Suppression subtractive hybridization library construction and identification of epidermal bladder cell related genes in the common ice plant, *Mesembryanthemum crystallinum* L.

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ABSTRACT

Mesembryanthemum crystallinum L., a halophytic species, displays modified trichomes, epidermal bladder cells (EBC), on the surfaces of its aerial organs. EBCs serve to sequester excessive salt from underlying metabolically active tissues. To elucidate the molecular determinants governing EBC development in the common ice plant, we constructed a cDNA-based suppression subtractive hybridization library and identified genes differentially expressed between the wild-type and the EBC-less mutant. After hybridization, 38 clones were obtained. Among them, 24 clones had homology with plant genes of known functions, whose roles might not be directly related to EBC-morphology, while 14 clones were homologous to genes of unknown functions. After confirmation by northern blot analysis, 12 out of 14 clones of unknown functions were chosen for semi-quantitative RT-PCR analysis, and the results revealed that three clones designated as MW3, MW21, and MW31 preferentially expressed in the EBC-less mutant, whereas the other two designated as WM10 and WM28 preferentially expressed in the wild type. Among these genes, the expression of a putative jasmonate-induced gene, designated as WM28 was completely suppressed in the EBC-mutant. In addition, the deletion of C-box cis-acting element was found in the promoter region of WM28 in the EBC-less mutant. Overexpression of WM28 in *Arabidopsis* resulted in increased trichome number due to the upregulation of key trichome-related genes *GLABRA*1 (*GL*1), and *GLABRA*3 (*GL*3). These results demonstrate that WM28 can be an important factor responsible for EBC formation, and also suggest the similarity of developmental mechanism between trichome in *Arabidopsis* and EBC in common ice plant.

Abbreviations: EBC: epidermal bladder cell; CAM: crassulacean acid metabolism; CaMV35S: cauliflower mosaic virus; RT-PCR: reverse transcription-PCR; qPCR: quantitative PCR; RACE: rapid amplification of cDNA ends; SSH: suppression subtractive hybridization

Introduction

Salinity stress imposes two major physiological stresses on plants: osmotic stress and ionic injury. Osmotic stress is related to the adverse effect of excess salt on water status of a plant. Increased soil salt concentrations around a plant's roots leads to the disruption of its water uptake, which inhibits cell expansion (Munns & Tester, 2008), results in the closure of stomata, and consequently reduces the rate of photosynthesis (Chaves et al., 2002). High external ionic concentrations disrupt ion homeostasis, leading to the excessive accumulation of toxic ions such as Na^+ and Cl^- (Nui et al., 1995), and have adverse effects on cell membrane integrity, enzyme activities, nutrient uptake, and photosynthesis (Tester & Davenport, 2003).

Halophytes are defined as plants that naturally inhabit saline environments and that tolerate salt concentrations of 200 mM or more, concentrations that would kill approximately 99% of other species. Halophytes have developed numerous strategies for adaptation to growth under high salinity concentrations, including osmotic adjustment through compartmentation of toxic and excess ions into epidermal bladder cells (EBCs), accumulation of compatible solutes, succulence, and secretion via salt-accumulating organs, such as salt glands (Flowers & Colmer, 2008; Shabala, 2013). EBCs or salt glands are found in about 50% of halophytes (Flowers & Colmer, 2008). About 10 times larger in diameter and capable of sequestering 1000-fold more salt than typical epidermal cells, the functions of EBCs

likely include the sequestration of excessive salts away from the more metabolically important mesophyll cells (Barkla et al., 2016; Shabala, 2013). In addition to sodium sequestration, EBCs likely serve as water reservoirs or as a secondary epidermis to reduce leaf water evaporation and prevent excessive UV damages (Adams et al., 1998).

Mesembryanthemum crystallinum L., the common ice plant, a facultative halophytic Crassulacean acid metabolism (CAM) plant native to the Namibian desert in southern Africa, exhibits tolerance to drought and to high salinity concentrations equivalent to that found in seawater (ca.500 mM). EBCs on the leaf and shoot surfaces of the ice plant are a modified trichome, characterized by a large central fluid-filled vacuole of 500 μm in diameter (Adams et al., 1998). Our previous study on the growth performance of wild-type and EBC-less mutant ice plants showed that under 400 mM salt stress condition, the leaf water content of wild type was maintained at relatively high levels, and attained a dry weight that was almost 2-fold greater compared to that of the EBC-less mutant. Moreover, the aerial tissues of wild-type plants had a Na^+ and Cl^- content that was about 1.5-fold higher than the EBC-less mutant. The Na^+ and Cl^- ion contents were partitioned into the EBCs of the wild type, resulting in the lower concentration of these toxic ions in the underlying photosynthetically active tissues (Agarie et al., 2007). The findings indicated that EBCs contributed to salt-tolerance by serving as a water reservoir and as a reservoir for salt sequestration, thus maintaining ion homeostasis in the common ice plant.

The molecular mechanisms controlling EBC development in the common ice plant have not yet been fully understood. The EBC has been thought to be a modified trichome. We have elucidated the mRNA abundance for orthologues of trichome development-related genes of *Arabidopsis* in the common ice plant and some of them showed different expression patterns between the wild type and EBC-mutant, indicating that the common ice plant has mechanism in common with *Arabidopsis* that underlies trichome formation. Here, we constructed a cDNA-based suppression subtractive hybridization (SSH) library from which to isolate genes encoding proteins with functions related to EBC-development in the common ice plant. The SSH library has been used to isolate and characterize genes that are preferentially expressed in one of two samples being compared (Diatchenko et al., 1996). Compared to microarray analysis, the SSH method is relatively inexpensive and can be performed in the absence of sequence information. Moreover, this approach is a powerful way to identify novel genes and genes with low abundance transcript (Huang et al., 2007). Here, the molecular genetic determinants of EBC development were elucidated by analyzing the expression of 12 clones derived from a cDNA-based SSH screening experiment comparing wild-type and EBC-less mutant plants. Moreover, we have overexpressed the cDNA corresponding to WM28, a differentially expressed transcript that was undetectable in the EBC mutant. Transgenic *Arabidopsis* plants overexpressing WM28 under the control of a strong constitutive promoter showed a greater number of trichomes than control lines suggesting a role for this cDNA in controlling EBC development in the common ice plant.

Materials and methods

Plant materials

Wild-type common or crystalline ice plant (*M. crystallinum* L.), and an EBC-less mutant (which has undergone seven generations of self-pollination) following fast-neutron irradiation of seeds (Agarie et al., 2007) were used for the present study. Seeds from wild-type plants and the EBC-less mutant were surface-sterilized in 2% sodium hypochlorite solution. The seeds were sown on germination medium (pH 5.7) containing 4.3 g L^{-1} Murashige and Skoog (MS) basal salt, 100 mg L^{-1} inositol, 10 mg L^{-1} thiamine-HCl, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine HC1, and 30 g L^{-1} sucrose, and 7 g L^{-1} type A agar (Sigma, St Louis MO, USA). One-week-old seedlings were transferred into 1 L expanded polystyrene foam cups, irrigated daily with a 0.5 × Hoagland's solution No. 2 (Hoagland & Arnon, 1983), and grown in a growth chamber on a 12-h (26 °C) light/ 12 h dark (18) cycle with a light intensity of 300 μmols m^{-2} s^{-1} with 50% relative humidity.

Arabidopsis thaliana (ecotype *Col*-0) seeds were surfaced sterilized and sown on half-strength MS basal medium with Gamborg's vitamins, 10 g L^{-1} sucrose, and 8 g L^{-1} type A agar. Seeded plates were stratified at 4 °C for 2–3 days to synchronize the germination. One-week-old seedlings were transplanted to the soil, irrigated twice a week, and grown at 22 °C on a 16-h (light)/ 8 h (dark) cycle with light intensity of ca. 100–120 μmols m^{-2} s^{-1} for plant transformation. To quantify the trichome number, the seed plates were incubated at 22 °C under constant light with a light intensity of ca. 100–120 μmols m^{-2} s^{-1} for 2 weeks. For each transgenic line, at least five individual third leaves of 14-day-old plants were used for trichome number quantification.

SSH cDNA library construction and screening

Total RNAs were isolated from leaf of 8-week-old plant for the wild type and the EBC-mutant, using modified acid guanidinium thiocyanate phenol chloroform method, as described by Chomczynski and Sacchi (1987). Total RNA concentrations were measured at 260 nm (OD$_{260}$) using spectrophotometer (GeneQuant 1300 RNA/DNA calculator, GE Healthcare Life Sciences, Piscataway, NJ).

The double-stranded cDNAs were synthesized from total RNA using SMART™ PCR cDNA synthesis kit from Clontech (Clontech laboratories, Inc., Mountain View, CA). The SSH experiment was performed using PCR-select™ cDNA Subtraction Kit (Clontech laboratories, Inc.) according to the manufacturer's instructions. For the forward subtraction cDNA library, cDNA obtained from the wild type was used as tester and the EBC-mutant-derived cDNA was used as driver. In the reverse subtraction, the tester and driver populations were interchanged. Therefore, the clones that were expected to be highly expressed in the EBC less-mutant were isolated from the first library, whereas the clones that were expected to be highly expressed in the wild type were identified from the second library. The SSH-libraries enriched for differentially expressed clones were cloned into pGEM-T Easy Vector (Promega, Inc., Madison, WI). The vector was then introduced in *Escherichia coli* strain JM109 with the heat-shock procedure as described by the vendor. The plasmids were extracted using the alkaline lysis (Birnboin & Doly, 1979) and sequenced by ABI PRISM 310 Genetic analyzer with reactions prepared using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Life Technologies, Inc., Grand Island, NY) according to the manufacturer`s instructions.

Semi-quantitative and real-time RT-PCR

To examine the steady-state mRNA abundance of putative clones for EBC development in both the wild-type plants and the EBC mutant, total RNA were extracted from leaves and stems (on which EBC are present) of 8- and 15-week-old plants. RT-PCR was conducted using cDNA strands synthesized from total RNA and primers specific for each gene (Supplemental Table 1). PCR was performed as follows: denaturation of 2 min at 94 °C, followed by 18–40 cycles of 94 °C for 30 s, primer-specific annealing temperature for 45 s, 72 °C for 80 s, followed by final elongation of 72 °C for 10 min. The internal control ubiquitin (*Ubq*) gene (*Accession*, TC7894) reactions were performed as described above with 18 cycles using an annealing temperature of 56 °C. The amplified products were electrophoresed on 1.2% agarose gels and stained in ethidium bromide solution to visualize the DNA amplification products with UV light.

To quantify the WM28 transgene expression levels in transgenic plants, semi-quantitative PCR was performed using the primers of WM28_*F*1 and WM28_*R*1 (Supplemental Table 2) as follows: denaturation of 2 min at 94 °C, followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, followed by final elongation of 72 °C for 5 min. The endogenous control *ACTIN*2 (AB026654) was used as internal control. The relative transgene expression was calculated from three biological replicates.

Real-time PCR was used to analyze the relative mRNA transcript abundance of endogenous trichome-related genes in the *Arabidopsis* transformants. Real-time PCR were performed using the StepOne System (Applied Biosystems) with SYBR GreenER™ (Invitrogen, Van Allen Way Carlsbad, CA). PCR amplification employed at 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for, 15 s and 60 °C for 1 min, and a dissociation stage of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15s) for *GL*1 (At3g27920), *GL*3 (At5g41315), *EGL*3 (At1g63650), *TTG*1 (At5g24502), *GL*2 (At1g79840), *CPC* (At2g46410), *TRY* (At5g53200), and *ACTIN*2 as recommended by the manufacturer. The relative expression level of *GL1, GL3, EGL3, TTG1, GL2, CPC,* and *TRY* transcript levels were calculated by the ΔΔCT method (Livak & Schmittgen, 2001) after being normalized to the endogenous control *ACTIN2*. Relative transcript abundance was calculated from three biological replicates. The primers for *GL1*, (*GL1_F* and *GL1_R*); for *GL3*, (*GL3_F* and *GL3_R*); for *EGL3*, (*EGL3_F* and *EGL3_R*); for *TTG1*, (*TTG1_F* and *TTG1_R*); for *GL2*, (*GL2_F* and *GL2_R*); for *CPC*, (*CPC_F* and *CPC_R*); for *TRY*, (*TRY_F* and *TRY_R*); and for *ACTIN2*, (*ACT2_F* and *ACT2_F*) (Sun et al., 2015; Tominaga-Wada et al., 2013) are shown in Supplemental Table 2.

Rapid amplification of cDNA ends

The 3′ ends of cDNAs were amplified with 3′ Rapid amplification of cDNA ends (RACE) using the SMART™ RACE cDNA amplification (Clontech laboratories, Inc.) according to the manufacturer's instructions using specific primers for each of the candidate putative orthologues for ice plant (Supplemental Table 3). PCR amplifications were performed using 35 cycles of 45 s at 94 °C, 45 s at 61 °C, and 120 s at 72 °C, followed by final extension at 72 °C for 10 min. The 5′ cDNA ends were amplified with 5′ RACE system from CapFishing™ Full-length cDNA kit (SmartBio Inc., Seoul, Korea) following the manufacturer's instructions using specific primers for each from the putative orthologues from common ice plant (Supplemental Table 3). These PCR amplifications were performed using 35 cycles of 40 s at 94 °C, 40 s at 63 °C, and 90 s at 72 °C, followed by final extension at 72 °C for 10 min. To fill the gap in the assembled cDNA sequences, primer walking was carried out and the PCR conditions were performed as follows: denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, followed by 45 s at the annealing temperature indicated by nucleotide composition of the primers, 72 °C for 80 s, followed by final extension of 72 °C for 10 min.

Promoter isolation

Genomic DNA was extracted from leaf tissues of wild-type and the EBC-less mutant using cetyl-trimethyl ammonium bromide protocol (Allen et al., 2006). Based on the cDNA sequence of WM28 (Supplemental Table 4 for gene specific

primer sequence), 5' flanking fragments upstream of the coding region were isolated from the wild type and the EBC mutant using Fusion Primer and Nested Integrated PCR (FPNI-PCR) as described by Wang et al. (2011). To search for their putative regulatory elements and binding motifs, the isolated promoters were subject to *NSITE-PL* search analysis (Solovyev et al., 2010) using the Softberry website (http://www.softberry.com/berry.phtml).

Expression vector construction and introduction into plants

The cDNA fragment of a SSH clone WM28 from the ice plant was amplified by PCR, and then cloned into a commercial binary vector pRI-101-AN (Takara Bio Inc., Shiga, Japan) at the *Bam*HI site under the cauliflower mosaic virus (*CaMV35S*) promoter using the In-Fusion® HD Cloning Kit (Clontech laboratories, Inc.).

The resultant construct was then introduced into *Agrobacterium tumefaciens* strain GV3101 using the Freeze/thaw method (Kan et al., 2006). Plant transformation was performed using a floral dip method as described by Clough and Bent (1998), and then screened on MS agar medium supplemented with Kanamycin 50 mg L^{-1}. At least 10 transgenic lines were isolated and the presence of transgene was confirmed by PCR using neomycin phosphotransferase (*NPTII*), a selectable marker for kanamycin resistant gene. The primers for WM28, (WM28-*inf_F* and WM28-*inf_R*); and *NPT II*, (*NPT II_F* and *NPT II_R*) are shown Supplemental Table 5.

Results

Characterization of the subtracted cDNA library

Subtractive cDNA libraries were constructed from wild-type and the EBC-less mutant plants using cDNA-based SSH PCR. After subtraction, the remaining cDNA was cloned into pGEM-T Easy Vector, and a total of 60 clones from the SSH library were obtained. PCR was then used to identify the clones containing inserts. As a result, the remaining 38 clones, with insertions ranging from 200 to 600 bp, were obtained. Of all 38 clones obtained, 21 clones (designated as MW) were expected to be highly expressed in the mutant and the other 17 clones (designated as WM) were expected to be highly expressed in the wild type. Homologs to the total 38 SSH-derived cDNA clones were identified from the expressed sequenced tag DFCI *M. crystallinum* gene index (ftp://occams.dfci.harvard.edu/pub/bio/tgi/data/Mesembryanthemum_crystallinum/). The search results revealed that of the 38, 24 clones had homology with plant genes of known function and 14 clones were homologous to gene of unknown functions (Tables 1 and 2). The known clones encoded proteins with functions related to photosynthesis, including genes encoding Rubisco small/large subunits, phosphonomutase, and pyruvate orthophosphate dikinase, whose roles might not be related directly to EBC development. Therefore, 14 genes of unknown functions, whose roles might be related to EBC development in the common ice plant, were subsequently subject to northern blot analysis (data not shown). As a result, the differential mRNA of 12 genes of unknown function was confirmed and subsequently reconfirmed using RT-PCR expression analysis. These genes were categorized into two groups: genes whose transcripts were highly expressed in EBC-less mutant and genes whose transcripts were highly expressed in wild-type plants. These two groups were designated as MW and WM, respectively.

Expression of unknown function genes isolated by SSH

The MW group included five genes of unknown function, namely MW3, MW11, MW21, MW29, and MW31,

Table 1. SSH-derived genes expected to be highly expressed in the EBC-less mutant compared to the WT.

Clone	Plant	Accession number	Putative identity	*E*-value
MW1	*M. crystallinum*	TC4866	rubisco small subunit	4.90e-64
MW2	*M. crystallinum*	TC4880	chlorophyll a/b-binding protein	8.20e-74
MW3	*Gossypium hirsutum*	TC25196	unknown protein	4.70e-51
MW4	*M. crystallinum*	TC6280	homologue to rubisco large subunit	5.70e-39
MW5	*M. crystallinum*	TC5165	50S ribosomal protein L24	3.80e-69
MW9	*M. crystallinum*	TC4866	rubisco small subunit	7.50e-65
MW10	*M. crystallinum*	TC6280	homologue to rubisco large subunit	1.40e-93
MW11	*M. crystallinum*	TC6599	unknown protein	1.10e-80
MW14	*M. crystallinum*	TC6280	homologue to rubisco large subunit	3.70e-51
MW16	*M. crystallinum*	TC6335	chlorophyll a/b-binding protein	1.10e-88
MW18	*M. crystallinum*	TC6280	homologue to rubisco large subunit	2.90e-81
MW19	*M. crystallinum*	TC6280	homologue to rubisco large subunit	3.70e-95
MW20	*M. crystallinum*	TC4867	rubisco small subunit	3.20e-35
MW21	*M. crystallinum*	CA834497	unknown protein	2.20e-16
MW22	*M. crystallinum*	TC7486	unknown protein	1.10e-52
MW23	*M. crystallinum*	TC6280	homologue to rubisco large subunit	4.70e-90
MW24	*M. crystallinum*	TC4881	similar to rubisco activase	3.30e-58
MW29	*Glycine max*	TC187838	unknown protein	0.026
MW30	*M. crystallinum*	TC6315	carboxyphosphonoenolpyruvate phosphonomutase homolog	1.30e-93
MW31	*M. crystallinum*	BE577461	unknown protein	1.60e-62
MW32	*Glycine max*	TC187838	unknown protein	0.069

Note. M. crystallinum – Mesembryanthemum crystallinum.

Table 2. SSH-derived genes expected to be highly expressed in the WT compared to the EBC-less mutant.

Clones	Plant	Accession number	Putative identity	*E*-value
WM2	*M. crystallinum*	TC6280	homologue to rubisco large subunit	2.70e-84
WM3	*M. crystallinum*	TC6280	homologue to rubisco large subunit	2.40e-74
WM4	*Helianthus annuus*	NP524595	partial mRNA for peroxin6 (pex6 gene)	2.80e-50
WM5	*Vitis vinifera*	TC21076	similar to peptide deformylase	3.00e-16
WM6	*M. crystallinum*	CA835839	Unknown protein	3.10e-19
WM7	*M. crystallinum*	TC6280	homologue to rubisco large subunit	2.30e-72
WM8	*M. crystallinum*	AW266449	unknown protein	1.00e-30
WM10	*M. crystallinum*	TC5712	unknown protein	4.30e-63
WM12	*M. crystallinum*	CA837401	unknown protein	8.70e-86
WM13	*Bos taurus*	TC209408	unknown protein	3.50e-01
WM15	*M. crystallinum*	TC6280	homologue to rubisco large subunit	8.60e-53
WM16	*M. crystallinum*	TC5712	unknown protein	9.70e-62
WM17	*M. crystallinum*	TC4935	pyruvate orthophosphate dikinase	3.60e-76
WM18	*M. crystallinum*	TC5577	phosphoglyceromutase	1.30e-41
WM22	*M. crystallinum*	TC6280	homologue to rubisco large subunit	4.80e-91
WM24	*M. crystallinum*	TC6280	homologue to rubisco large subunit	7.00e-89
WM28	*M. crystallinum*	TC6305	unknown protein	1.10e-77
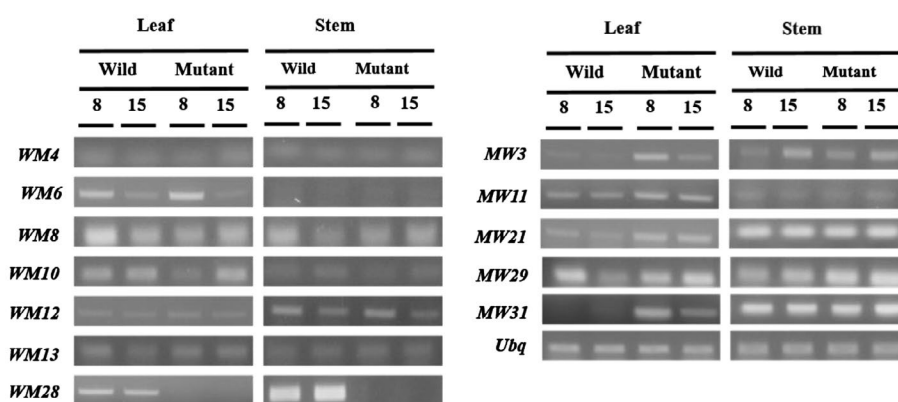
Figure 1. Transcript abundance of SSH-unknown function clones in leaves and stems of the wild type and the EBC mutant (8, 8-week-old plants, 15, 15-week-old plant).

whose transcript levels were analyzed by RT-PCR in leaf and stem of wild-type plant and the EBC-less mutant (Figure 1). No clear differences were observed in the transcript abundance patterns of MW11 and MW29 in either leaves or stems of both the wild-type plants and the EBC-less mutant. MW3 and MW21 transcripts were expressed more strongly in the leaves of the mutant, but were significantly reduced in the leaves of wild-type plants. The transcripts of MW31 were preferentially expressed in the leaves of the mutant, but absent in the wild-type plants. The WM group consisted of seven genes of unknown function, which were designated as WM4, WM6, WM8, WM10, WM12, WM13, and WM28. Significant differences in transcript abundance were found between wild-type plants and the EBC-less mutant for both WM10 and WM28 (Figure 1). The transcript abundance of WM10 was lower in leaves of the EBC-less mutant of eight weeks. The transcript abundance of WM28 was undetectable and possibly absent in both the leaves and stems of the EBC mutant.

Sequence analysis

The nucleotide sequence of the full-length cDNA of SSH-derived genes, MW3, MW21, MW31, WM10, and WM28, which were differentially expressed between the EBC mutant and the wild-type plant, were determined (Table 3). We found that the MW3 ortholog (KT223763) was 1081 bp in length and shared 89% overall nucleotide identity to the stem-specific TSJT1-like genes conserved in *Beta vulgaris subsp. vulgaris* (Table 3). Moreover, the amino acid sequence of MW3 included a conserved Gn_AT_II superfamily domain for specific DNA binding (Larsen et al., 1999) and a glutaminase domain that catalyzes an amide-nitrogen transfer from glutamine to an appropriate substrate. Full-length MW21 (KT454784) was 2074 bp and showed overall 68% sequence identity to serine/threonine and tyrosine kinase HT1-like genes conserved in *Gossypium raimondii* (Table 3). In addition, the amino acid sequence of MW21 included a conserved PKc-like superfamily DNA-binding domain (Johnson, 2009). These protein kinases

Table 3. Full-length cDNAs of SSH-derived clones and homologous genes.

Clones	Nucleotides acids (bp)	Homologous gene	Score	E value	Plant
MW3(*McTSTJ*1)	1081	Stem-specific protein TSJT1	487	6e-170	*Beta vulgaris subsp. vulgaris* (XM_010677329)
MW21(*McHT*1)	2074	Serine/threonine and tyrosine kinase HT1	688	0.0	*Gossypium raimondii* (XM_012589723)
MW31(*McNSA*2)	1118	Ribosomal biogenesis NSA2	478	4e-166	*Vitis vinifera* (XM_002266127)
WM10	856	similar to unknown protein	82	1e-15	*Beta vulgaris subsp. Vulgaris*(XM_010687500)
WM28	961	Jasmonate-induced protein	93	7e-14	*Suaeda glauca* (KP006442)

Note. The nucleotide sequences reported in this study were deposited under GenBank accession number as following: *McTSTJ*1 (KT223763), *McHT*1 (KT454784), *McNSA*2 (KT366264), WM10 (KT366266), and WM28 (KT366265).
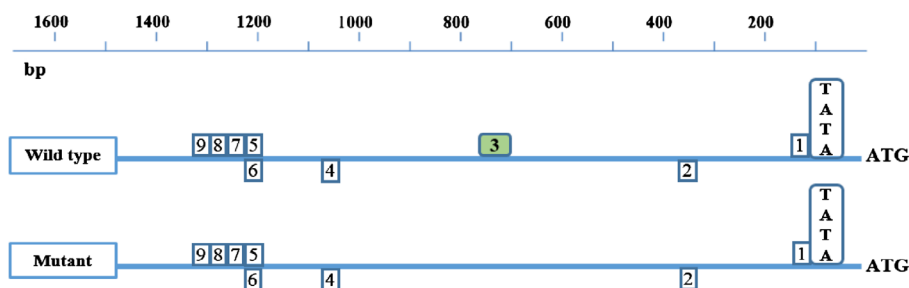

Figure 2. The WM28 promoter architecture isolated from the wild type and the EBC mutant. Nucleotides number relative to their start codon show on the top of the graph. The boxes (with number) show the position of different regulatory elements in the two promoter regions. The green box indicates the regulatory elements absent from the promoter isolated from the EBC mutant.

catalyze ATP-dependent phosphorylation of serine, threonine, and tyrosine residues on target proteins (Rudrabhatla et al., 2006). MW31 (KT366264) was 1118 bp in length and showed homology with the ribosomal protein S8e/ ribosomal biogenesis NSA2-like gene in *Vitis vinifera* (88% identity) (Table 3). The WM10 ortholog (KT366266) was 856 bp in length, and was similar to unknown proteins in *Beta vulgaris subsp. Vulgaris*. WM28 ortholog (KT366265) was 961 bp in length, contained G/AAVVY and Y/WLVAW amino acid sequences, which are highly conserved in jasmonate-induced genes reported in *Suaeda glauca, Beta vulgaris subs. vulgaris* (XM010673399), *Atriplex canescens* (P42764), *and G. raimondii* (XM012602058) (Table 3).

Isolation and characterization of promoters for WM28

As shown by the transcript expression analysis (Figure 1), the transcript abundance of WM28 was undetectable or possibly absent in both leaves and stems of the EBC-less mutant. This result suggests that the absence of WM28 transcript in the mutant might arise from changes in the cis-acting elements of the 5`-regulatory region of this gene. The sequence analysis illustrated that two promoters of 1530 and 1532 bp from the start codon were bp download isolated from wild-type plants and the EBC-less mutant, respectively. Using the program *NSITE-PL* (Solovyev et al., 2010), the putative transcription factor binding sites and

regulatory elements were identified for these two isolated promoters. We confirmed that the two promoters shared common regulatory elements, except that the promoter isolated from the mutant lacked a C-box, a cis-acting element, which might result in the observed reduction of WM transcript abundance in the EBC-less mutant (Figure 2 and Table 4).

Production and characterization of transgenic Arabidopsis expressed with a SSH-clone, WM28

Common ice plant EBC, as singled-cell, non-glandular trichomes, are thought to have arisen via molecular mechanisms similar to those underlying *Arabidopsis* trichome formation. In the present study, we have isolated from common ice plant a putative jasmonate-induced gene (WM28). The transcript abundance of WM28 was undetectable in the EBC-less mutant. This result suggests that WM28 might be involved in EBC development or trichome initiation. Thus, we have generated and characterized the *Arabidopsis* transgenic lines that overexpressed with the common ice plant EBC-related gene, WM28. The WM28 was expressed under the cauliflower mosaic virus (*CaMV35S*) promoter. The resultant construct was designated *CaMV35S::WM28* (Figure 3(A)).

We have isolated 10 transgenic lines and confirmed the presence of the transgenes using PCR for the kanamycin resistance gene (*NPT II*) (Figure 3(B)). Semi-quantitative

Table 4. Putative transcription factor binding sites and regulatory elements in the upstream genomic sequence of WM28 promoters isolated from the wild type and EBC-less mutant.

Box	Position on the strand and sequence consensus	Putative transcription factor binding sites	Position*	Presence in promoters Wild type	Mutant
1	(+) CTATAAATAC	*Triticum aestivum* /GENE: Amy2/54/RE: Inr element	−112	√	√
2	(−) CCAATGATGAGA	*Arabidopsis thaliana* /GENE: AtCRC/RE: EL1 /BF: LFY	−368	√	√
3	(+) ATGAACATAGTaaaTAA	*Lycopersicon esculentum* /GENE: PG/RE: C-box /BF: fruit nuclear protein extracts	−782	√	
4	(−) TaTAAAtTAAGTAGTTATGa	*Arabidopsis thaliana* /GENE: CPC/RE: WBSI /BF: WER	−1073	√	√
5	(+) ACCTACCAAaC	*Zea mays* /GENE: a1/RE: C1 PBS/P /BF: C1	−1234	√	√
6	(−) TGGTAGGTGAGAg	*Nicotiana tabacum* /GENE: LTR-Tto1/RE: 13 bp-box /BF: unknown nuclear factor	−1239	√	√
7	(+) TCTCACCTACCA	*Various plants* /GENE: chsA/RE: box 1 /BF: Unknown nuclear factor	−1238	√	√
8	(+) CACCTACC	*Eucalyptus gunnii* /GENE: EgCAD2/RE: MYBa /BF: MYB	−1235	√	√
9	(+) TCTCACCTACC	*Glycine max* /GENE: Synthetic oligonucleotides/RE: GmMYB92 BS3 /BF: GmMYB92	−1238	√	√

Notes. (+): On the strand; (−): on the reverse-strand; Position*: The location of cis-acting elements in the WM28 promoter isolated from the wild type.
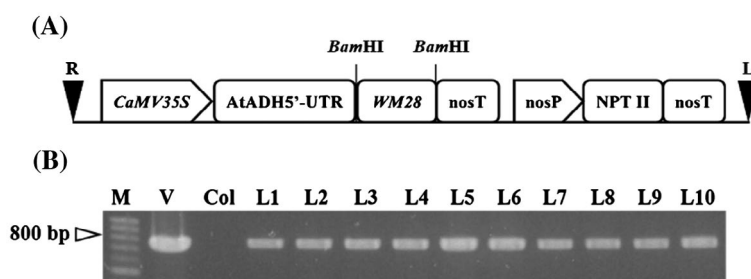

Figure 3. Production of WM28-transgenic *Arabidopsis*. (A) Binary vector *CaMV35S::*WM28 for overexpression of WM28 driven by constitutive promoter *CaMV35S*. *CaMV*35S: cauliflower mosaic virus promoter; AtADH5′-UTR: 5′ non-coding region of *Arabidopsis* Alcohol dehydrogenase gene (translational enhancer); NPTII: Selectable marker gene in plant; nosP and nosT: Promoter or terminator, respectively, for expression of selectable marker gene in plant; R: right border; L: left border. (B) PCR analysis of selectable marker gene (NPTII) in WM28 overexpressed lines. Agarose gel electrophoresis of PCR amplification was performed with primers of the *NPTII* gene (780 bp); M, DNA size marker. V, plasmid DNA. Col, nontransformed *Arabidopsis* (ecotype *Col-0*). L1–L10, transformants.

PCR was used to detect the transcript level of WM28 in transgenic lines; among all the 10 lines tested, Line #9 showed the highest WM28 transcript level (Figure 4). Overexpression transgenic *Arabidopsi*s showed a phenotype of increased trichome number compared to the wild type (Figure 5). The average number of trichome of the wild type was 55 ± 4.0, while that of the 10 transgenic lines was about 20–40% higher than that of the wild type, showing correlation with WM28 transcript abundance in transgenic lines (Figure 4).

The ice plant SSH-clone WM28 acts in the upstream for cascade of GL1 and GL3 expression in regulation of trichome initiation

The transgenic *Arabidopsis* expressed with a putative jasmonate-induced gene (WM28) from the ice plant resulted in increased trichome formation. We speculated that WM*28* might be involved in the transcriptional regulation of trichome-formation-related genes. Therefore, we further analyzed the transcript abundance changes

of trichome-related genes in the WM28 transgenic plants and the wild type using RT-qPCR. The results showed that among all trichome-related genes tested, *GLABRA1* (*GL1*), *GLABRA3* (*GL3*), and *GLABRA2* (*GL2*) showed increased mRNA abundance in the WM28 transgenic plant compared to the wild type, while the mRNA abundance of other genes *TRANSPARENT TESTA GLABRA1* (*TTG1*), *Enhancer of GLABRA3* (*EGL3*), *CAPRICE* (*CPC*), and *TRYPTCHON* (*TRY*) were remained largely unchanged (Figure 6). These results might help explain the increased trichome number in the transgenic plants.

Discussion

Mesembryanthemum crystallinum L., is a facultative CAM halophytic plant that can tolerate high salinity concentration equivalent to those found in sea water (ca. 500 mM NaCl). EBCs are found on the surfaces of the common ice plant shoots and leaves and function by storing water, sequestering salt, and maintaining ion homeostasis within underlying photosynthetically active tissues of the plant
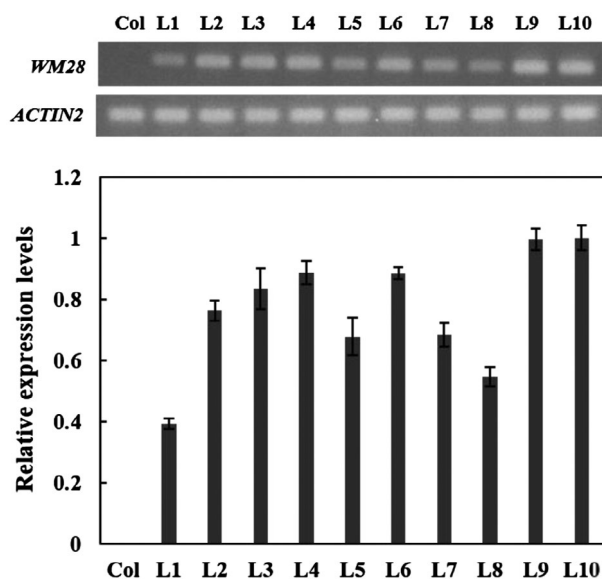
Figure 4. WM28 transcript abundance in *CaMV35S::*WM28 transgenic *Arabidopsis*. Col, wild type (ecotype *Columbia*) was used a negative control. The amplification of WM28 and the endogenous control *ACTIN2* in the ten transgenic lines and wild type (ecotype *Col-0*) were performed using semi-quantitative RT-PCR. The transcript abundance of WM28 in transgenic independents was calculated from three biological replicates. The relative mRNA abundance of WM28 in each line was plotted and compared to the mRNA in line # 9 as positive control (the highest mRNA level among all lines tested).

(Agarie et al., 2007). The molecular mechanisms governing EBC development in the common ice plant remain unknown. Here, by employing cDNA-based SSH PCR, we have identified candidate genes responsible for EBC development in the common ice plant. Moreover, the mechanisms controlling trichome number were investigated by expressing a candidate gene WM28 in *Arabidopsis*.

The transcript abundance analysis of SSH-derived clones indicated that three genes (MW3, MW21, and MW31) were preferentially expressed in the mutant, and the other two (WM10 and WM28) were preferentially expressed in the wild-type plants (Figure 1). Sequence analysis indicated that MW3 is similar to stem-specific protein TSJT1-like gene in other species (Table 3) and shared a conserved domain for specific DNA-binding protein (Gn_AT_II superfamily) with Wali7, a protein of unknown function that is induced by aluminum in wheat (Richards et al., 1994). Recently, Lei et al. (2013) reported the overexpression of the Wali7 protein resulted in a short root hair phenotype due to the decreased meristematic activity and impaired stem cell division.

Moreover, in the present study, MW3 (*McTSTJ1*) transcript abundance was increased in the EBC mutant (Figure 1), suggesting that *McTSTJ1* might be related to the impaired EBC development in the EBC mutant. Our sequence analysis confirmed that MW21 (*McHT1*) showed similarity to kinase protein families composed of
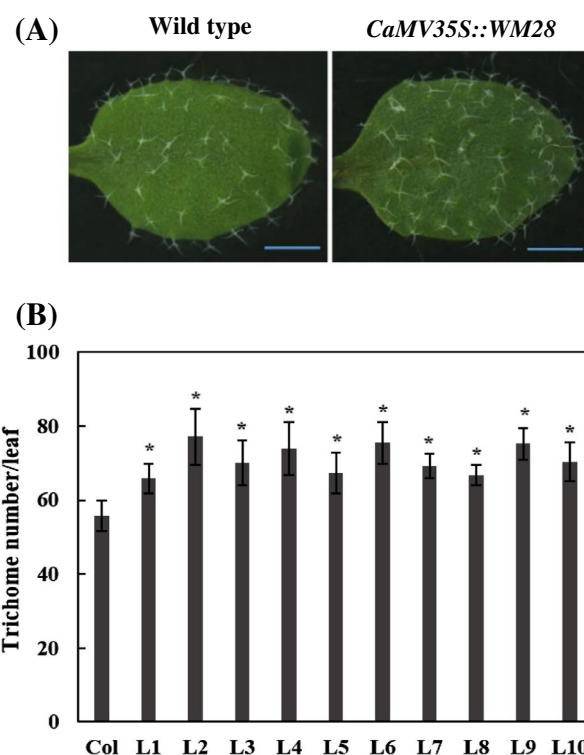

Figure 5. Trichome Phenotype of 14-day-old *CaMV35S::*WM28 transgenic *Arabidopsis*. (A) Trichome formation on the third leaves of 14-day-old plants of Col, wild type (ecotype *Col-0*) and *CaMV35S::*WM28 transgenic *Arabidopsis*. (B) Trichome number on the third leaf of transgenic lines (L1–L10). Five leaves of 14-day-old plants were counted for trichome number for each line. Asterisks represent a significant difference between transgenic and wild-type plants at $p < 0.05$. Bar indicated 1 mm.
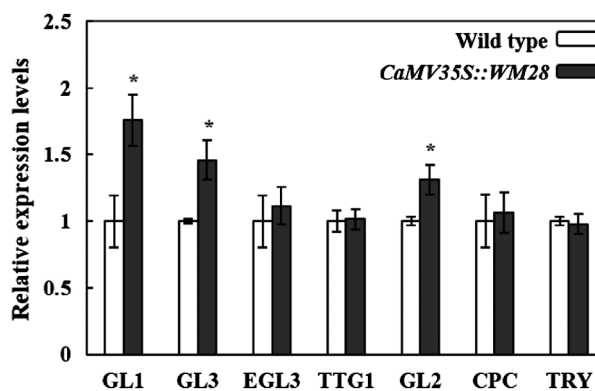

Figure 6. Relative mRNA abundance changes of trichome-related genes in *CaMV35S::*WM28 transgenic *Arabidopsis*. The relative expression of positive regulators (*GL*1, *GL*3, *EGL*3, *TTG*1, and *GL*2) and negative regulators (*CPC* and *TRY*) for trichome development was calculated using RT-qPCR. The experiment results were calculated from at least three biological replicates. Error bar indicates the standard error. Asterisks represent a significant differences between transgenic and wild-type plants at $p < 0.05$.

non-overlapping groups: the protein tyrosine kinases and the serine/threonine kinases (Rudrabhatla et al., 2006). In animal protein, the protein tyrosine phosphorylation act

as an on-off switch in numerous pathways, which play important role in regulation of cell growth, differentiation, and oncogenesis (Rudrabhatla et al., 2006). Although no studies on the function of dual tyrosine-serine/threonine kinase in trichome or fiber development plants have been conducted, several studies have examined the roles of these dual-specificity kinases in other processes in plants (Parthibane et al., 2012). The present study shows that *McHT1* was preferentially expressed in the EBC-less mutant (Figure 1); however, the exact function of this gene in cell proliferation and differentiation and the possibility of its involvement in the impaired EBC development in the mutant must await further study.

The amino acid sequence of MW31 showed homology to ribosomal protein S8e/ ribosomal biogenesis NSA2-like genes in other species (Table 3). Ribosomal biogenesis is necessary for the production of proteins required for cell proliferation and growth (Manzano et al., 2013). MW31 (*McNSA2*) transcript abundance was differentially expressed between the wild-type and the EBC-less mutant (Figure 1). Whether the differential transcript abundance of *McNSA2* is functionally related to deficits in production of others protein or the impairment of EBC development in the common ice plant remains to be tested.

WM10 is weakly similar to protein of unknown function in *Beta vulgaris subsp. vulgaris* (Table 3). Therefore, the result of transcript abundance and sequence analysis raises the possibility that WM10 encodes a novel gene product that might be responsible for EBC development in the common ice plant. The transcription analysis showed that WM28, a putative jasmonate-induced gene, was undetectable or possibly absent in both leaves and stems of the EBC-less mutant (Figure 1). The results presented here suggest that the 5` flanking regions of WM28 could transcriptionally control the observed differences in mRNA abundance between wild-type plants and the EBC-less mutant. Isolation and sequence analysis of this 5` upstream regions from the mutant revealed that it lacked a C-box element, which might explain why WM28 was not transcribed at detectable levels in the EBC mutant (Figure 2 and Table 4). This result, along with the lack of transcription from this gene, might explain the loss of EBC development in the EBC-less mutant.

Salt-bladders are characterized as modified hair and trichome along with glandular hairs, thorns, and surface glands (Adams et al., 1998; Shabala, 2013). EBCs or salt bladders of the common ice plant are also characterized as singled-cell non-glandular trichomes. EBCs might have developed from molecular mechanisms that are related to the underlying mechanisms that mediate *Arabidopsis* trichome formation. Constitutive over-expression of the common ice plant WM28 in *Arabidopsis* plants resulted in increased trichome density and increased relative mRNA

abundance of the trichome positive regulators, *GL1* and *GL3* (Figure 6). Sequence analysis showed that WM28 contained amino acid sequences common to those of jasmonate-induced genes conserved in halophyte species such as *Suaeda glauca* and *Beta vulgaris subs. vulgaris*. Recently, much evidence suggests that jasmonic acid can increase the trichome-density (Kobayashi et al., 2010; Qi et al., 2014; Traw & Bergelson, 2003), by acting upstream of the GL1-GL3/EGL3-TTG1 activator complex, regulating trichome initiation via *GL2* (Kobayashi et al., 2010). Taken together, these results suggested that the ice plant WM28 acts as transcriptional factor upstream of positive regulators of trichome complex, *GL1* and *GL3*, possibly through jasmonate signaling pathways and positively regulate trichome initiation in *Arabidopsis*, via *GL2*, part of the main machinery for trichome initiation (Rerie et al., 1994; Wang et al., 2010).

In conclusion, this study provides initial insight into the molecular mechanism underlying EBC development in the common ice plant and suggests that EBCs of the common ice plant have evolved in a manner similar to that of trichomes in *Arabidopsis*.

Disclosure statement

No conflict of interest was reported by the authors.

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