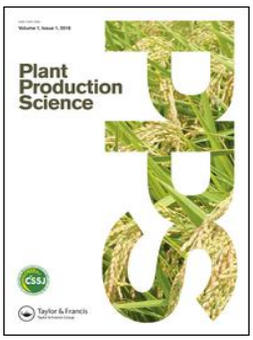

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MYB and HD-ZIP IV homologs related to trichome formation are involved in epidermal bladder cell development in the halophyte *Mesembryanthemum crystallinum* L.

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ABSTRACT

The common ice plant, *Mesembryanthemum crystallinum L.*, a halophytic new functional leafy vegetable crop, develops epidermal bladder cells (EBCs) on the surfaces of its aerial organs. Our previous studies of the physiological characteristics of the wild-type and the EBC-less mutant indicated that EBCs sequester salt and maintain ion homeostasis within photosynthetically active tissues. The EBC has been thought to be a modified trichome; however, molecular mechanisms governing EBC development in the common ice plant have not been fully understood. Here, we have analyzed the steady-state mRNA abundance of nineteen cotton fiber-related gene homologs and eight *Arabidopsis* trichome development-related genes, and found that a MYB transcription factor homolog (*McMYB2*) and a *GLABRA2*-like gene (*McC4HDZ*) were preferentially expressed in wild-type plants, whereas a putative *TRIPTYCHON* (*McTRY*)- and *CAPRICE*-like gene (*McCPC*) were preferentially expressed in the EBC-mutant. The full-length cDNA sequences of these homologs were determined, and constructs containing *McC4HDZ* and *McMYB2* were introduced into an *Arabidopsis* trichome-less mutant and wild-type plants. Overexpression of *McMYB2* in wild-type *Arabidopsis* increased trichome number, associated with activation of the trichome development-related gene, *GLABRA2* (*GL2*). Moreover, overexpression of *McC4HDZ* partially complemented trichome development in the trichome-less mutant of *gl2-1*, and resulted in increased trichome number in wild-type *Arabidopsis*, associated with the upregulation of key trichome-positive regulators *GLABRA1* (*GL1*) and *TRANSPARENT TESTA GLABRA1* (*TTG1*). These results suggest that *McMYB2* and *McC4HDZ* could be functional in *Arabidopsis* trichome formation, implying that EBCs of the common ice plant and trichomes of *Arabidopsis* may share some molecular mechanisms in their development.

Abbreviations: AGPC, acid guanidinium thiocyanate phenol chloroform; EBC, epidermal bladder cell; CAM, crassulacean acid metabolism; CaMV35S, cauliflower mosaic virus; CTAB, hecadecyltrimethylammonium bromide; RT-PCR, reverse transcription-PCR

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Introduction

Soil salinization is an increasingly important factor limiting plant growth and crop productivity. Estimates indicate that salinity decreases the average yields of major crops by more than 50% (Bray et al., 2000). About 10% (930 million hectares) of global land surface is affected by both natural and human-induced salt accumulation, which is attributable to poor drainage, vegetation clearance and inadequate irrigation (Shabala, 2013).

Halophytes naturally inhabit saline environments and tolerate salt concentrations of 200 mM or more at which approximately 99% of other plant species would die (Flowers & Colmer, 2008; Shabala, 2013). Halophytes have developed numerous strategies to adapt to high salinity. These adaptive mechanisms include osmotic adjustment through compartmentation of ions into vacuoles, accumulation of compatible solutes, succulence, and secretion of salt into specialized epidermal cells.

Mesembryanthemum crystallinum L., the common ice plant, is a leafy vegetable crop (Agarie et al., 2009) that exhibits extreme tolerance to high salinity concentrations equivalent to seawater (ca. 500 mM NaCl). Epidermal

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Supplemental data for this article can be accessed here.

bladder cells (EBCs), which is a specialized epidermal cell on the surface of the leaf and stem of the common ice plant, are a unique component of salinity tolerance of the species. Comparative characterization of wild-type plants and an EBC-less mutant showed that EBCs allow the common ice plant to adapt to salt stress conditions by storing water, sequestering salt, and maintaining ion homeostasis within underlying photosynthetically active tissues (Agarie et al., 2007).

EBC has been thought to be a modified trichome. Trichomes are appendages that typically originate from the epidermal cells of aerial plant organs. Different plant species may have different types of trichomes and some plant species might possess more than one type of trichomes. *Arabidopsis*, a model for the molecular study of trichome patterning, has unicellular, non-glandular trichomes that usually have three branches, whereas cotton, a model for the study of fiber development was single-celled trichome on seed surfaces that arise from the outer integument of ovules (Li et al., 2002).

A number of genes involved in the cell fate determination and trichome formation in *Arabidopsis* have been identified (Schellmann & Hulskamp, 2005). Trichome patterning in *Arabidopsis* is regulated through cell interaction networks involving a number of positive and negative regulators (Grebe, 2012; Schellmann & Hulskamp, 2005). *GLABRA3* (*GL3*), *GLABRA1* (*GL1*) and *TRANSPARENT TESTA GLABRA1* (*TTG1*) are initially expressed in all leaf trichome progenitors (Shabala, 2013). During trichome initiation, the R2R3 MYB factors, *GL1*, *MYB5* and *MYB23* form redundant complexes to regulate the leaf trichome initiation, branching, and extension (Kirik et al., 2005; Li et al., 2009). The R2R3 MYB factors interact with a bHLH protein (*GL3* and *EGL3)* and a WD-40 protein (*TTG1*) to form a complex to regulate trichome formation via *GL2* (Grebe, 2012; Payne et al., 2000; Rerie et al., 1994). The activator complex also triggers the transcription of genes for its inhibitors, *TRIPYCHON* (*TRY*) and *CAPRICE* (*CPC*) and four other homologs of these genes and the translated proteins could then move into adjacent cells (Grebe, 2012). These R3 single-repeat proteins then inhibit GL1-GL3-TTG1 activator complexes in the neighboring cells, thereby preventing trichome development (Grebe, 2012; Serna & Martin, 2006).

Cotton fibers are unicellular trichomes that are derived from the outer integument cells of the ovule. Development of cotton fibers takes place from 0 to 60 d post-anthesis (DPA) that including fiber initiation, elongation, biosynthesis of secondary cell walls, and maturation (Lee et al., 2007; Li et al., 2002). Genes for several transcription factors that function in fiber development, including *GaMYB2*, *GhMYB25*, *GhMYB109*, and a putative homeodomain transcription factor *GhHD1*, which are preferentially expressed in early fiber initiation, have been identified (Suo et al., 2003; Wang et al., 2004; Wu et al., 2006). Additionally, comparison of expression in the plants of wild-type and a fiber deficient mutant (*fl*) using hybridized filter arrays at 5 DPA, identified a number of genes involved in cell structure development, long-chain fatty acid biosynthesis, and sterol biosynthesis, including an RD22-like protein (*GhRDL*), a putative acyltransferase (*GhACY*), a *Fiddlehead* homolog (*GhFDH*), two tubulin components (*GhTUA* and *GhTUB1*), the fiber protein E6, a cellulose synthase catalytic subunit *GhCesA-5*, and a 24-sterol-C-methyltransferase *GhSMT* (Li et al., 2002).

The complete molecular mechanisms controlling EBC development in the common ice plant have not yet been determined. In our previous study, using a cDNA based-suppression subtractive hybridization (SSH), we have identified genes differentially expressed between the wild type and EBC-less mutant, and have shown that a SSH-derived clone WM28, a putative jasmonate-induced gene, positively regulates trichome number in *Arabidopsis* by increase of the expression of genes for the components of trichome development-acting complex such as *GL1* and *GL3* (Roeurn et al., 2016). Here, we elucidated the other genetic determinants of EBC development in the common ice plant by analyzing the expression of the common ice plant homologs of genes known to be involved in the development of cotton fibers and *Arabidopsis* trichomes. We investigated the steady-state mRNA abundance of nineteen fiber development-related homologs, and eight trichome development-related homologs in wild-type and EBC-less mutant plants. The full-length cDNAs of genes that were differentially expressed between wild-type and EBC-less mutant plants were also isolated and sequenced. Moreover, genes for the common ice plant homolog of cotton R2R3 MYB factor, *McMYB2*, and the common ice plant homolog of *Arabidopsis GL2*, *McC4HDZ*, which encodes an HD-Zip IV protein, were introduced into *Arabidopsis* to dissect their functions in plant trichome and EBC-formation.

Materials and methods

Plant materials

Wild-type *M. crystallinum* L., and an EBC-less mutant of the common ice plant (Agarie et al., 2007) were used for the present study. The plants were cultured as described previously (Roeurn et al., 2016). Seeds were surface-sterilized in sodium hypochlorite solution and sown on MS basal medium with Gamborg's vitamins and 30 g L^{-1} sucrose (0.7% Agar). Seedlings were cultured in a growth chamber at 26 °C during the day and 18 °C during night with 12-h (light)/12-h (dark) cycle with a light intensity of 300 µmol m^{-2} s^{-1} and 50% relative humidity.

Wild type of *Arabidopsis thaliana* (ecotype *Col-0*) and the *gl2-1* mutant in Landsberg *erecta* (Koornneeff et al., 1982) were obtained from the Arabidopsis Biological Resource Center and were cultured as described previously (Roeurn et al., 2016). The seeds were surfaced sterilized and sown on 1/2 MS basal medium with Gamborg's vitamins and 10 g L^{-1} sucrose (0.8% agar), and were vernalized at 4 °C for 2–3 d before germinating at 22 °C under constant light with a light intensity of ca. 100–120 µmol m^{-2} s^{-1}. Seedlings were cultured at 22 °C under a 16-h (light)/ 8-h (dark) cycle with relative humidity of 60–70%. For trichome analysis, the seeds germinated on the plates were incubated at 22 °C under constant light with a light intensity of ca. 100–120 µmol m^{-2} s^{-1} for 2 weeks.

DNA and RNA extraction

Genomic DNA was extracted using a CTAB (hexadecyltrimethylammonium bromide) method (Allen et al., 2006). Total RNA was isolated from the common ice plant or *Arabidopsis* plants using a modified acid guanidinium thiocyanate phenol chloroform (AGPC) method, as described by Chomczynski and Sacchi (1987). To remove contaminating genomic DNA, the RNA samples were treated with DNase (Takara Bio Inc., Shiga, Japan) according to the manufacturer`s instructions. Total RNA concentrations were measured at 260 nm (OD_{260}) using a spectrophotometer (GeneQuant 1300 RNA/DNA Calculator, GE Healthcare Life Sciences, Piscataway, NJ).

End-point semi-quantitative and real-time RT-PCR

To examine the steady-state mRNA abundance of putative homologs for EBC development in both the wild-type plants and the EBC-less mutant, total RNA was extracted from the leaves and stems of 8- and 15-week-old plants. End-point semi-quantitative reverse (RT)-PCR was conducted using cDNAs synthesized from the total RNA isolated and primers specific for each homologs (Supplemental Table 1). The PCR cycle consisted of denaturation at 94 °C for 2 min, followed by 18–40 cycles, which were adjusted for each genes individually to detect the differences in mRNA abundance among genes tested, denaturing at 94 °C for 30 s, annealing at a primer-specific annealing temperature for 45 s, and extension at 72 °C for 80 s, followed by an additional extension step for 10 min at 72 °C. The steady-state mRNA abundance of *McMYB2* and *McC4HDZ* in transgenic *Arabidopsis* plants were also analyzed by same method with slightly modified PCR conditions such that the annealing and extension were conducted at 60 °C for 30 s and 72 °C for 1 min, respectively using the specific primers (Supplemental Table 2). The amplified PCR products were analyzed by agarose electrophoresis containing 1.2% agarose in Tris-Acetate-EDTA buffer and stained with ethidium bromide solution to visualize DNA fragments with UV.

The mRNA transcript levels of endogenous trichome-related genes in *Arabidopsis* transformants were performed using real-time PCR. The PCR was performed using the StepOne System (Applied Biosystems) with SYBR GreenER™ (Invitrogen, Van Allen Way Carlsbad, CA). PCR amplification was employed at 50 °C for 2 min, at 95 °C for 2 min, followed by 40 cycles of denaturing at 95 °C for 15 s and annealing at 60 °C for 1 min, and followed by a dissociation stage at 95 °C for 15 s, at 60 °C for 1 min, and at 95 °C for 15 s as recommended by the manufacturer. The relative expression of the transcript levels of trichome-related genes was calculated by $\triangle$CT method (Livak & Schmittgen, 2001) after being normalized to the internal control of gene encoding actin (*ACTIN2*). The primers for the genes were designed according to Tominaga-Wada et al. (2013) and Sun et al. (2015) which were shown in Supplemental Table 2.

RACE

The 3′ ends of cDNAs were amplified by 3′ RACE using the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA) according to the manufacturer's instructions using primers specific for each of the candidate putative homologs from the common ice plant (Supplemental Table 3). The 5′ ends of cDNAs were amplified by 5′ RACE using the CapFishing™ Full-length cDNA Kit (Seegene Inc., Seoul, Korea) according to the manufacturer's instructions using primers specific for each of the putative homologs from the common ice plant (Supplemental Table 3).

PCR products were purified by a QIAEX II Gel Extraction Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions, and cloned into the pGEM-T Easy Vector (Promega, Inc., Madison, WI). The vector was introduced into *Escherichia coli* strain DH5α using the heat-shock procedure as described by the manufacturer. The plasmids were isolated using the alkaline lysis method (Birnboin & Doly, 1979) and sequenced by ABI PRISM 310 Genetic Analyzer using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Life Technologies, Inc., Grand Island, NY).

Expression vector construction and plant transformation

The full length cDNA fragments of the R2R3 MYB transcription factor, *McMYB2* and the HD-Zip IV protein, *McC4HDZ* were amplified by PCR, and then cloned into

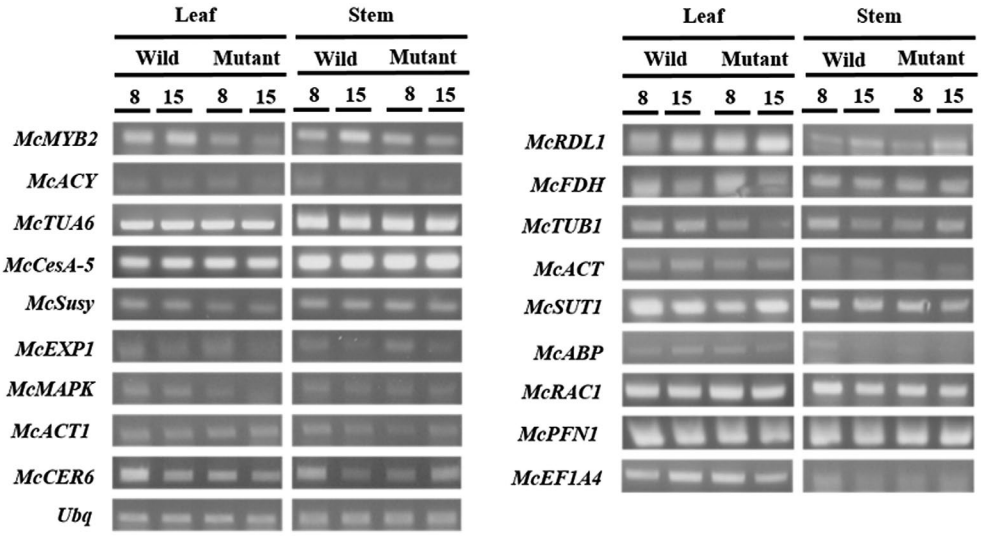

Figure 1. Transcript abundance of the putative cotton fiber development-related homologs in the leaves and stems of wild type and the EBC-less mutant of the common ice plants (8, 8-week-old plants, 15, 15-week-old plants).

Table 1. Cotton fiber-development related genes and their homologs in the common ice plant.

Cotton developmental stage	Cotton genes	Accession number	Common ice plant orthologs[a]	Homology (%)
Fiber initiation	*GaMYB2*	AY641990	BE034349	60
	GaRDL1	AY626160	BG269414	66
Fiber initiation and elongation	*GhACY*	AF191025	BE035266	69
	GhFDH	AF512539	TC10060	73
	GhTUA6	AY207316	BE035618	67
	GhTUB1	AF165925	DY032429	81
	GhCesA-5	AY305723	TC9344	80
	GhACT	AI729533	TC8112	72
	GhSuSy	DQ122189	TC9620	73
Fiber elongation	*GhSUT1*	DQ174250	TC8096	86
	GhEXP1	AY072824	BE035905	62
	GhABP	AY072823	TC8661	68
	GhMAPK	AY072822	BE130411	77
	GhRac1	BM356394	TC7912	82
	GhACT1	BM356393	TC10566	77
	GhPFN1	BM356396	BE034816	73
	GhCER6	BM356395	TC8550	84
	GhEF1A4	8U7358	TC8367	74

[a]They were obtained from NCBI and DFCI database using TBLAST search algorithm against cotton fiber genes reported in Lee et al. (2007).

the commercial binary vector pRI-101-AN (Takara Bio Inc., Shiga, Japan) at the *Bam*HI site under the control of cauliflower mosaic virus promoter (*CaMV*35S) using the In-Fusion® HD Cloning Kit (Clontech Laboratories) using primers (Supplemental Table 4).

The resultant constructs were then introduced into *Agrobacterium tumefaciens* strain GV3101 using a freeze/thaw method (Kan et al., 2006). Transformation of *Arabidopsis* plants, *Arabidopsis thaliana* (ecotype *Col-0*), and the *gl2-1* mutant, were performed using a floral dip method as described by Clough and Bent (1998), and then screened on the selection medium supplemented with 50 mg L^{-1} Kanamycin.

Results

Transcript abundance of cotton fiber development-related homologs in the common ice plant

The relative steady-state mRNA abundances of the homologs of fiber development-related genes were analyzed in wild-type common ice plants and the EBC-less mutant (Figure 1). The nucleotide sequences of homologs, which were selected based on the report by Lee et al. (2007), were partially obtained from the common ice plant gene index (ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/Mesembryanthemum_crystallinum/) using the TBLAST search algorithm against cotton-fiber development genes in GenBank (http://www.ncbi.nlm.nih.gov/Blast.cgi). Nineteen nucleotide sequences for the common ice plant homologs of cotton genes related to fiber initiation, fiber development, and fiber elongation were retrieved (Table 1).

MYB transcription factors (*GaMYB2*) and RD22-like 1 (*GaRDL1*) have been reportedly involved in fiber initiation in cotton (Wang et al., 2004). The transcription abundance of *McMYB2*, a homolog of *GaMYB2* was relatively low in the leaves and stems of both the 8- and 15-week-old EBC-less mutant (Figure 1). However, no changes in the transcript abundance of *McRDL1*, a homolog of *GaRDL1*, were observed between wild-type plants and the EBC-less mutant. The genes *GhACY*, *GhFDH*, *GhTUA6*, *GhTUB1*, *GhCesA-5*, *GhACT*, and *GhSuSy* have been suggested to be involved in fiber initiation and elongation (Li et al., 2002; Taliercio & Boykin, 2007). The transcript abundance of the common ice plant homolog of cotton genes including *McACY*, *McFDH*, *McTUA*, *McCesA*, *McACT*, and *McSuSy* showed no significant differences in expression level

Table 2. *Arabidopsis* trichome development-related genes and their homologs in the common ice plant.

Trichome developmental stage	*Arabidopsis* genes	Accession number	Common ice plant orthologs[a]	Homology (%)
Pattern formation	*TTG1*	NM_18073	TC5440	74
	GL2	NM_106633	BE036426	66
	TRY	NM_124699	BE036810	67
	CPC	NM_130205	CA836861	58
Branch formation	*AN*	NM_100033	BE034659	73
	TFCA	AF48684	BE034749	77
Growth direction	*WRM*	NM_113614	TC4942	59
	CRK	NM_11640	TC5320	71

[a]They were obtained from NCBI and DFCI database using TBLAST search algorithm against *Arabidopsis* genes reported in Schellmann and Hulskamp (2005).

homologs of *GLABRA2* (*GL2*), *TRANSPARENT TESTA GLABRA1* (*TTG1*), *TRIPTYCHON* (*TRY*), and a *CAPRICE*-like gene (*CPC*) which are related to trichome patterning; *ANGUSTIFOLIA* (*AN*) and *TUBULIN FOLDING COFACTOR A* gene (*TFCA*) which are related to trichome branch formation; and homologs of *WURN* (*WRM*) and *CROOKED* (*CRK*) which are related to patterning of trichome growth direction (Table 2). These homologs have been designated as *McGL2* (*McC4HDZ*), *McTTG1*, *McTRY*, *McCPC*, *McAN*, *McTFCA*, *McWRM*, and *McCRK*, respectively.

The transcription profiles of these homologs in leaves and stems of both 8- and 15-week-old wild-type and the EBC-less mutant plants are illustrated in Figure 2. *GL2*, which encodes a homeobox transcription factor (Rerie

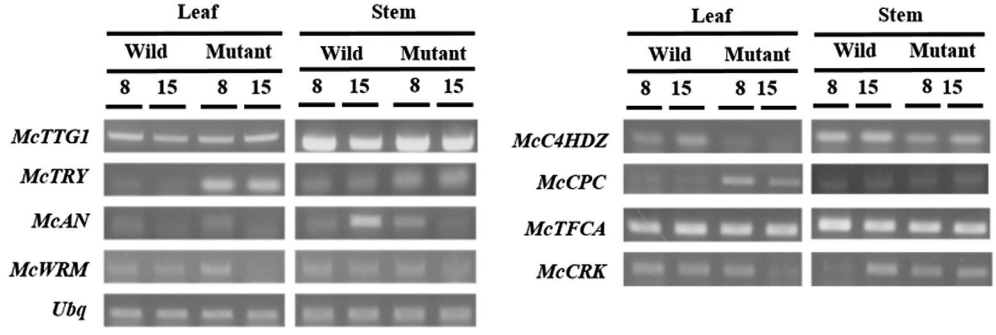

Figure 2. Transcript abundance of the putative trichome development-related homologs in the leaves and stems of wild type and the EBC-less mutant of the common ice plants (8, 8-week-old plant, 15, 15-week-old plant).

between the wild-type plants and the EBC-less mutant. One exception was a putative homolog of *Beta tubulin 1* (*McTUB1*) that exhibited reduced transcript expression in leaves, but increased expression in stems of the 15-week-old mutant compared to the wild type. There were no significant differences in the transcript abundance of the common ice plant homologs of genes related to fiber elongation such as *McSUT1*, *McEXP1*, *McABP*, *McMAPK*, *McRAC1*, *McACT1*, *McPFN1*, *McCER6*, *and McEF1A4* between wild-type and EBC-less mutant plants.

Transcript abundance of the common ice plant homologs of Arabidopsis trichome development-related genes

Arabidopsis trichome development-related genes were identified in the TAIR database based on the report of Schellmann and Hulskamp (2005) and the information of nucleotide sequences for these genes was retrieved. To obtain the homologs of *Arabidopsis* trichome development-related genes from the common ice plant, the TBLAST algorithm was used to search the NCBI and DFCI databases using *Arabidopsis* query sequences. As a result, eight homologs of the common ice plant for trichome development-related genes were obtained, including

et al., 1994), and *TTG1*, which encodes a WD40 protein (Galway et al., 1994), were suggested to be positive regulators of trichome pattern formation in *Arabidopsis* (Schellmann & Hulskamp, 2005). Our results indicated that *McGL2*, which was designated *McC4HDZ* based on the result of sequence analysis as described below, was preferentially expressed in wild-type plants, but its expression was reduced in the EBC mutant, whereas *McTTG1* was expressed to a similar degree in both wild-type and EBC-less mutant plants. In contrast, both negative regulators *TRY* and *CPC* (Schellmann et al., 2002) were expressed preferentially in the mutant compared to wild-type plants (Figure 2). The *AN* and *TFCA* genes are reportedly involved in trichome branch formation in *Arabidopsis* (Schellmann & Hulskamp, 2005). In the common ice plant, *McAN* transcripts were expressed at higher levels in the stems of 8-week-old EBC-less mutants than in the wild type, but expressed at extremely low levels in the stems of 15-week-old mutant plants. The expression of *McTFCA* transcript remained at similar abundances in both wild-type and EBC-less mutant plants. Both *WRM* and *CRK*, which are thought to be related to the direction of trichome growth, showed slightly lower expression in the leaves of 15-week-old mutants than in the wild-type plants.

Table 3. Full-length cDNAs of the common ice plant homologs of cotton fiber and *Arabidopsis* trichome development-related genes.

Orthologs	Nucleotide acids (bp)	Homologous genes	Score	*E*-value	Plant
McMYB2	1393	R2R3 MYB factor (AtMYB5)	196	1e−58	*Arabidopsis thaliana* (NM_112200)
		MYB transcription factor (*GbMYB2*)	197	8e−62	*Gossypium barbadense* (FJ198052)
McC4HDZ	2392	*GL2*	853	0.0	*Arabidopsis thaliana* (XM_106633)
McCPC (*McZFP*)	1089	Zinc finger protein-like gene	220	4e−66	*Beta vulgaris subsp. vulgaris* (XM_010687926)
McTRY (*McEF1A*)	1817	*EF1A*-like gene	795	0.0	*Cicer arietinum* (XM_012719757)

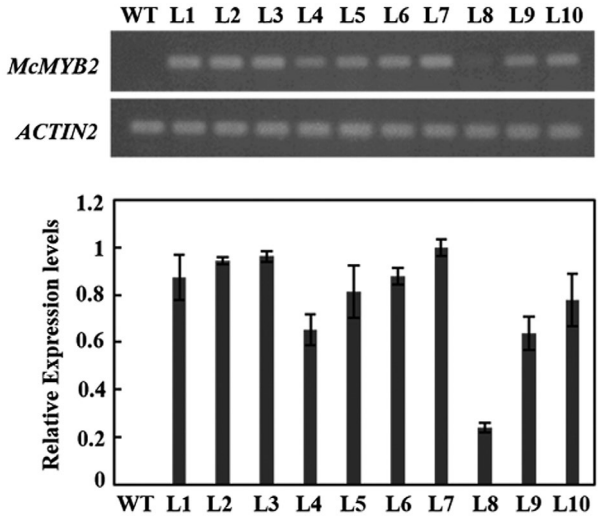

Figure 3. The transcript abundance of *McMYB2* in the *McMYB2*-transgenic *Arabidopsis*. The amplification of *McMYB2* and the endogenous control *ACTIN2* in the *McMYB2*-transgenic lines (L1, L2, L3, L4, L5, L6, L7, L8, L9, and L10), and wild type (WT) were performed using the end-point semi-quantitative RT-PCR. *McMYB2* transcript in transgenic plants were calculated from three biological replicates. The relative mRNA abundance of *McMYB2* in each line was expressed as relative values, with the value of line 7 being 1, which showed the highest mRNA level among lines tested.

Sequence analysis

The nucleotide sequences of the homologs that showed clear differences in the transcript abundance between the wild-type and the EBC-less mutant were determined (Table 3). *McMYB2* (KT223761) was 1393 bp in length, and a homology search using BLASTX at NCBI showed that the predicted amino acid sequence of *McMYB2* encodes an R2R3 MYB protein containing an R2 DNA-binding domain from residue 14 to 60 and an R3 DNA-binding domain from residue 66 to 112. Sequence alignment and phylogenetic analysis revealed that *McMYB2* is similar to *GbMYB2* (40% identity), *AtMYB5* (39% identity), and *AtMYB7* (39% identity) (Supplemental Figure 1 and Table 3). The 2392-bp full-length cDNA of *McGL2* (hereafter designated *McC4HDZ,* Genbank accession number KT223762) encodes a Class IV Homeodomain Leucine Zip (HD-Zip IV) protein that contains an HD-Zip region and a START domain, which are highly conserved in the HD-Zip III and IV protein families (Nakamura et al., 2006). The sequence alignment and phylogeny confirmed that *McC4HDZ* is closely related to *Arabidopsis GL2* (66% identity) (Supplemental Figure 2 and Table 3). The full-length 1089-bp cDNA of *McCPC* (KT366262) (hereafter designated *McZFP*) encodes a putative protein of 230 amino acids, with 53% overall sequence identity to a Zinc Finger Protein-like gene previously reported in *Beta vulgaris subsp. vulgaris* (Table 3). The 1817-bp Full-length *McTRY* (KT366263) (hereafter designated *McEF1A*) encodes a putative protein of 448 amino acids that is similar to the elongation factor 1-alpha *(EF1A)* homolog of *Cicer arietinum* (88% identity) (Table 3).

Characterization of transgenic Arabidopsis overexpressing the common ice plant R2R3 MYB transcription factor, McMYB2

The sequence and predicted functional similarity of the common ice plant *McMYB2* to *Arabidopsis AtMYB5* and *Gossypium barbadense GbMYB2* suggested that *McMYB2* might function in trichome formation in *Arabidopsis*. To test this idea, *McMYB2* was introduced into *Arabidopsis thaliana* (ecotype *Col-0*) under the *CaMV*35S promoter.

In the isolated *McMYB2*-transgenic *Arabidopsis*, the expression of *McMYB2* estimated by semi-quantitative RT-PCR were varied among the 10 lines tested, and it was the highest in line 7 (Figure 3). All *McMYB2*-transgenic *Arabidopsis* lines showed significantly increased trichome density compared to the wild type. The average number of trichome per leaf in the wild type was 48.2 ± 4.8, while it ranged from 61 ± 4.1 to 75 ± 6.1 in the 10 transgenic lines (Figure 4).

Expression of trichome development-related genes in McMYB2 transgenic Arabidopsis

To check the involvement of *McMYB2* in the transcriptional regulation of trichome development, transcript levels of trichome development-related genes in the *McMYB2* transgenic line and wild-type *Arabidopsis* plants were determined by the real-time RT-PCR (Figure 5). The transcript levels of the positive regulators (*GL1*, *MYB5*, *MYB23*, *GL3*, *EGL3* and *TTG1*) and negative regulators (*CPC* and *TRY*) of trichome development-related genes remained unchanged. However, the expression of *GL2* transcript in the *McMYB2*-transgenic *Arabidopsis* was upregulated as

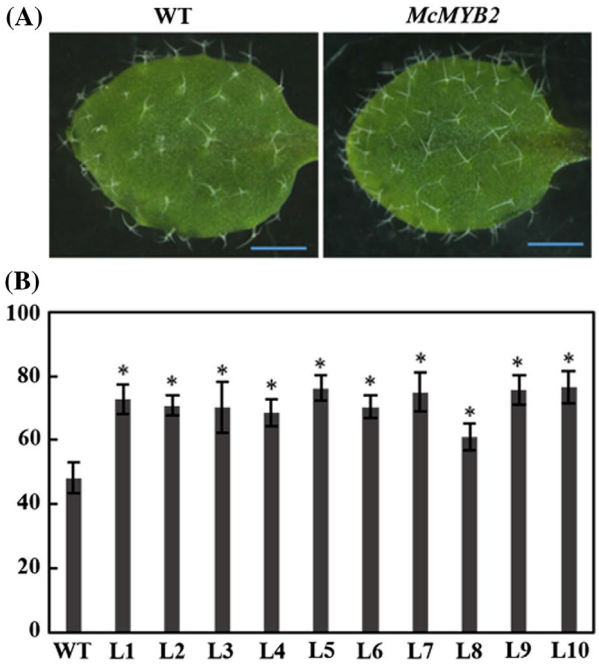

Figure 4. Trichome phenotype of 14-day-old *McMYB2*-transgenic *Arabidopsis*. (A) Trichome formation on the third leaves of 14-day-old plants of wild-type (WT) and *McMYB2*-transgenic *Arabidopsis* plants (*McMYB2*). (B) The number of trichomes on the third leaf of the wild type and the *McMYB2*-transgenic lines (L1, L2, L3, L4, L5, L6, L7, L8, L9, and L10). The number of trichomes were counted on the five leaves for each line. Asterisks represent significant differences between transgenic and wild type plants at $p < 0.05$. Bar indicates 1 mm.

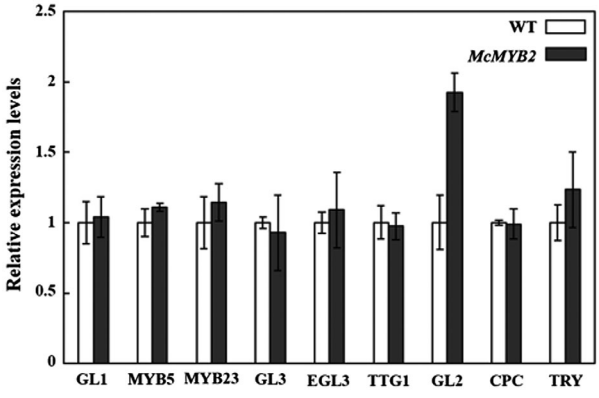

Figure 5. The mRNA abundance of trichome development-related genes in *McMYB2*-transgenic *Arabidopsis*. The relative expression of positive regulators (*GL1, MYB5, MYB23, GL3, EGL3, TTG1,* and *GL2*) and negative regulators (*CPC* and *TRY*) for trichome development in wild-type (WT) and *McMYB2*-transgenic *Arabidopsis* plants were calculated using the real-time RT-PCR. The representative transgenic line 7 was used. The experiment results were obtained from at least 3 biological replicates. Error bar indicates the standard error.

much as twofold relative to that in the wild type. Increased *GL2* transcript levels were confirmed in the *McMYB2* transgenic lines (Figure 6), indicating that increased expression of *GL2* might be attributed to the increased trichome number in the transgenic lines.

Characterization of transgenic Arabidopsis overexpressing the common ice plant HD-Zip IV protein, McC4HDZ

The sequence similarity between the *McC4HDZ* and *Arabidopsis GL2* suggested that *McC4HDZ* could function in *Arabidopsis* trichome formation. To test this idea, we introduced the *McC4HDZ* into the *gl2-1* mutant and wild-type plants of *Arabidopsis*.

The phenotype of the *gl2-1* transgenic *Arabidopsis* harboring *CaMV*35S::*McC4HDZ* was partially complemented, showing that the trichomes were developed in the *gl2-1* transgenic lines (Figure 7). To provide additional evidences that *McC4HDZ* is involved in trichome formation in *Arabidopsis*, we introduced *McC4HDZ* into *Arabidopsis thaliana* (ecotype *Col-0*). The end-point semi-quantitative PCR was conducted to estimate the transgene expression for the 10 transgenic lines. In all lines tested, transcript abundance of *McC4HDZ* was detected (Figure 8). *McC4HDZ* overexpression in *Arabidopsis* resulted in increased number of trichomes compared to the wild type. The trichome number of the wild type was 52 ± 4.2, while that of the 10 transgenic lines ranged from 65 ± 3.9 to 75 ± 5.0 (Figure 9).

Expression of trichome development-related genes in McC4HDZ transgenic Arabidopsis

To determine genes that *McC4HDZ* is involved in their transcriptional regulation in the trichome development-related genes, transcript levels of trichome development-related genes in *McC4HDZ*-transgenic and wild-type *Arabidopsis* plants were determined by real-time RT-PCR (Figure 10). Two representative *McC4DHZ*-transgenic *Arabidopsis* lines, *McC4HDZ-2* and *McC4HDZ-10*, were used in this experiment. The expression of mRNAs for the positive regulators of trichome development, *GL1*, *TTG1*, and *GL2*, was upregulated in the transgenic lines, while that of the negative regulators, *CPC* and *TRY*, remained unchanged (Figure 10).

Discussion

Several recent studies showed the contributions of EBCs to the adaptation of the common ice plant to salt stress including a mutational study on the role of EBCs in the salinity tolerance (Agarie et al., 2007), a proteomic analysis to identify the proteins expressed in response to salt stress (Cosentino et al., 2013), and a transcriptome analysis to detect the cell-type specific responses to salinity (Oh et al., 2015). In the previous study, we isolated WM28, a putative jasmonate-induced gene, an EBC development-related gene using a cDNA based-SSH and we showed

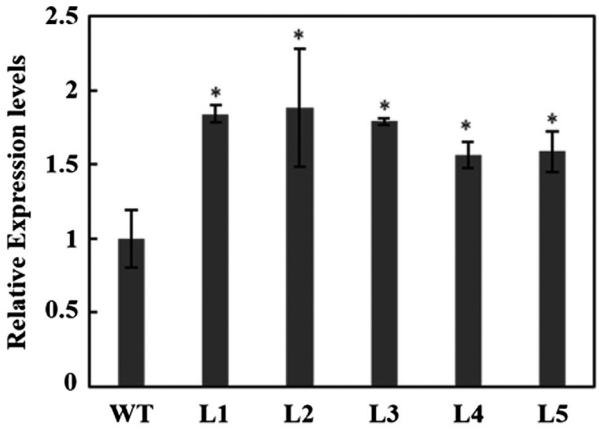

Figure 6. The mRNA abundance of *GL2* in *McMYB2*-transgenic *Arabidopsis*. The transcript levels of *GL2* in wild-type *Arabidopsis* (WT), and five *McMYB2*-transgenic lines (L1, L2, L3, L4, and L5) were calculated using the real-time RT-PCR. The data were obtained from three biological replicates. Asterisks represent significant differences between transgenic and wild type plants at $p < 0.05$. Error bar indicates the standard error.

that WM28 positively regulated trichome in *Arabidopsis* by increase of the expression of genes for trichome development-acting complex, *GL1* and *GL3* (Roeurn et al., 2016). In the present study, we attempt to further elucidate the molecular genetic basis of the development of EBCs in the common ice plant through the analysis of the common ice plant homologs of the trichome and fiber-development related-genes of *Arabidopsis* and cotton. Some homologs showed different abundance between the wild type and the EBC-less mutant, and we focused on the two positive regulators, an R2R3 MYB protein, *McMYB2* and a HD-ZIP IV protein, *McC4HDZ*.

The putative common ice plant homologs of trichome development-related gene, a *GLABRA2*-like gene (*McC4HDZ*) and a fiber-related *MYB* transcription factor gene (*McMYB2)* were expressed at lower levels in the EBC-less mutant (Figures 1 and 2). In *Arabidopsis*, the positive regulator *GL2* encodes a homeodomain protein that triggers trichome formation (Rerie et al., 1994). Likewise, the MYB transcription factor *GaMYB2* from cotton has been reported to be a functional homolog of the positive regulator of trichome development, *GLABRA1* (*GL1*) in *Arabidopsis* (Wang et al., 2004). Therefore, the reduced transcript abundance of these homologous genes in the EBC-less mutant might be related to the impairment of EBC development, suggesting that *McC4HDZ* and *McMYB2* would be positively involved in the development of EBCs in the common ice plant. Moreover, sequence homology comparisons and phylogenetic analysis revealed the *McMYB2* was similar to *AtMYB5* of *Arabidopsis* and *GbMYB2* of cotton, which have been reported to be involved in trichome formation in *Arabidopsis* (Huang et al., 2013; Li et al., 2009). *McC4HDZ* also shared 66% nucleotide sequence identity with *Arabidopsis GL2* (Table 3). These results suggest that *McMYB2* and *McC4HDZ* may serve as positive regulators of EBC development in the common ice plant.

McMYB2 is a R2R3 MYB factor, and the R2R3 MYBs have been suggested to play important roles in trichome development in *Arabidopsis* (Serna & Martin, 2006). The homologous proteins from cotton can also function in the trichome formation in *Arabidopsis* (Huang et al., 2013; Serna & Martin, 2006; Wang et al., 2004). For example, Huang et al. (2013) reported that the constitutive expression of *GbMYB2*, R2R3 MYB factor of cotton whose nucleotide sequences were similar to *AtMYB5* of *Arabidopsis*, resulted in increased number of trichomes in *Arabidopsis*. To confirm the function of the R2R3 MYB protein, the common ice plant R2R3 MYB factor, *McMYB2* was introduced into *Arabidopsis thaliana*. The increased expression of *McMYB2* resulted in the increased number of trichomes in the transgenic *Arabidopsis* (Figure 4). In *Arabidopsis*, it has been suggested that R2R3 MYBs (*GL1*, *MYB5*, and *MYB23*) interact with a bHLH protein (*GL3* and

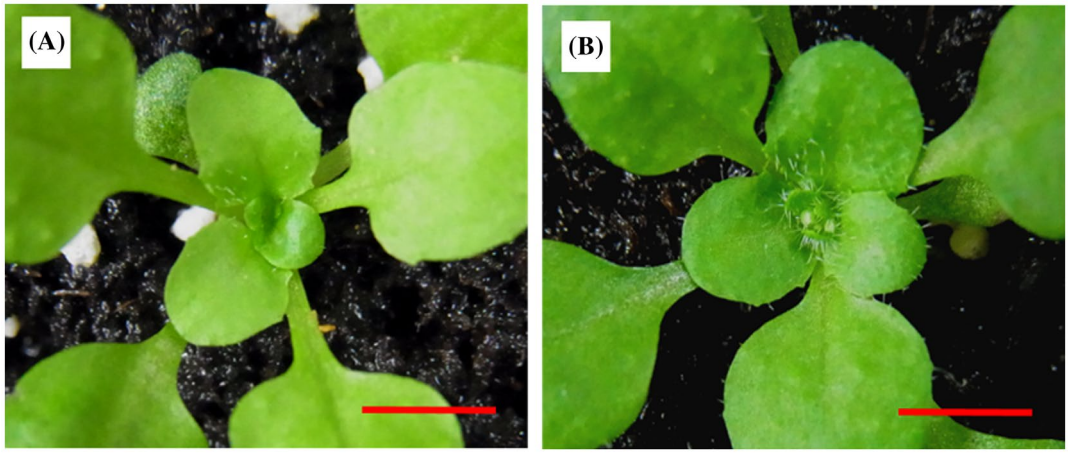

Figure 7. Trichome phenotype of the *gl2-1* transgenic plant harboring *CaMV*35S::*McC4HDZ*. (A) the *gl2-1* mutant. (B) the *gl2-1* transgenic *Arabidopsis* harboring *CaMV*35S::*McC4HDZ*. The photographs were taken from 20-day-old plant. Bar indicated 5 mm.

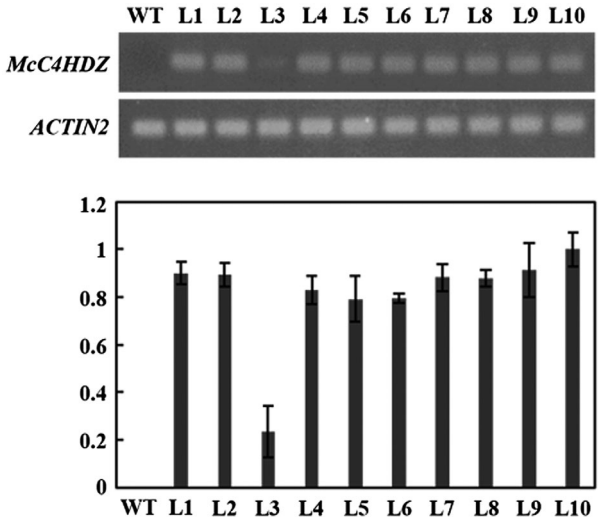

Figure 8. The transcript abundance of *McC4HDZ* in the *McC4HDZ*-transgenic *Arabidopsis*. The amplification of *McC4HDZ* and the endogenous control *ACTIN2* in the *McC4HDZ*-transgenic lines (L1, L2, L3, L4, L5, L6, L7, L8, L9, and L10), and wild type (WT) was performed using the end-point semi-quantitative RT-PCR. The transcripts of *McC4HDZ* in the transgenic plants were calculated from three biological replicates. The relative mRNA abundance of *McC4HDZ* in each line was expressed as relative values, with the value of line 10 being 1, which showed the highest mRNA level among all lines tested.

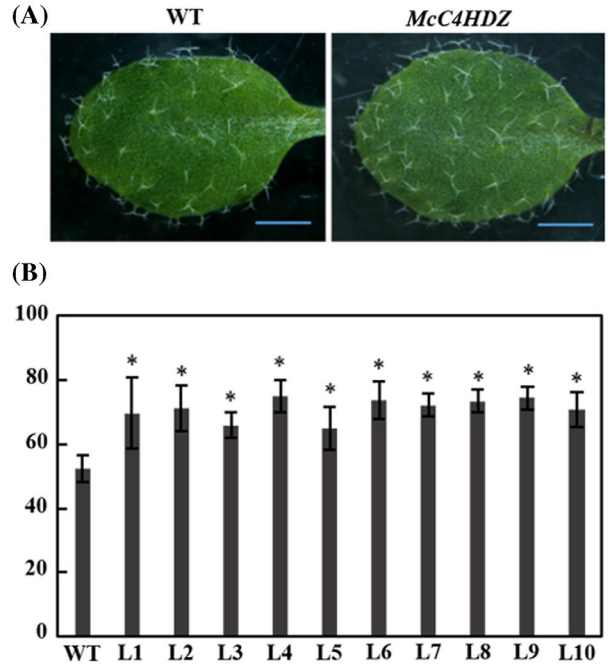

Figure 9. Trichome phenotype of *McC4HDZ*-transgenic *Arabidopsis*. (A) Photograph of wild-type (WT) and *McC4HDZ*-transgenic *Arabidopsis* showing trichome formation on the third leaf of 14-day-old plants of wild-type (WT) and *McC4HDZ*-transgenic *Arabidopsis* plants. (B) The number of trichomes on the third leaf of the wild type and the *McC4HDZ*-transgenic lines (L1, L2, L3, L4, L5, L6, L7, L8, L9, and L10). The number of trichome on the five leaves of 14-day-old plants were counted. Asterisks represent significant differences between transgenic and wild type plants at $p < 0.05$.

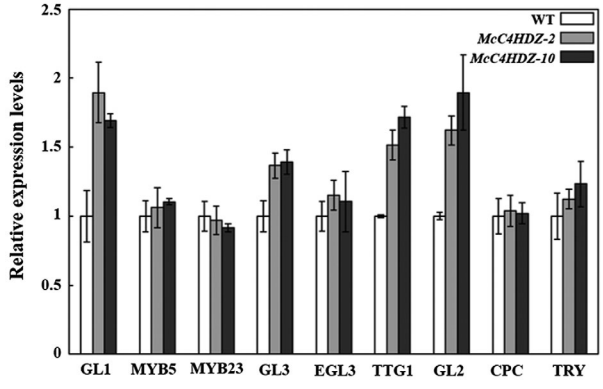

Figure 10. The mRNA abundance of trichome development-related genes in the *McC4HDZ*-transgenic *Arabidopsis*. The relative expression of positive regulators (*GL1, MYB5, MYB23, GL3, EGL3, TTG1,* and *GL2*) and negative regulators (*CPC* and *TRY*) for trichome development in wild-type (WT) and *McMYB2*-transgenic *Arabidopsis* plants were calculated using the real-time RT-PCR. Two representative transgenic line, *McC4HDZ*-2 (line 2) and *McC4HDZ*-10 (line 10) were used. The data were obtained from at least 3 biological replicates. Error bar indicates the standard error.

EGL3) and a WD-40 protein (*TTG1*) to form a complex that induces the expression of *GL2* and trichome formation (Grebe, 2012; Payne et al., 2000; Rerie et al., 1994). The expression of *GL2* transcripts in *McMYB2*-transgenic plants was upregulated compared to the wild type. These results suggested *McMYB2* could interact with a GL3/EGL3-TTG1 complex, which regulates trichome formation via *GL2* (Figure 11).

GL2, a member of the class IV HD-Zip proteins, was previously reported as a key regulator of trichome development in *Arabidopsis* (Rerie et al., 1994). The expression of the *McC4HDZ* of the common ice plant in the *gl2* mutant partially complemented and restored the *gl2* phenotype (Figure 7). This result indicates that the role of *McC4HDZ* could be similar to that of *GL2*, but not the same. Because the expression of *GL2* was induced by the activation complex GL1-GL3-TTG1 in *Arabidopsis*, but the expression of endogenous *GL1* and *TTG1* was upregulated in the transgenic *Arabidopsis* harboring *McC4HDZ* (Figure 10), indicating that the *McC4HDZ* functioned in the upstream in the cascade of the transcriptional regulation in the trichome development, which induced *GL2* expression. Recently, several reports indicated the existence of a transcriptional factor other than *GL2* that is involved in the trichome development in *Arabidopsis*. For example, the *Arabidopsis HDG11*, a gene encoding a HD-Zip IV protein, recovered trichome formation in the *gl2* mutant via a proposed unknown transcriptional mechanism or activation of *MYB23* (Khosla et al., 2014). Ishida et al. (2007), reported that the *TRANSPARENT TESTA GLABRA2* (*TTG2*),

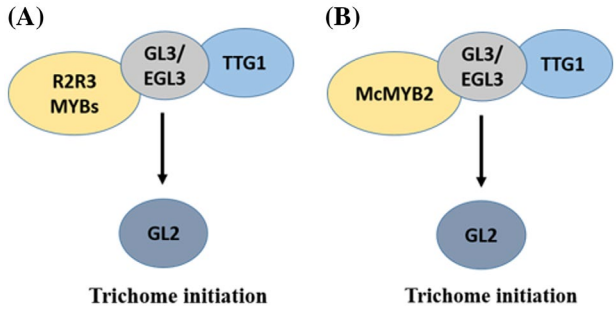

Figure 11. The proposed model for the involvement of *McMYB2* in trichome formation in *Arabidopsis*. (A). A proposed model of trichome initiation in *Arabidopsis* leaf in the previous study (Grebe, 2012). (B) The proposed role of *McMYB2* involved in the trichome initiation of *Arabidopsis* leaf in the present study.

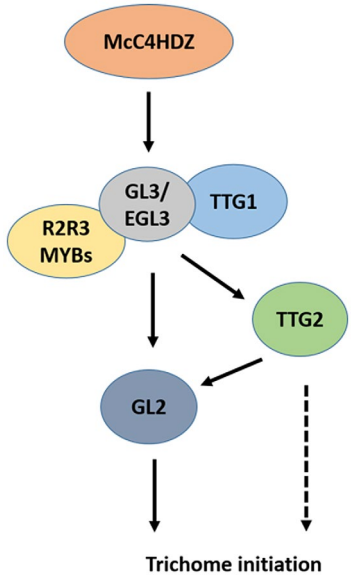

Figure 12. The proposed model for the involvement of *McC4HDZ* in trichome initiation.

a WRKY transcription factor was activated by the GL1-GL3-TTG1 complex and acted as positive regulator in the trichome formation. This gene induced trichome formation directly not through *GL2* in *Arabidopsis*. The homology of predicted amino acid sequences of the common ice plant McC4HDZ and TTG2 were 4%, indicating that *McC4HDZ* is not homologous gene of *TTG2*. Therefore, the common ice plant HD-Zip IV protein might complement of the *gl2* mutant phenotype through the enhanced *TTG2* expression or unknown genes other than *GL2* (Figure 12).

Taken together, our results show that the common ice plant homologs of fiber and trichome development-related genes, *McMYB2* and *McC4HDZ* could function as the EBC development. Our study showed that EBC development in the common ice plant shares molecular mechanisms at least partially similar to that of the trichome development in *Arabidopsis*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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