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RESEARCH PAPER

The identification of *Cucumis sativus Glabrous 1* (*CsGL1*) required for the formation of trichomes uncovers a novel function for the homeodomain-leucine zipper I gene

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Abstract

The spines and bloom of cucumber (*Cucumis sativus* L.) fruit are two important quality traits related to fruit market value. However, until now, none of the genes involved in the formation of cucumber fruit spines and bloom trichomes has been identified. Here, the characterization of trichome development in wild-type (WT) cucumber and a spontaneous mutant, *glabrous 1* (*csgl1*) controlled by a single recessive nuclear gene, with glabrous aerial organs, is reported. Via map-based cloning, *CsGL1* was isolated and it was found that it encoded a member of the homeodomain-leucine zipper I (HD-Zip I) proteins previously identified to function mainly in the abiotic stress responses of plants. Tissue-specific expression analysis indicated that *CsGL1* was strongly expressed in trichomes and fruit spines. In addition, CsGL1 was a nuclear protein with weak transcriptional activation activity in yeast. A comparative analysis of the digital gene expression (DGE) profile between *csgl1* and WT leaves revealed that *CsGL1* had a significant influence on the gene expression profile in cucumber, especially on genes related to cellular process, which is consistent with the phenotypic difference between *csgl1* and the WT. Moreover, two genes, *CsMYB6* and *CsGA20ox1*, possibly involved in the formation of cucumber trichomes and fruit spines, were characterized. Overall, the findings reveal a new function for the HD-Zip I gene subfamily, and provide some candidate genes for genetic engineering approaches to improve cucumber fruit external quality.

Key words: Bloom, cucumber, HD-Zip I, map-based cloning, spine, trichome.

Introduction

Cucumber (*Cucumis sativus* L.) is an economically important crop cultivated worldwide (Huang *et al.*, 2009). The cucumber fruit is classified as a pepo, a hard-shelled berry with a specialized epidermis (the exocarp) covered with thick cuticle, spines, tubercules, and trichomes (Roth, 1977). The spine and bloom are two very important fruit quality traits affecting the market value of cucumber. Cucumber fruit spines are multicellular, non-glandular trichomes similar in shape and structure to leaf trichomes (Guan, 2008; Chen *et al.*, 2014). When spines are combined with tubercules, cucumber fruits have a characteristic warty trait. Compared with warty fruit, smooth fruit, which has no fruit spines and tubercules, are more important for the breeding of the fresh-eaten cucumber types, as they are easy to clean, package, transport, and

store (Zhang *et al.*, 2010; Yang *et al.*, 2014). The bloom, giving cucumber a coarse outer appearance, is a fine white powder on the fruit surface primarily composed of silica (SiO_2) secreted by the multicellular and glandular trichomes (bloom trichomes) (Yamamoto *et al.*, 1989; Samuels *et al.*, 1993). Moreover, bloomless cucumber is increasingly popular because of its more attractive and distinctly shiny appearance. Despite their importance in breeding for cucumber external quality, little is known about the molecular basis of the cucumber fruit spines and bloom trichomes.

In *Arabidopsis*, trichomes are unicellular, non-glandular, and usually have three branches. Trichome development in this model plant has been intensively studied, and the interplay of transcriptional regulators and hormone action has been shown to be at the heart of this developmental process (Ishida *et al.*, 2008). In particular, the transcriptional activation of *GL2* (Rerie *et al.*, 1994) by a regulatory complex formed from GLABRA1 (GL1) (Oppenheimer *et al.*, 1991), TRANSPARENT TESTA GLABRA1 (TTG1) (Walker *et al.*, 1999), and GLABRA 3/ENHANCER OF GLABRA3 (GL3/EGL3) (Payne *et al.*, 2000; Zhang *et al.*, 2003) is a key event in the initiation of trichomes. Similar to *Arabidopsis* trichomes, cotton fibres are also unicellular structures of epidermal origin, and the development of cotton fibres and *Arabidopsis* trichomes share a similar mechanism (Suo *et al.*, 2003; Wang *et al.*, 2004; Humphries *et al.*, 2005; Serna and Martin, 2006; Machado *et al.*, 2009; Zhang *et al.*, 2010; Walford *et al.*, 2012). However, the trichomes of *Antirrhinum* and solanaceous species are multicellular and some are glandular trichomes. Previous studies demonstrated that trichomes in *Antirrhinum* and solanaceous species develop through a transcriptional regulatory network that differs from those regulating trichome formation in *Arabidopsis* (and perhaps cotton) (Payne *et al.*, 1999; Serna and Martin, 2006; Yang *et al.*, 2011).

Homeodomain-leucine zipper (HD-Zip) proteins are unique to the plant kingdom, and contain two indispensable conserved domains: the homeodomain (HD) and the leucine zipper (Zip) (Schena and Davis, 1992; Henriksson *et al.*, 2005). They can be divided into four groups, I–IV, according to distinctive features of DNA-binding specificities, gene structures, additional common motifs, and physiological functions (Henriksson *et al.*, 2005; Ariel *et al.*, 2007). The HD-Zip IV proteins play pivotal roles in regulating trichome development in numerous plants. Studies on *Arabidopsis* demonstrated that members of the HD-Zip IV group, such as GL2 (Rerie *et al.*, 1994; Khosla *et al.*, 2014), PROTODERMAL FACTOR2 (PDF2) (Abe *et al.*, 2003), HOMODOMAIN GLABROUS2 (HDG2), HDG11, and HDG12 (Khosla *et al.*, 2014) mainly regulated trichome formation and epidermal cell differentiation. HD-Zip IV proteins that affect trichome development in other species are Woolly (Wo) in tomato (Yang *et al.*, 2011), GhHD-1 (Walford *et al.*, 2012) and GbML1 (Zhang *et al.*, 2010) in cotton, and OCL4 in maize (Vernoud *et al.*, 2009). However, it remains unknown whether trichome development in plants is regulated by the proteins of the other three subgroups, especially for HD-Zip I. The HD-Zip I genes have been demonstrated to be involved in diverse biological processes, including abiotic stress responses (Hanson *et al.*, 2001; Himmelbach *et al.*, 2002; Hjellstrom *et al.*, 2003; Johannesson *et al.*, 2003; Olsson *et al.*, 2004; Henriksson *et al.*, 2005), meristem regulation (Saddic *et al.*, 2006), and photomorphogenesis (Aoyama *et al.*, 1995; Wang *et al.*, 2003). To date, none of the HD-Zip I genes controlling the differentiation and maintenance of epidermal cell fate has been identified in plants.

The cucumber *glabrous 1* mutant (*csgl1*), with glabrous aerial organs, is an ideal material to study the molecular mechanism of the formation of trichomes, fruit spines, and tubercules in cucumber (Cao and Guo, 1999; Cao *et al.*, 2001). Genetic analysis demonstrated that the phenotypes of *csgl1* were controlled by a single recessive nuclear gene, and *csgl1* was epistatic to the *Tuberculate fruit* gene (*Tu*) (Cao *et al.*, 2001; Yang *et al.*, 2014). Although *Tu* was recently identified, *csgl1* is still unknown. Therefore, it was very important to identify *csgl1* to obtain insight into development of cucumber fruit tubercules, spines, and bloom trichomes. In this study, it was found that *CsGL1* was required for the further differentiation of cucumber trichomes, but not for their initiation. *CsGL1*, encoding a HD-ZIP I protein, was identified by map-based cloning. This is a newly discovered function for HD-ZIP I proteins. Moreover, two possible key genes, *CsMYB6* and *CsGA20ox1*, involved in cucumber trichome and fruit spine formation, were further identified through digital gene expression (DGE) profiling. The results provided a solid foundation for further studies on cucumber fruit external quality traits related to market value.

Materials and methods

Plant materials and growing conditions

The *csgl1* is a spontaneous mutant and was isolated from a north China-type cucumber cultivar 'Daqingba' with a warty fruit trait [wild type (WT)]. An F_2 population for map-based cloning was constructed from a cross between the *csgl1* and a European greenhouse-type inbred line ZG. Tissues for gene expression analysis were taken from the *csgl1* and WT. All plants were grown in a greenhouse under natural sunlight at Shandong Agricultural University, Tai' an, China.

Scanning electron microscope (SEM) analysis

Leaves from *csgl1* and the WT were pre-fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.0) for >4 h, rinsed three times (15 min each) with phosphate buffer, and fixed overnight with 1% OsO_4 in phosphate buffer at 4 °C. The fixed samples were then washed three times (15 min each) in the phosphate buffer, dehydrated through an ethanol series (50, 70, 80, 90, 95, and 100%) for 15 min at each step, incubated in a 1:1 (v:v) ethanol–isoamyl acetate mixture for 30 min, and then transferred to pure isoamyl acetate for 1 h. Finally, the samples were dried to critical point with liquid CO_2, and coated with gold–palladium before they were mounted for observation under an SEM (JSM-6610LV).

Map-based cloning of CsGL1

For map-based cloning of *CsGL1*, a mapping population was constructed from a cross between *csgl1* and ZG. The individual plants showing a glabrous phenotype in the F_2 progeny were selected for mapping. There were 20 simple sequence repeat (SSR), two

sequence-tagged site (STS), and three cleaved amplified polymorphic sequence (CAPS) polymorphic markers used for mapping *CsGL1*, and the primer sequences are listed in Supplementary Table S1 available at *JXB* online.

DNA and RNA isolation and gene expression analysis

Cucumber genomic DNA was extracted from leaves by the modified cetyltrimethylammonium bromide (CTAB) method of Murray and Thompson (1980). For tissue-specific analysis, total RNA was prepared from different tissues with an RNAprep pure Plant Kit (TIANGEN, China), according to the manufacturer's instructions. Foliar trichomes were isolated from leaves as described by Marks *et al.* (2008). The extracted RNA was treated with RNase-free DNase I (Fermentas, Canada) to eliminate genomic DNA contamination according to the protocols recommended by the manufacturer. First-strand cDNA was synthesized by using 1 μg of total RNA and the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Japan).

Semi-quantitative reverse transcripton–PCR (RT–PCR) and quantitative real-time PCR (qRT-PCR) were performed to analyse gene expression. qRT-PCR was carried out using the RealMasterMix (SYBR Green) kit (TIANGEN, China) and the PCR amplification was quantified according to the manufacturer's protocol. Amplification was performed on an iCycler iQ™ multicolour real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and the analysis of each type of sample was repeated four times. The analysis of relative mRNA expression data was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each expression profile was independently verified in three replicate experiments performed under identical conditions. All primers used in this analysis are listed in Supplementary Table S2 at *JXB* online.

Subcellular localization of CsGL1

The full coding sequence (CDS), except for the stop codon, was cloned into the pAT7-GFP (green fluorescent protein) vector between *Sal*I and *Spe*I sites. The *CsGL1–GFP* fusion was driven by the 35S promoter. The control vector pA7-GFP and the *Cauliflower mosaic virus* (CaMV) 35S::CsGL1–GFP fusion construct were bombarded into onion epidermal cells using Biolistic PDS-1000 (Bio-Rad). Samples were observed with a Leica TCSST2 confocal laser microscope.

Transcriptional activation analysis of CsGL1

The sequence, including the nuclear localization signal (NLS) and GAL4 activation domain (AD) sequence, was amplified from the pGADT7 vector (Clontech) by PCR. The PCR product was ligated into the pGBKT7 vector (Clontech) fused with the GAL4 DNA-binding domain (BD). The recombinant vector, pGBKT7-NLSAD, was used as a positive control. In contrast, the pGBKT7 vector was used as a negative control. To determine which part of CsGL1 was important for the transcriptional activation, the full-length or partial CDS [the deletion of amino acids 1–64 (ΔN1-64), 1–121 (ΔN1-121), and 1–175 (ΔN1-175) at the N-terminus; and 169–240 (ΔC169-240) and 80–240 (ΔC80-240) at the C-terminus] of *CsGL1* was fused with the BD in the pGBKT7 vector. The recombinant constructs pGBKT-CsGL1, pGBKT-CsGL1 ΔN1-64, pGBKT-CsGL1 ΔN1-121, pGBKT-CsGL1 ΔN1-175, pGBKT-CsCsGL1 ΔC169-240, and pGBKT-CsGL1 ΔC80-240 were each transformed into yeast strain AH109. The transformed yeast cells were diluted 10^0, 10^1, and 10^2, and grown on synthetic defined (SD) plates lacking tryptophan and histidine (SD/-Trp-His) and lacking tryptophan, histidine, and adenine (SD/-Trp-His-Ade) with α-gal and on control plates lacking only tryptophan (SD/-Trp). All experiments were performed according to the manufacturer's user manual. Photographs of the plates were taken after 3–4 d at 30 °C. All primers used in this analysis are listed in Supplementary Table S3 at *JXB* online.

DGE analysis

The leaves of *csgl1* and the WT were harvested, snap-frozen immediately in liquid nitrogen, and stored at –80 °C until further processing. Two independent replicates were collected (each replicate consisted of leaves from three individuals). Total RNAs were extracted from the samples using TRIzol reagent (Invitrogen, USA) and treated with DNase I (Fermentas, Canada) according to the manufacturers' instructions. A 20 μg aliquot of the total RNA was used for Illumina DGE tag profiling processed by BioMarker Technologies. Library construction was performed according to Illumina instructions and sequenced on an Illumina HiSeq™ 2500 sequencer. More than 5.7 million clean tags were obtained in each sample. All clean tags were mapped to the cucumber genome (http://www.icugi.org/cgi-bin/ICuGI/genome/home.cgi?ver=2&organism=cucumber&cultivar=Chinese-long) (Huang *et al.*, 2009) using TopHat2 (Trapnell *et al.*, 2009), with no more than one nucleotide mismatch allowed. The clean tags mapped to reference sequences from multiple genes were filtered. The remaining clean tags were designed as perfect clean tags. The number of perfect clean tags for each gene was calculated and then normalized in reads per kilobase of exon model per million mapped reads (RPKM) using the method of Mortazavi *et al.* (2008). Differentially expressed genes (DEGs) were defined using IDEG6 (Romualdi *et al.*, 2003), with FDRs (false discovery rates) <0.01 and $|\log_2 \text{RPKM}| \geq 1$ as a threshold. The DEGs were assigned functional categories based on the MapMan annotation (Thimm *et al.*, 2004).

Agrobacterium*-mediated cucumber transformation*

The full-length CDS of *CsGA20ox1* was inserted into the expression vector pBI121 between the *Bam*HI and *Sac*I sites. The resultant plasmid (pBI121-*CaMV35S::CsGA20ox1*) was transformed into the *Agrobacterium tumefaciens* strain LBA4404 using the freeze–thaw method (Holsters *et al.*, 1978). The overexpression vector pBI121-*CaMV35S::CsGA20ox1* was transformed into the WT using the cotyledon transformation method (Wang *et al.*, 2013).

Results

CsGL1 *controls the formation of cucumber trichomes, including foliar trichomes, fruit spines, and bloom trichomes*

All aerial parts of the *csgl1* mutant were glabrous, including leaves, stems, tendrils, floral organs, and fruits (Fig. 1). To characterize this phenotype further, the epidermis of leaves and fruits in *csgl1* and the WT were observed under an SEM (Fig. 2). In the WT, there were many trichomes on the epidermis of leaves (Fig. 2D, E). In contrast, there were no trichomes on the epidermis of leaves in *csgl1* instead of papillae (Fig. 2A, B). Similarly, there were no spines on the epidermis of *csgl1* fruits (Fig. 2C). The glandular trichomes (bloom trichomes) secreting bloom on the fruit epidermis of *csgl1* were much smaller than those of the WT (Fig. 3). The high-resolution images (Fig. 3) showed that the development of bloom trichomes stopped at the initiation stage. However, root growth and development did not significantly differ between *csgl1* and the WT (Supplementary Fig. S1 at *JXB* online).

CsGL1 *encodes a HD-Zip I protein*

To decipher the molecular defects in *csgl1*, a map-based cloning approach was used to isolate *CsGL1*. First, rough mapping was performed using 26 individuals with glabrous

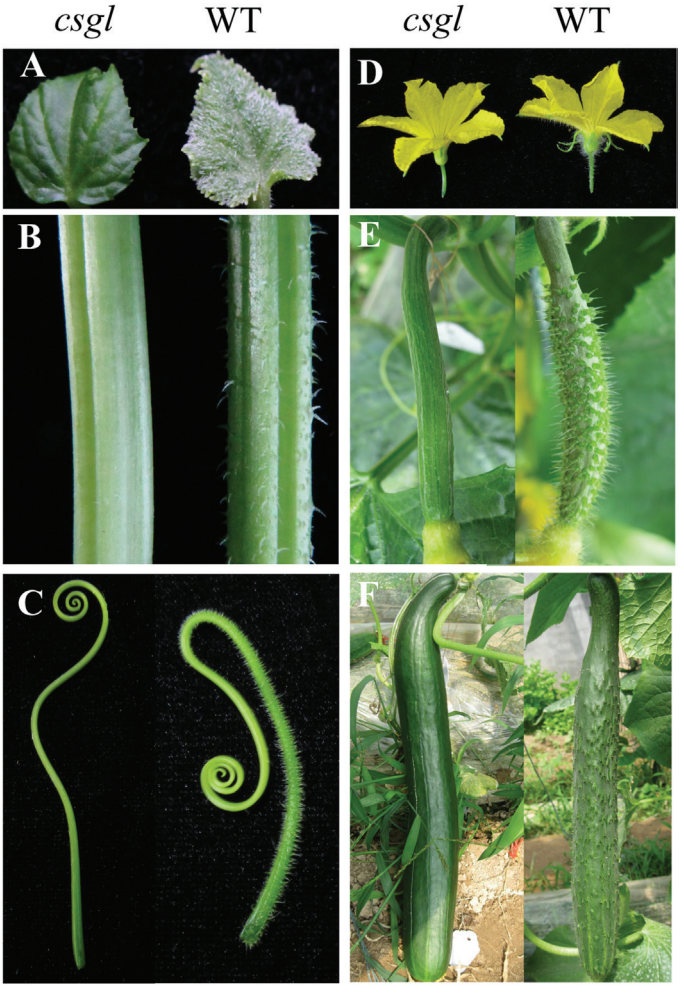

Fig. 1. Phenotypic characterization of *csgl1* and the WT. (A) Leaves; (B) stems; (C) tendrils; (D) male flowers; (E) fruits at 0 d after anthesis; (F) fruits at commodity maturity stage. (This figure is available in colour at *JXB* online.)

phenotype in 100 F_2 plants from the cross between *csgl1* and ZG, and *CsGL1* was mapped in a region between markers SSR21054 and SSR117 on chromosome 3 (Fig. 4A). Secondly, 32 recombinant lines were screened out with SSR21054 and SSR117 markers from 2400 plants of the mutant phenotype in the above F_2 population. Thirdly, two STS and three CAPS polymorphic markers were developed by comparing the difference in genomic sequences between *csgl1* and ZG and analysed in the 32 recombinant plants. *CsGL1* was finally narrowed to a region of 79.7 kb between STS-2 and CAPS-1. Based on the annotation in the Cucurbit Genomics Database (CuGenDB), a total of 13 predicted genes were located in this region (Fig. 4A). All 13 genes were sequenced and compared between *csgl1* and the WT. There was a 2649 bp fragment deletion from –189 to 2460 bp of the start codon in *Csa3G748220* in the *csgl1* mutant compared with the WT (Fig. 4B, C). The mRNA abundance of *Csa3G748220* was then analysed by RT–PCR. As a result of the sequence deletion, *Csa3G748220* was not expressed in *csgl1* (Fig. 4D). Moreover, there was no difference in the other 12 gene sequences between *csgl1* and the WT (data not shown). Therefore, the results strongly indicated that *Csa3G748220* was the candidate for *CsGL1*.

CsGL1 was predicted to encode a protein of 240 amino acids with the conserved HD (amino acids 65–120) and Zip domains (amino acids 121–164) (Fig. 4B). CsGL1, being a member of the clade ε of the HD-Zip I subfamily, shared high similarity with AtHB22 and AtHB51 from *Arabidopsis* (Liu *et al.*, 2013). A previous study showed that *CsGL1*, similarly to some members of the HD-Zip I genes, was also induced by abiotic stress [i.e. abscisic acid (ABA), NaCl, and low temperature] (Liu *et al.*, 2013). However, the role of *CsGL1* in development of cucumber trichomes and fruit spines revealed a novel function for the HD-ZIP I protein in plants.

CsGL1 *is preferentially expressed in foliar trichomes and fruit spines*

The expression patterns of *CsGL1* in different cucumber tissues, especially in foliar trichomes and fruit spines, were analysed by qRT-PCR. *CsGL1* was faintly expressed in leaves, male flowers, and ovaries, and barely in roots, stems, and tendrils. It was remarkable that *CsGL1* was highly expressed in foliar trichomes and fruit spines (Fig. 5A).

CsGL1 is a nuclear protein with weak transcriptional activation activity

To examine the subcellular distribution of CsGL1, it was fused with GFP (*35S::CsGL1-GFP*). Confocal imaging showed that the CsGL1–GFP fusion protein localized exclusively in the nuclei of onion (*Allium cepa*) epidermal cells in a transient expression assay (Fig. 5B). As a control, the GFP protein was found in both the nucleus and cytoplasm. Therefore, CsGL1 had a typical feature of a transcription factor that localizes in the cell nucleus.

To investigate whether CsGL1 possesses transcriptional activation activity, the GAL4 DNA BD–CsGL1 fusion protein was assayed in yeast for its ability to activate transcription of the GAL4 upstream sequence-driven His and LacZ reporter gene expression. Expression of the CsGL1-BD (GAL4-binding domain) fusion protein in yeast resulted in low expression of the reporter genes (Fig. 5C), revealing that CsGL1 had weak activity as a transcription activator in yeast. Deletion analysis showed that the Zip motif was required for transcriptional activation, and the AD was localized in the last 66 C-terminal residues. The truncated C-terminal protein with 122–240 amino acids clearly showed strong transcriptional activation activity. These results indicated that a repression domain was localized in the 121 residues of the N-terminus (Fig. 5C).

Characterization of CsMYB6 *and* CsGA20ox1*, two DEGs in DGE profiles between* csgl1 *and the WT*

To elucidate further the mechanism by which *CsGL1* regulates the formation of cucumber trichomes, including foliar trichomes, fruit spines, and bloom trichomes, DGE profile analysis was performed to identify genes differentially expressed in leaves between the *csgl1* and the WT. There were 470 DEGs between the WT and *csgl1*. Of these, there were

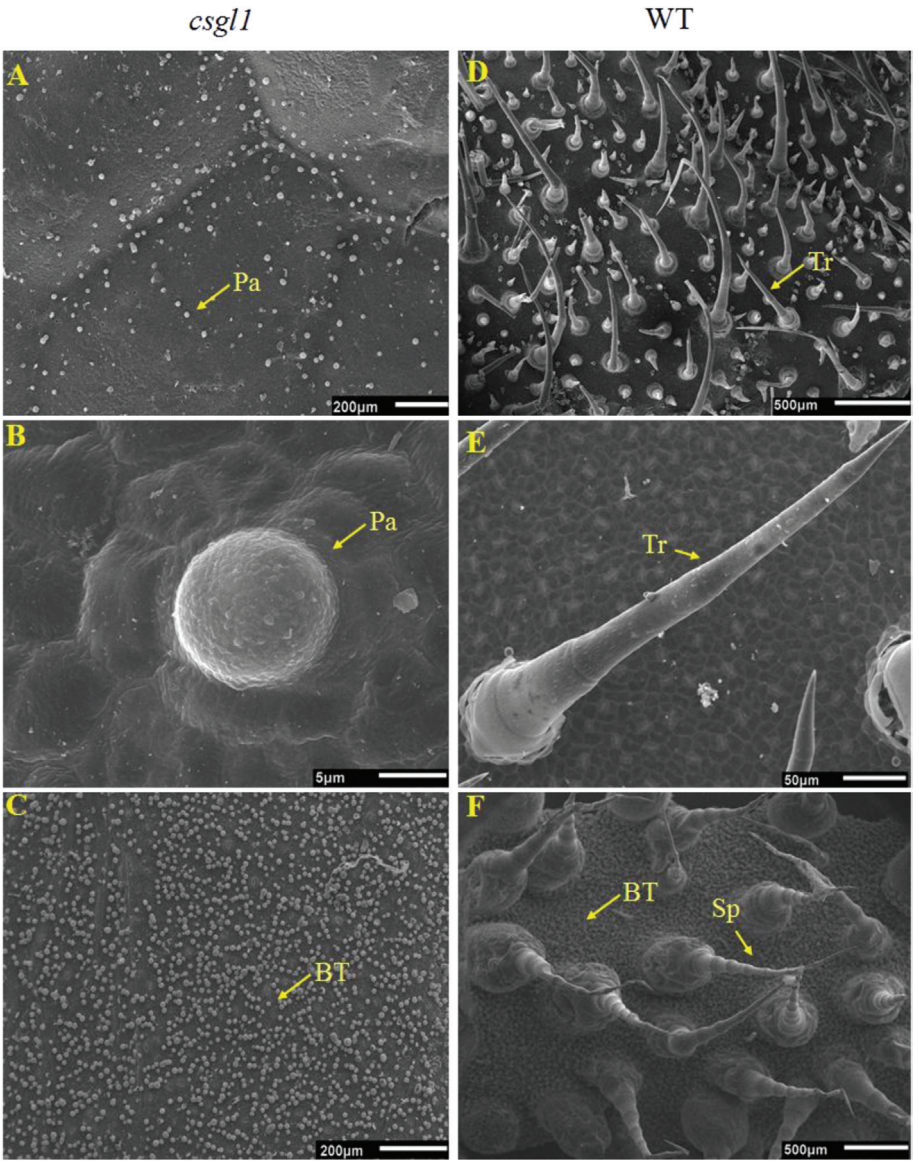

Fig. 2. SEM images of the epidermis of leaves, and fruits at 0 days after anthesis (DAA) from *csgl1* (A–C) and the WT (D–F). (A, D) Epidermis of leaves from *csgl1* (A) and the WT (D). (B, E) Papillae and trichome on the epidermis of leaves from *csgl1* (B) and the WT (E). (C, F) Epidermis of 0 DAA fruits from *csgl1* (C) and the WT (F). Tr, trichome; pa, papilla; BT, bloom trichome; Sp, spine.

269 down-regulated genes and 201 up-regulated genes in *csgl1*, implying that *CsGL1* had a significant impact on the global gene expression profile in cucumber (Supplementary Table S4 at *JXB* online).

To analyse the functions of DEGs identified by DGE profiling, a total of 390 DEGs were annotated and divided into 24 broad functional categories (Fig. 6A) and their percentage distribution is illustrated in Fig. 6B and C. The top five categories of DEGs encoded proteins related to: cellular process (350 genes), metabolic process (335), response to stimulus (286), biological regulation (206), and developmental process (183) (Fig. 6; Supplementary Table S5 at *JXB* online). These results implied that *CsGL1* significantly affected the expression of genes related to cellular process, consistent with the phenotypes of the foliar trichomes, fruit spines, and bloom trichomes in *csgl1* and the WT.

The *R2R3MYB* transcription factor plays a key role in the development of trichomes in plants (Larkin *et al.*, 1993; Payne *et al.*, 1999; Wang *et al.*, 2004; Schellmann *et al.*, 2007; Machado *et al.*, 2009). A previous study showed that CsMYB6 (Csa3G824850) was grouped together with two *Arabidopsis* R2R3MYB proteins, AtMYB16 (MIXTA), proposed to control the shape of petal epidermal cells, and AtMYB106 (NOK), a negative regulator of trichome branching (Baumann *et al.*, 2007; Jakoby *et al.*, 2008; Li *et al.*, 2012). This implied that *CsMYB6* might function in the formation of cucumber trichomes. Moreover, in the present study, *CsMYB6* was down-regulated in *csgl1* with an 11.3-fold change (Supplementary Table S4 at *JXB* online), which was confirmed by qRT-PCR (Fig. 7), indicating that *CsMYB6* might play an important role in the development of foliar trichomes and fruit spines in cucumber.

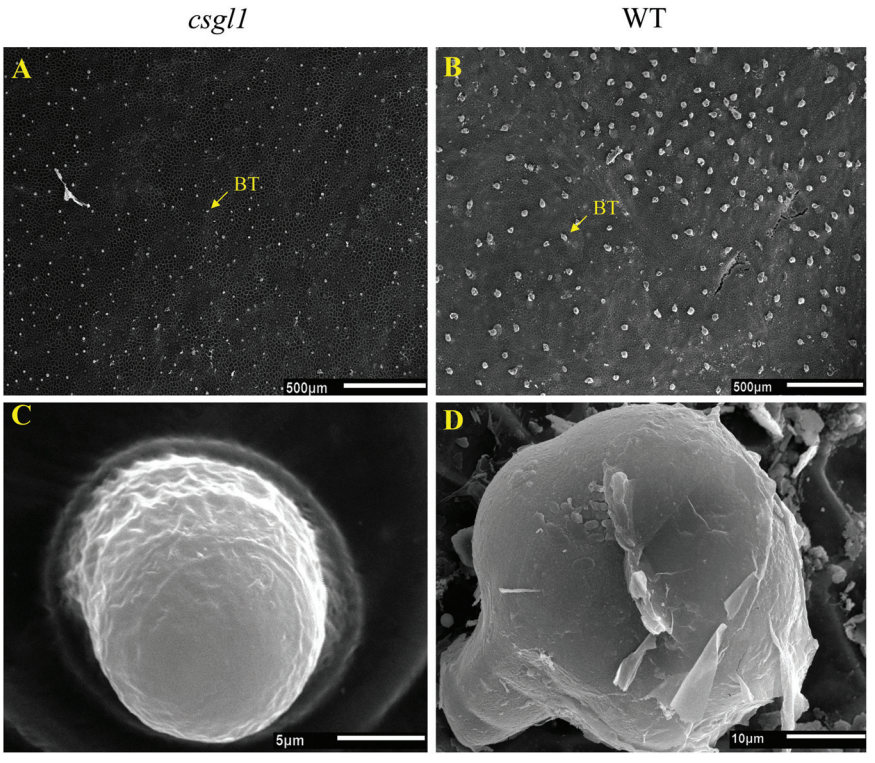

Fig. 3. SEM images of bloom trichomes on epidermis of fruits at the commodity maturity stage from *csgl1* (A, C) and the WT (B, D). BT, bloom trichome.

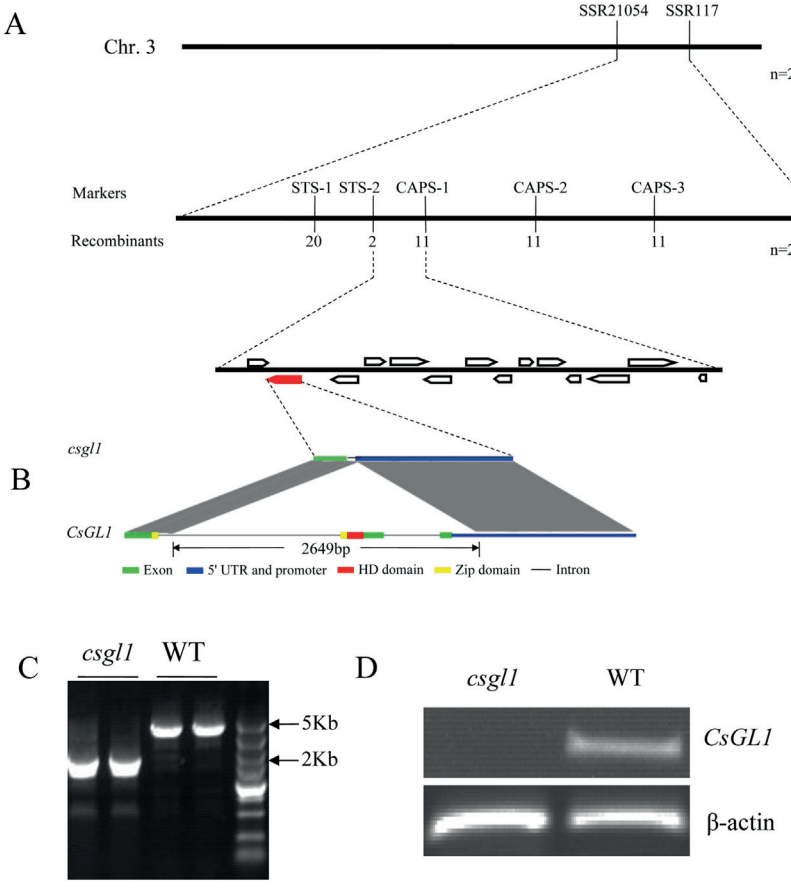

Fig. 4. Map-based cloning of the *CsGL1* gene. (A) Fine mapping of the *CsGL1* locus. *CsGL1* was first mapped to a region between the markers SSR21054 and SSR117 on chromosome 3 using 26 recessive individuals with glabrous phenotype in 100 F_2 plants from the cross between *csgl1* and ZG. Then it was narrowed to a 79.7 kb region between STS-2 and CAPS-1 using 2400 recessive mutants from the above F_2 population. Thirteen open reading frames (ORFs) were predicted in the mapped region. (B) Gene structure and sequence alignment of *Csa3G748220* alleles between *csgl1* and the WT. The promoter region upstream of the start codon, exons, HD domain, Zip domain, and introns are indicated by a blue box, green boxes, red box, yellow box, and single lines, respectively. (C) PCR analysis shows the deletion of the *CsGL1* genomic sequence in *csgl1*. (D) The expression of *CsGL1* in *csgl1* and the WT analysed by RT–PCR. (This figure is available in colour at *JXB* online.)

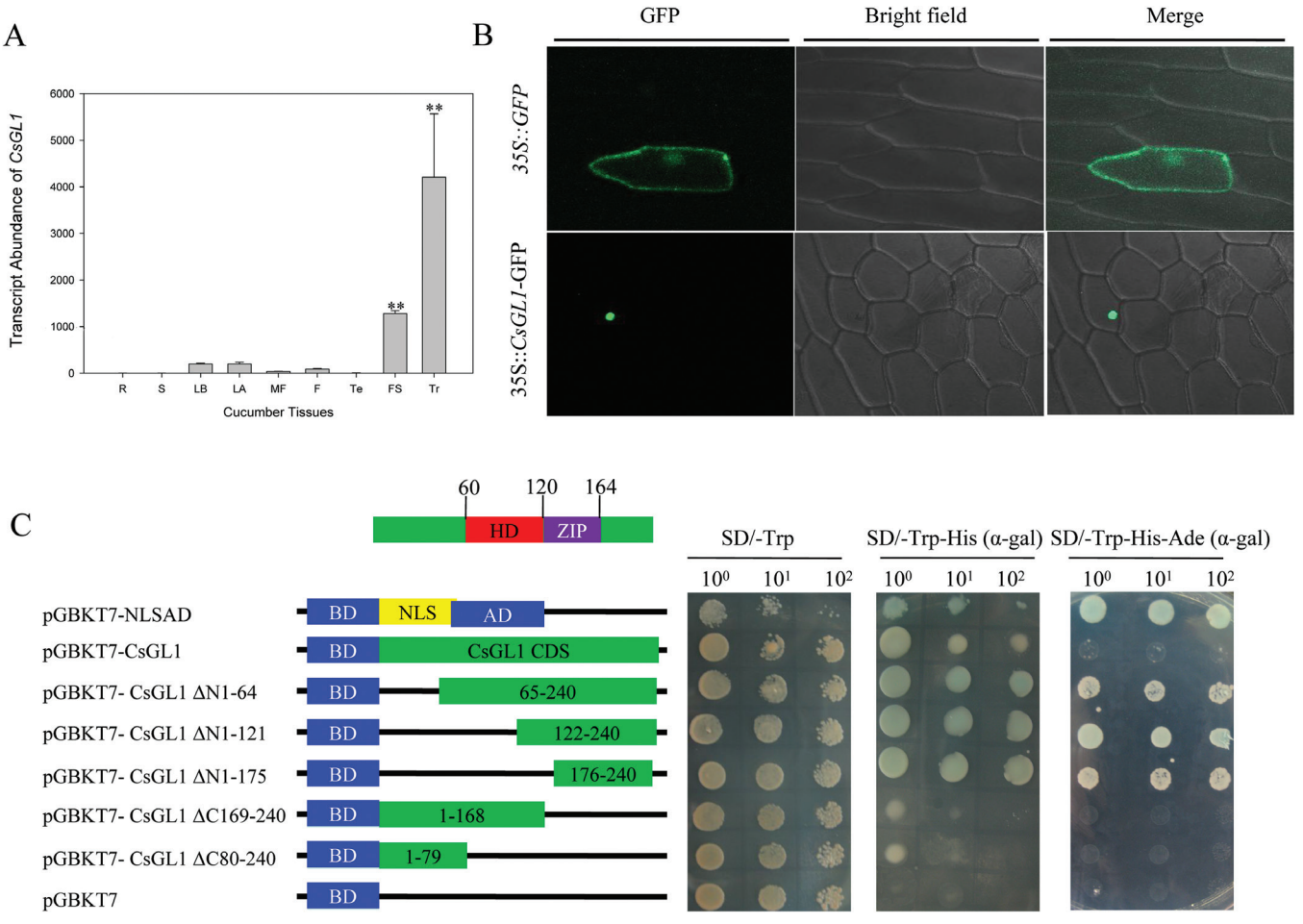

Fig. 5. Spatial expression pattern, subcellular localization, and transcriptional activation activity analysis of CsGL1. (A) Relative transcript abundances of *CsGL1* in different tissues of cucumber plants. R, roots; S, stems; LB, leaves before trichome removal; LA, leaves after trichome removal; MF, male flowers; F, fruits at 0 DAA; Te, tendrils; FS, fruit spines; Tr, trichomes. Values are the mean ±SD (n=3) (** and * indicates significant differences between *csgl1*and the WT at P=0.01 and 0.05, respectively). (B) *GFP* and the *CsGL1–GFP* fusion gene under the control of the CaMV *35S* promoter were expressed transiently in onion epidermal cells. Eight transformed cells were examined and all of them were observed with nuclear localization of CsGL1–GFP. (C) Schematic diagrams of various constructs used for transactivation activity assay. The GAL4 DNA-binding domain was fused with different parts of CsGL1 and transformed into yeast strain AH109 containing the His3 and LacZ reporter genes. Three independent experiments were performed and each showed similar patterns. BD, GAL4 DNA-binding domain; AD, GAL4 activation domain; NLS, nuclear localization signal. (This figure is available in colour at *JXB* online.)

Trichome formation is known to be influenced by gibberellins (GAs) in maize (Evans and Poethig, 1995) and *Arabidopsis* (Chien and Sussex, 1996; Telfer *et al.*, 1997). In addition, many studies have demonstrated a relationship between HD-Zip I genes and GA metabolism (Sakamoto *et al.*, 2001; Rosin *et al.*, 2003; Jasinski *et al.*, 2005; Son *et al.*, 2010). For example, accumulation of mRNA for GA 20-oxidase1, a key biosynthetic enzyme of GAs, decreased in lines overexpressing *POTH1*, a potato HD-Zip I gene (Rosin *et al.*, 2003). ATHB12, a HD-Zip I protein in *Arabidopsis*, negatively regulates the expression of *AtGA20ox1* (Son *et al.*, 2010). In the present study, *Csa5G172270* was up-regulated in *csgl1* with a 3.22-fold change (Supplementary Table S4 at *JXB* online) and was also confirmed by qRT-PCR (Fig. 7). *Csa5G172270* was annotated as an orthologue of *Arabidopsis thaliana GA20ox1* in the CuGenDB and, here it was named *CsGA20ox1* after confirmation by phylogenetic analysis. To confirm further the roles of *CsGA20ox1* in the development of trichomes in cucumber, transgenic plants expressing the CDS of *CsGA20ox1* under control of the CaMV *35S* promoter were generated in a WT background. Five independent T_1 lines were obtained and four of them (L2, L3, L4, and L5), with significantly increased expression of *CsGA20ox1*, were chosen for further observation (Fig. 8A). All four transgenic lines had shorter fruit spines (Fig. 8B) compared with the WT, indicating that *CsGA20ox1* was a negative regulator of growth of cucumber fruit spines.

Discussion

CsGL1 *controls the formation of cucumber trichomes*

csgl1 is a single recessive mutant and had no trichomes on leaves, stems, tendrils, and floral organs (Fig. 1). Instead, many papillae were found on the epidermis of the mutant leaves, with the papillae density similar to the trichome density of the WT (Fig. 2; Supplementary Fig. S2 at

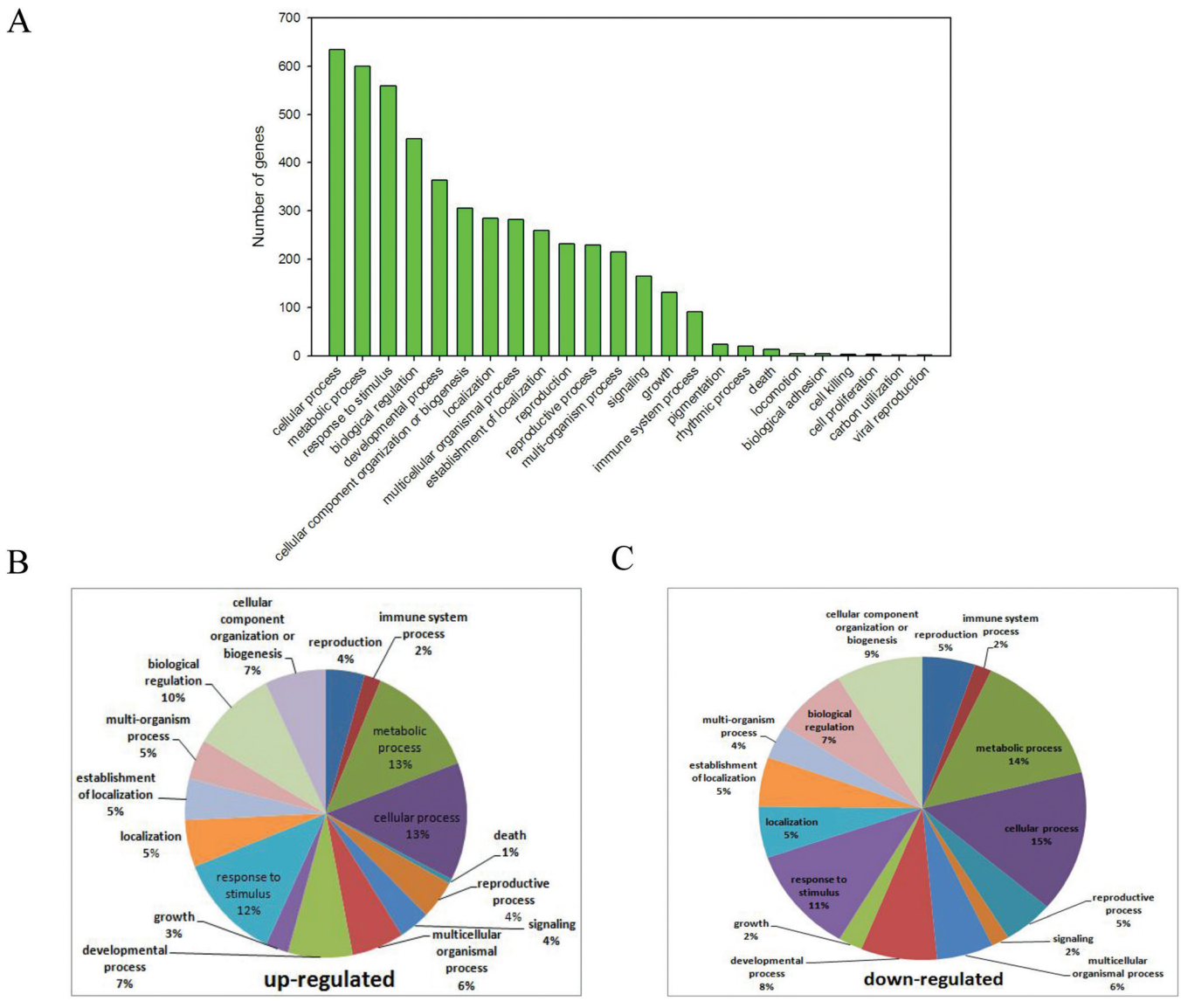

Fig. 6. Distribution of the 390 DEGs between *csgl1* and the WT among their functional categories. (A) The 390 DEGs were separated into 24 functional categories. (B) Percentage distribution of the functional categories of the up- and down-regulated genes in the *csgl1* mutant. (This figure is available in colour at *JXB* online.)

JXB online), suggesting that *CsGL1* was very probably involved in foliar trichome development but not initiation. Similarly, bloom trichome initiation was not affected, but the expansion and differentiation were greatly curtailed in *csgl1* (Fig. 3). Additionally, the silicified glandular trichomes and silicified epicuticular wax fragments surrounding them caused the WT cucumber fruit to appear dull (bloom) (Fig. 3B, D; Yamamoto *et al.*, 1989; Mitani *et al.*, 2011), while the surface of *csgl1* fruit was much cleaner and shinier than that of the WT because of the developmental defect of bloom trichomes in *csgl1* (Figs 1E, F, 3). A previous study indicated that *csgl1* was epistatic over the tuberculate fruit gene *Tu*, and *Tu* was not expressed in the *csgl* mutant (Yang *et al.*, 2014), which led to a lack of tubercules and spines on fruit of *csgl1*. However, root growth and development was not affected in *csgl1* (Supplementary Fig. S1), consistent with the lack of expression of *CsGL1* in roots (Fig. 5A), and this result was confirmed by RNA-seq of roots from *csgl1* and the WT (data not shown). These results suggest that *CsGL1* was required for the formation of trichomes in cucumber, especially fruit spines and bloom trichomes.

Characterization of CsGL1 *uncovered a novel function for the HD-Zip I gene in plants*

Genetic analysis in *Arabidopsis* has established a regulatory pathway that controls trichome initiation and development. However, the structures of cucumber trichomes are totally different from those of *Arabidopsis* (Fig. 2; Marks, 1997). Therefore, exploration of the mechanisms involved in trichome and fruit spine formation in cucumber can provide new insights into understanding the diversity of cell differentiation.

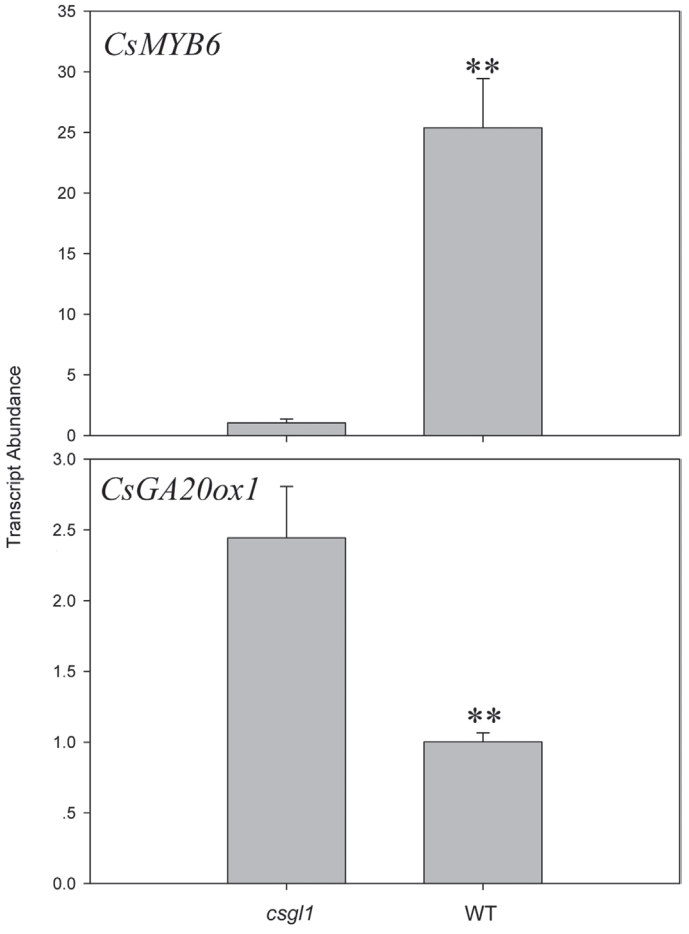

Fig. 7. Relative transcript abundance of *CsMYB6* and *CsGA20ox1* in *csgl1* and the WT. Values are the mean ±SD (*n*=3) (** indicates significant differences between *csgl1*and the WT at *P*=0.01 level).

HD-Zip homeobox proteins, defined by the conserved HD and adjacent leucine zipper motifs, are unique to plants but are related to other eukaryotic HD proteins (Sessa *et al.*, 1993; Henriksson *et al.*, 2005). Homeobox genes contain a highly conserved homeobox DNA sequence of 180 bp, encoding a protein which folds into a characteristic DNA-binding structure of helix–loop–helix–turn–helix, and are involved in developmental processes (Lin *et al.*, 2008). To date, for HD-Zip genes, only members of the HD-Zip IV subfamily have been reported to be involved in trichome formation, including *GL2* in *Arabidopsis* (Rerie *et al.*, 1994; Khosla *et al.*, 2014), *Woolly* (*Wo*) in tomato (Yang *et al.*, 2011), *Outer Cell Layer 4* (*OCL4*) in maize (Vernoud *et al.*, 2009), and *GhHD-1* in cotton (Walford *et al.*, 2012). However, no HD-Zip I gene has been identified for trichome initiation or development in plants. In the present study, *CsGL1* was identified as a HD-Zip I transcription factor that was essential for trichome and fruit spine formation in cucumber. Sequencing confirmed that the HD domain and part of the Zip domain of *CsGL1* were absent in *csgl1*, resulting in its glabrous phenotype (Figs 1–3).

To obtain information about the evolutionary relationship of *CsGL1* and its orthologues, an unrooted Neighbor–Joining (NJ) phylogenetic tree using bootstrap analysis (1000 replicates) was built from alignments of CsGL1 complete protein sequences and other HD-Zip I complete protein sequences from *Arabidopsis*, maize, grape, rice, poplar, and tomato. Phylogenetic analysis revealed that CsGL1 was clustered into clade ε, which was exclusively from eudicots (cucumber, *Arabidopsis*, grape, poplar, and tomato) (Supplementary Fig. S3 at *JXB* online). The closest homologue of *CsGL1* in *Arabidopsis* is *AtLMI1* (*ATHB51*; Liu *et al.*, 2013; Supplementary Fig. S3) which is a meristem identity regulator and has not been shown to be involved in trichome formation (Saddic *et al.*, 2006). In addition, *CsGL1* was highly expressed in cucumber trichomes and fruit spines, while *AtLMI1* was not expressed in trichomes of *Arabidopsis* (Saddic *et al.*, 2006), suggesting functional diversification among homologous genes in different plant species. This also suggested that trichome formation in cucumber may be regulated by a mechanism distinct from that characterized in *Arabidopsis*. Moreover, it would be interesting to see whether the *CsGL1* homologues in other species such as tomato (*Solyc09g008810.2.1*), poplar (*PtrHox29*), and grape (*Vv00021713001* and *VvXM 002283895*) are also involved in trichome formation.

CsGL1 might regulate the expression of CsMYB6 *and* GA20ox1 *indirectly*

Like other homeodomain proteins, CsGL1 is thought to function as a transcription factor regulating many downstream target genes controlling specific developmental processes. To facilitate identification of other key genes involved in trichome formation and regulatory pathways controlled by CsGL1, a comparative DGE profiling was performed between the leaves of the WT and *csgl1*. There were 470 DEGs with 201 up-regulated and 269 down-regulated genes detected in *csgl1*. In addition, consistent with the suggested function of *CsGL1*, the functional category ‘cellular process’ was over-represented among the DEGs (Fig. 6).

In plants, many *R2R3MYB* transcription factor genes were reported to have a role in trichome formation, such as *GL1* (Larkin *et al.*, 1993) and *MYB106* (Jakoby *et al.*, 2008; Gilding and Marks, 2010) in *Arabidopsis*. Other *R2R3MYB* genes that affect trichome development in other species are *MIXTA* (Noda *et al.*, 1994), *GaMYB2* (Wang *et al.*, 2004), *GhMYB25* (Machado *et al.*, 2009), *GhMYB109* (Suo *et al.*, 2003), and *PtaMYB186* (Plett *et al.*, 2010). In cucumber, *CsMYB6* was the homologue of *Arabidopsis AtMYB106*, a repressor of trichome outgrowth and branching, and of the poplar *PtaMYB186*, a positive regulator of trichome initiation (Jakoby *et al.*, 2008; Gilding and Marks, 2010; Plett *et al.*, 2010; Li *et al.*, 2012). Interestingly, *CsMYB6* was down-regulated in *csgl1* compared with the WT (Supplementary Table S4 at *JXB* online). Moreover, Chen *et al.* (2014) found that *CsMYB6* was one of the DEGs between the *tiny branched hair* (*tbh*) mutant (a spontaneous mutant, found because of its glabrous phenotype with hairless foliage and smooth fruit surface) and the WT, indicating that *CsMYB6* was involved in the multicellular fruit spine development in cucumber. These results indicate that *CsMYB6* might be involved in the formation of cucumber trichomes.

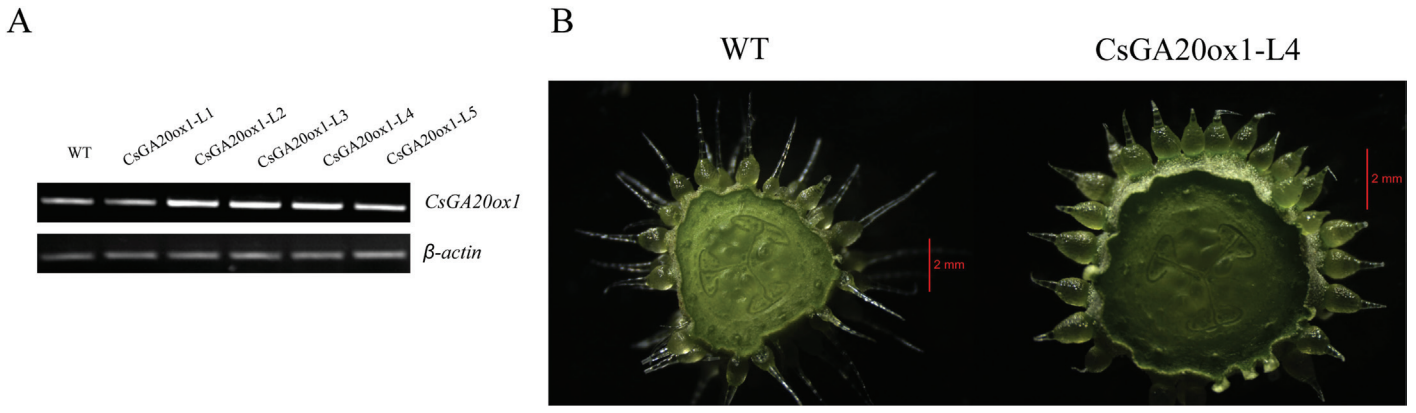

Fig. 8. Identification of *CsGA20ox1* overexpression transgenic lines (CsGA20ox1-L1, CsGA20ox1-L2, CsGA20ox1-L3, CsGA20ox1-L4, and CsGA20ox1-L5) and its phenotype analysis. Semi-quantitative RT-PCR analysis of *CsGA20ox1* transcript abundance in transgenic plants. Analysis of the length of fruit spines in the WT and *CsGA20ox1* overexpression transgenic lines. (This figure is available in colour at *JXB* online.)

CsGA20ox1 is a gene putatively encoding GA 20-oxidase, which catabolizes active GA (Huang *et al.*, 1998; Coles *et al.*, 1999), and was present in the DEGs identified through DGE profiling. Therefore, it was very probably involved in trichome formation in cucumber. In plants, a growing body of evidence suggests that HD-Zip I protein negatively regulates the expression of *GA20ox1* (Sakamoto *et al.*, 2001; Hay *et al.*, 2002; Jasinski *et al.*, 2005; Son *et al.*, 2010). As the transcript abundance of *CsGA20ox1* was up-regulated in *csgl1* (Fig. 7; Supplementary Table S4 at *JXB* online), it is speculated that *CsGL1* also negatively regulated the expression of *CsGA20ox1* in cucumber. Moreover, overexpression of *CsGA20ox1* reduced the length of cucumber fruit spines (Fig. 8B). These results indicated that *CsGL1* very probably decreased *CsGA20ox1* expression in cucumber, and further studies will be needed.

For the reasons mentioned above, it is hypothesized that *CsMYB6* and *CsGA20ox1* were very likely to be involved in the formation of cucumber trichomes and fruit spines. What is the relationship of *CsGL1* with *CsMYB6* or *CsGA20ox1*? Does CsGL1 directly regulate the expression of *CsMYB6* or *CsGA20ox1*? Many studies have demonstrated that HD-Zip I proteins bind to 9 bp DNA sequences with dyad symmetry, CAATNATTG, through the combined HD-Zip domains (Sessa *et al.*, 1993). Therefore, the sequence CAATNATTG was scanned for in the promoter regions of *CsMYB6* and *CsGA20ox1*. However, no CAATNATTG *cis*-element was found. Moreover, no direct interaction was found between *CsGL1* and the promoter of *CsMYB6* or *CsGA20ox1* using yeast one-hybrid assays (data not shown), indicating that CsGL1 may not directly regulate *CsMYB6* or *CsGA20ox1* expression by binding to their promoter regions. The possibility cannot be excluded that CsGL1 may bind to some other control region of *CsMYB6* or *CsGA20ox1* that has not been checked. Alternatively, CsGL1 may control the expression of *CsMYB6* and *CsGA20ox1* indirectly via other regulators. Further work will be directed to study whether and how CsGL1 regulates the expression of *CsMYB6* and *CsGA20ox1*.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Root phenotype of *csgl1* and the WT.

Figure S2. The density of trichomes or papillae in leaves of *csgl1* and the WT.

Figure S3. Phylogenetic tree of CsGL1 and other HD-Zip I proteins from *Arabidopsis*, maize, grape, rice, poplar, and tomato.

Table S1. The primers of SSR, STS, and CAPS polymorphic markers used in map-based cloning.

Table S2. The primers used for gene expression analysis.

Table S3. The primers of the full-length or partial sequence of the *CsGL1* CDS used for transcriptional activation analysis.

Table S4. DEGs in leaves between WT and *csgl1* libraries (WT versus *csgl1*).

Table S5. Functional categories of the DEGs between WT and *csgl1* leaf libraries (WT versus *csgl1*).

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