following norms: first, SNP-index equal to 1; second, the mutation mode is homozygous, meaning that mutation should occur in the mutant instead of the wild type; third, mutation should be the typical EMS mutagenized type, including mutation from C to T or from G to A; fourth, mutation should be located in the exon region with nonsynonymous mutation or in the alternative splicing site of the intron of the gene. Then six SNPs with SNP-index of 1 in the bulk of mutant were screened (Table 1). Among the six candidate SNPs, SNP 18506237 was located in the intronic region without an alternative splice site and can be eliminated. Among the remaining five SNPs, three SNPs (18670038, 22022883, and 21578651) were located in downstream and UTR3. SNP 18277305 was located in the exon with a non-synonymous amino acid change, and the SNP-23376757 were located in splicing site of intron (Table 1, Fig. 3B), which indicated that genes harboring these two SNPs might be the candidate gene of *Csvl* mutant in cucumber.

To further validate the candidate SNP, the genotyping analysis in F<sub>2</sub> population plants was conducted by using the mutation information of two SNPs (18277305 and 23376757) with KASP technique. The results indicated that SNP 18277305 of *Csa6G405290* was co-segregated with phenotyping data in the F<sub>2</sub> population. The genotype of plants with mutant phenotype was T: T, and plants with wild type phenotype was C:T or C:C (Supplemental Table 4). However, SNP 23376757 was not co-segregated with the phenotype in F<sub>2</sub> population plants (Supplemental Table 4). These results demonstrated that *Csa6G405290*, encoding chorismate synthase, which contains SNP 18277305, was identified to be the candidate gene of the cucumber *Csvl* mutant. In this study, the candidate gene of cucumber variegated leaf mutant is named *Cscs*.

### Sequence and expression analysis of *Cscs* gene

The DNA sequence of *Cscs* (*Csa6G405290*) was 5392 bp



**Fig. 3.** Scatter plot of SNP-index for *Csv1* mutant and the structure diagram of *Cscs* gene. A: Distribution of SNP-index on seven chromosomes of cucumber in mutant pool. The red line indicates the distribution of average SNP-index; the blue line represents the threshold. B: Two SNPs located on cucumber chromosome 6. Black triangles indicate non-synonymous SNP-18277305; white triangles indicate SNP-23376757 located in the splicing site. C: The structure of predicted *Csa6G405290*. Rectangles represent exons; solid lines represent introns; arrow indicates the position of SNP 18277305.

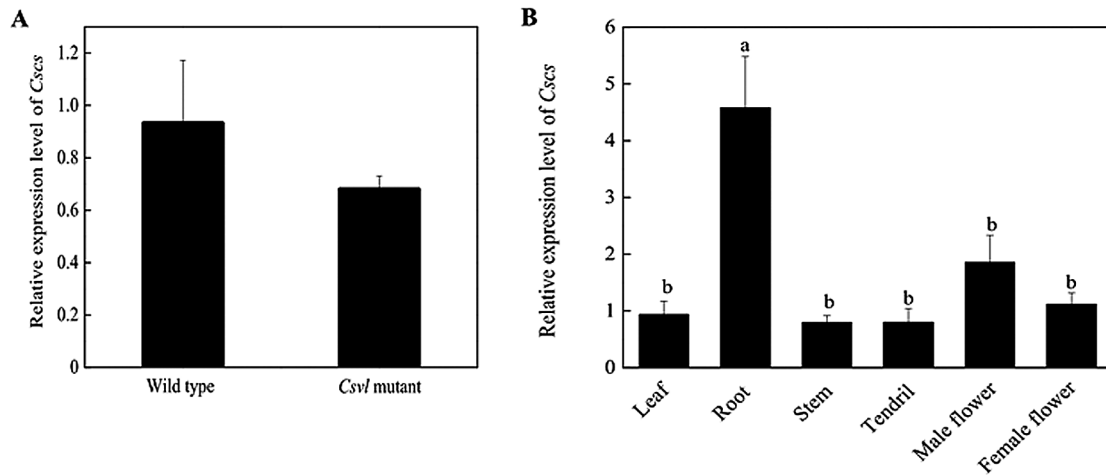
**Table 1.** Analysis of the candidate SNPs for the *Csv1* mutant

chromosome	Position	Gene	Reference	Alteration	Wild type	Reference depth	Alteration depth	SNP_index	Type	Amino acid change	Annotation
Chr. 6	18506237	Csa6G406550	C	T	0/0	0	15	1	intron		
Chr. 6	18670038	Csa6G408170	C	T	0/0	0	15	1	downstream		
Chr. 6	21578651	Csa6G452640	C	T	0/0	0	12	1	UTR3		
Chr. 6	22022883	Csa6G476670	G	A	0/0	0	20	1	downstream		
Chr. 6	23376757	Csa6G490810	G	A	0/0	0	11	1	splicing		Nodulation protein H
Chr. 6	18277305	Csa6G405290	C	T	0/0	0	12	1	exon	P to S	Chorismate synthase

in length containing 13 exons and 12 introns by consulting Cucurbit Genomics Database (<http://cucurbitgenomics.org/organism/2>) (Fig. 3C). A transition from proline (Pro) to serine (Ser) was occurred in the ninth exon of *Cscs* gene due to a C-to-T substitution (Fig. 3C). *Cscs* encoded the chorismate synthase catalyzed the dephosphorylation of 5-enolpyruvylshikimate 3-phosphate (EPSP) to generate the

chorismate (Braus 1991).

*Cscs* protein contains three characteristic domains, ranging from 70<sup>th</sup> to 85<sup>th</sup> amino acids, 177<sup>th</sup> to 193<sup>th</sup> amino acids and 383<sup>th</sup> to 399<sup>th</sup> amino acids, respectively (Supplemental Fig. 2). Most species have these three characteristic regions, which are mainly used for binding to the substrates EPSP and NADPH in higher plants, bacteria and other



**Fig. 4.** The relative expression of *Cscs* in cucumber. A: The relative expression level of *Cscs* in leaves of wild type and *Csvl* mutant. *t* test was conducted for statistical analysis ( $p$  value < 0.05). B: The relative expression of *Cscs* in different organs of wild type. Letters indicate the significant difference of traits between wild type and *Csvl* mutant with  $p$  value < 0.05. Data were presented as means  $\pm$  SD of four replicates. Tukey's test was conducted for statistical analysis.

microorganisms. Mutations occurred at certain site in the conserved domains can result in changes in enzyme activity (Ahn *et al.* 2004). Chloroplast transit peptide was found in *Cscs* protein by the prediction of TargetP (<http://www.cbs.dtu.dk/services/TargetP>).

Gene mutation may lead to changes in its expression. In order to detect whether the expression of *Cscs* was changed in mutant, its relative expression level was detected by qRT-PCR. The results show that the expression of *Cscs* in mutant was similar to wild type (Fig. 4A). In addition, *Cscs* expression in root of wild type plants was significantly higher than that of leaf, stem, female flower, male flower and tendrill (Fig. 4B).

#### Transcriptome profile analysis of *Csvl* mutant in cucumber

We tried to explore the molecular mechanism of variegation related genes from the transcriptional level and lay a foundation for the further study of variegation mutant in cucumber. A total of 183 differentially expressed genes related to variegated leaf in cucumber were identified by RNA-seq, within which 128 genes were significantly up-regulated and 55 genes were significantly down-regulated in the leaves of *Csvl* mutant as compared to wild type (Supplemental Table 5).

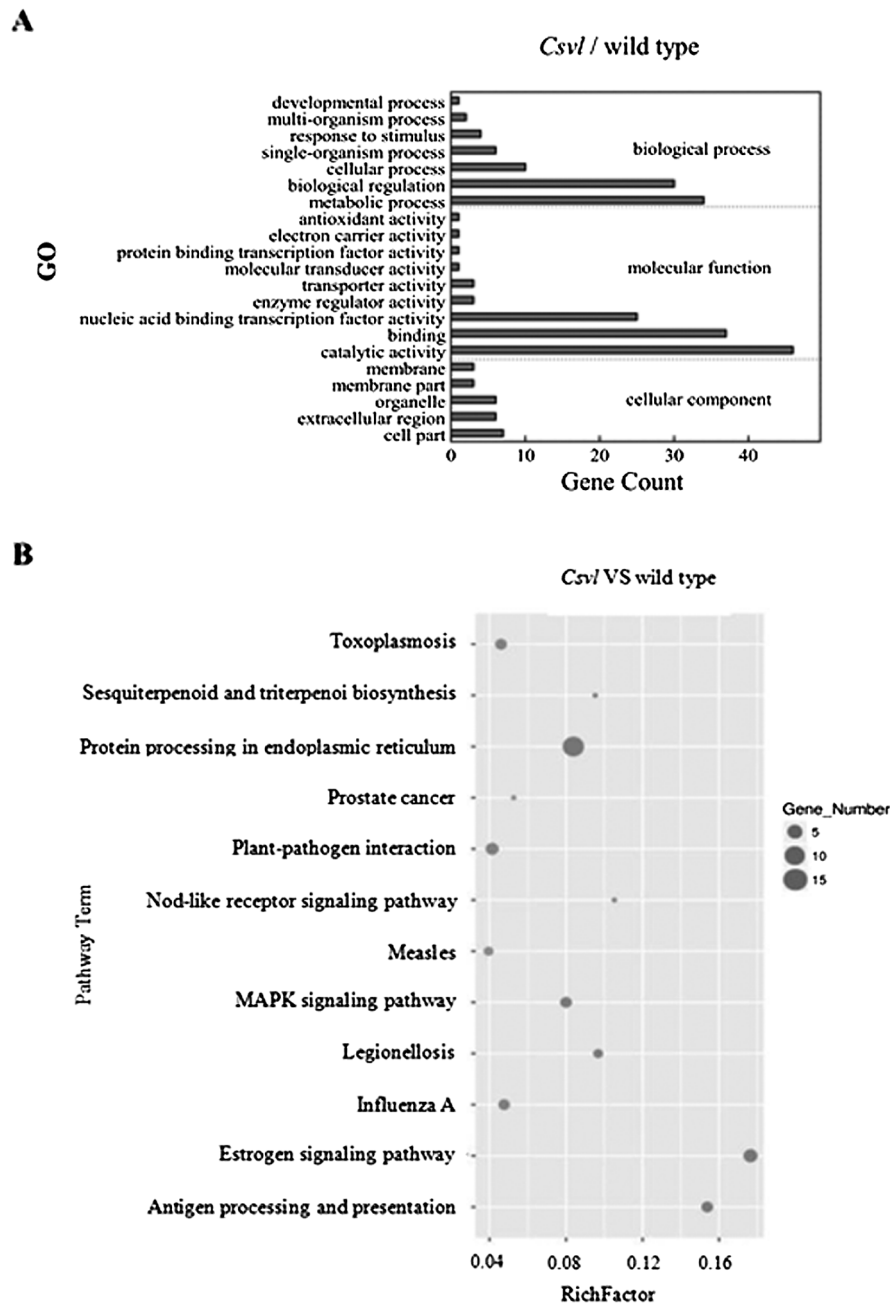
qRT-PCR were performed to verify the differentially expressed genes identified by RNA-Seq in the present study. The expression changes of the 18 differentially expressed genes showed that the results of qRT-PCR analysis were consistent with RNA-Seq data (Supplemental Table 6), indicating that the RNA-Seq data were reliable.

GO terms are used for functional classifications of differentially expressed genes obtained in this study, which include biological processes, cellular components, and molecular functions. Pathways analysis are derived from the KEGG database. Results showed that these genes were sig-

nificantly enriched in 21 GO terms and 12 KEGG pathways (*Csvl* versus wild type). The major biological groups of differentially expressed genes were functionally enriched in nucleic acid binding transcription factor activity, cellular process, catalytic activity, metabolic process, biological regulation, and binding (Fig. 5A). The main pathways of differentially expressed genes were enriched in estrogen signaling pathway, protein processing in endoplasmic reticulum, plant-pathogen interaction, antigen processing and presentation pathways, and MAPK signaling pathway (Fig. 5B).

Based on known function and functional predictions, some candidate important genes related to leaf variegation in cucumber were selected, such as genes encoding heat shock protein and zinc finger protein (Supplemental Tables 7, 8). Under normal growth and stress conditions, plants can produce heat shock proteins with molecular weight of 10–200 kD. Heat shock proteins were divided into five classes according to molecular weight, including HSP100, HSP90, HSP70, HSP60 and sHSPs (Waters *et al.* 1996). Some heat shock protein-related genes were identified as differentially expressed genes related to variegated leaf development in cucumber (Supplemental Table 7). These genes were predominantly up-regulated in *Csvl* mutant, such as HSP70 (*Csa2G070310*, *Csa5G514500*, *Csa5G512930*, *Csa7G312930*), DNAJ HSP (*Csa3G710780*, *Csa2G264570*), HSP90 (*Csa3G183950*), Hsp100 (*Csa1G039100*) and smHSPs (*Csa5G198120*, *Csa5G190530*, *Csa3G020090*, *Csa3G020080*, *Csa7G072770*, *Csa1G527870*, *Csa5G591720*, *Csa5G190560*, *Csa1G527860*). Zinc finger protein (ZFP) transcription factor, one of the important family of transcription factors in plants, acts a pivotal part in many biological processes, such as plant morphogenesis, transcriptional activation and stress (Laity *et al.* 2001, Takatsuji 1999). Some up-regulated genes related to ZFP were found





**Fig. 5.** The analysis of GO classification and KEGG enrichment of variegated leaf related genes in cucumber. A: The main GO classification of variegated leaf responsive genes in cucumber. The abscissa indicates the number of different genes in terms, and the ordinate indicates the enriched GO term ( $p$  values  $< 0.05$ ). B: KEGG enrichment scatter plot of differentially expressed genes related to leaf variegation in cucumber. Ordinate represents the pathway name; the abscissa represents the Rich factor, and the count of differentially expressed genes is represented by the size of the dots in this pathway.

as differentially expressed genes related to variegated leaf development in cucumber (**Supplemental Table 8**), such as *Csa6G303740* (C2H2 type), *Csa6G405920* (GATA type), *Csa6G499220* (C3HC4-type), *Csa6G152950* (AN1-like) and salt tolerance zinc finger (*Csa2G354820*, *Csa4G642460*, *Csa5G207940*).

## Discussion

Leaf color variation is mainly due to gene mutation affects the synthesis and degradation of chlorophyll, which in turn leads to changes in pigment content, composition and proportion (Beale 2005). In this study, reduced chlorophyll, carotenoid content (**Fig. 2**) and defective chloroplast (**Fig. 1C<sub>2</sub>-C<sub>5</sub>**) were contained in *Csvl* mutant. The *Cscs*



gene involved in the formation of cucumber leaf variegation was successfully identified, which encoded chorismate synthase. Chorismate synthase catalyzed the dephosphorylation of EPSP to generate the chorismate, which was an important intermediary metabolism substance (Chen *et al.* 2012, Herrmann 1995). Salicylic acid (SA), produced with chorismate as precursor is a phenolic plant hormone that involves in the regulation of photosynthetic pigment and photosynthetic machinery (Kusumi *et al.* 2006, Wildermuth *et al.* 2001). Under the environmental stress, moderate salicylic acid can avoid the decrease of the photosynthetic pigment content and maintain a high photosynthetic rate to ensure the normal growth in plant. Chloroplast is one of the major sites for ROS production in cells and ROS accumulation can cause oxidative damage to photosynthetic membranes (Apel and Hirt 2004, Wang *et al.* 2010). SA also mediately affects photosynthetic electron transport, indicating that SA plays a significant part in regulating the balance of redox reaction in the chloroplast (Janda *et al.* 2012). A benzoquinone derivative, plastoquinone, was also produced by a series of reactions to chorismate (Ksas *et al.* 2015). Plastoquinone acted as the electron mediator in the photosynthetic chain, and its absence in thylakoid caused the electron transfer of the photosynthetic chain to be blocked, and eventually the plastid was photooxidized (Norris *et al.* 1995).

Based on previous research, *Cscs* gene encoding chorismate synthase, which is the key enzyme in the chloroplast SA and plastoquinone biosynthetic pathway. Blocked SA and plastoquinone synthesis pathways due to *Cscs* gene mutation can affect the biogenesis of chloroplasts and photosynthetic pigments, and destroy the balance of redox reactions in the chloroplasts. Therefore, we hypothesized that mutation in *Cscs* caused insufficiency of chorismate, and then SA and plastoquinone level reduced in the chloroplasts of the *Csvl* mutant. SA protects the PSII reaction center and regulates the turnover of D1 protein in chloroplasts and reduce the damage of photosynthetic apparatus in leaves under high temperature and light stress (Sahu *et al.* 2002). PSII can not be repaired under excessive light, causing damage to the chloroplasts and rapid degradation of photosynthetic pigments. Meanwhile, the lack of plastoquinone may block the transfer of electrons from PS II to the cytochrome  $b_{6/f}$  complex in photosynthetic chain. The photoreaction proceed abnormally caused photooxidation damage of the PS II in the chloroplast, and abnormal photosynthetic pigment metabolism. In summary, a new mechanistic insight has been provided that *Cscs* gene mutation can lead to the formation of the variegated leaves in cucumber.

Transcriptional sequencing experiment also provided a theoretical basis for the molecular mechanism variegation formation. We analyzed differentially expressed genes from leaves of wild type and *Csvl*, which were heat shock protein and zinc finger protein. Heat shock proteins can be expressed in peroxidation (Wang *et al.* 2004). Previous studies indicated that chloroplast Hsp70 was involved in photoprotection and repair of photodamaged photosystem II (PS II)

(Schroda *et al.* 1999). DnaJ heat shock protein was a helper protein of HSP70 (Kampinga and Craig 2010). Some of the DnaJ-like zinc finger domain proteins were involved in the development or maintenance of plastid and regulate the redox reactions in PS I (Wang *et al.* 2016b). For example, the AtJ8 protein was found to be nearly absent from the Arabidopsis variegation (*var1*) mutant (Chen *et al.* 2011). Zinc finger proteins were also involved in photosynthetic physiology and pigment regulation. Salt-tolerant zinc finger protein gene (STZ) was found in Arabidopsis, which encode a C2H2 zinc finger protein with double zinc finger structure (Sakamoto *et al.* 2000). C2H2-type zinc finger protein also take part in the response to photooxidative stress and internal changes in leaves, such as palisade tissue, anthocyanin and chlorophyll content increased significantly (Lida *et al.* 2000). *Cscs* may also regulate the variegated leaf development in cucumber by interacting with these genes.

In conclusion, we can assume that the formation of variegated leaf is due to mutation of *Cscs* gene. In *Csvl*, mutation in the *Cscs* encoding the chorismate synthase hindered the synthesis and metabolism of the chorismate. Decreased levels of chorismate derivatives, such as SA and plastoquinone, result in abnormal redox reaction in chloroplast. Photooxidation damaged the construction of chloroplast and insufficient photosynthetic pigment content in *Csvl* mutant, and also induced down-regulation of photosynthesis-related genes in the nucleus, thus resulting in variegated leaf phenotype in *Csvl* mutant plants. In addition, *Cscs* may also regulate the variegated leaf development in cucumber by interacting with differentially expressed genes obtained from RNA-seq. The results obtained in the present study are important to explore the formation of the leaf variegation in cucumber from the perspective of *Cscs* encoding chorismate synthase.

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