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

**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
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). GGLABRA3 (GL3), GGLABRA1 (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-repete 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 GalMYB2, 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 acredyltransferase (Ghacy), a Fiddlehead homolog (GhFDH), two tubulin components (GhtUA and GhtUB), the fiber protein E6, a cellulose synthase catalytic subunit GhesA-5, and a 24-sterol-C-methyltransferase GhsM (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 (Koornneef et al., 1982) were obtained from the Arabidopsis Biological Resource Center and were cultured as described previously (Roeurn et al., 2016). The seeds were surface 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 (OD260) 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 △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
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).

| Cotton developmental stage | Cotton genes | Accession number | Common ice plant orthologs | Homology (%) |
|-----------------------------|--------------|------------------|---------------------------|--------------|
| Fiber initiation            | GaMYB2       | aY641900         | BE034349                  | 60           |
| Fiber initiation and elongation | GaRDL1    | aY626160         | BG269414                  | 66           |
|                             | GhACY        | AF191025         | BE035266                  | 69           |
|                             | GhFDH        | AF512539         | TC10060                   | 73           |
|                             | GHTUA6       | aY207316         | BE035618                  | 67           |
|                             | GHTUB1       | aY65925          | DY032429                  | 81           |
|                             | GhCesA-5     | aY305723         | TC9344                    | 80           |
|                             | GhACT        | aY729533         | TC8112                    | 72           |
|                             | GhSuSy       | dQ122189         | TC9620                    | 73           |
| Fiber elongation            |              |                  |                           |              |
|                             | GhSUT1       | dQ174250         | TC8096                    | 86           |
|                             | GhEXP1       | aY072824         | BE035905                  | 62           |
|                             | GhMAPK       | aY072822         | TC8661                    | 68           |
|                             | GhRac1       | BM356394         | TC7912                    | 82           |
|                             | GhACT1       | BM356393         | TC10566                   | 77           |
|                             | GhPFN1       | BM356396         | BE034816                  | 73           |
|                             | GhCER6       | BM356395         | TC8550                    | 84           |
|                             | GhEF1A4      | 8U7358           | TC8367                    | 74           |

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

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

| Trichome development stage | Arabidopsis genes | Accession number | Common ice plant orthologs | Homology (%) |
|---------------------------|------------------|------------------|---------------------------|-------------|
| Pattern formation         | TTG1             | NM_18073         | TCS440                    | 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        | TC9492                    | 59          |
|                           | CRK              | NM_11160         | TC5320                    | 71          |

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

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
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) |
| Mc4HDZ    | 2392                 | GL2              | 853   | 0.0     | Arabidopsis thaliana (XM_106633) |
| McPC (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) |

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 CaMV35S 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).
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 CaMV35S::*McC4HDZ* was partially complemented, showing that the trichomes were developed in the *gl2-1* transgenic lines (Figure 7). To provide additional evidence 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 $52 \pm 4.2$, while that of the 10 transgenic lines ranged from $65 \pm 3.9$ to $75 \pm 5.0$ (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 using real-time RT-PCR (Figure 10). Two representative *McC4DHZ*-transgenic *Arabidopsis* lines, *McC4DHZ-2* and *McC4DHZ-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
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).

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.

Figure 7. Trichome phenotype of the gl2-1 transgenic plant harboring CaMV35S::McC4HDZ. (A) the gl2-1 mutant. (B) the gl2-1 transgenic Arabidopsis harboring CaMV35S::McC4HDZ. The photographs were taken from 20-day-old plant. Bar indicated 5 mm.
a proposed unknown transcriptional mechanism or activation of MYB23 (Khosla et al., 2014). Ishida et al. (2007), reported that the TRANSPARENT TESTA GLABRA2 (TTG2), 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 Mc4HDZ 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 Mc4HDZ 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 Mc4HDZ (Figure 10), indicating that the Mc4HDZ 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

Figure 8. The transcript abundance of Mc4HDZ in the Mc4HDZ-transgenic Arabidopsis. The amplification of Mc4HDZ and the endogenous control ACTIN2 in the Mc4HDZ-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 Mc4HDZ in the transgenic plants were calculated from three biological replicates. The relative mRNA abundance of Mc4HDZ 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 Mc4HDZ-transgenic Arabidopsis. (A) Photograph of wild-type (WT) and Mc4HDZ-transgenic Arabidopsis showing trichome formation on the third leaf of 14-day-old plants of wild-type (WT) and Mc4HDZ-transgenic Arabidopsis plants. (B) The number of trichomes on the third leaf of the wild type and the Mc4HDZ-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 Mc4HDZ-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, Mc4HDZ-2 (line 2) and Mc4HDZ-10 (line 10) were used. The data were obtained from at least 3 biological replicates. Error bar indicates the standard error.
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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.
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