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Cloning and functional characterization of epidermis-specific promoter *MtML1* from *Medicago truncatula*

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ABSTRACT

Epidermis-specific promoters are necessary for ectopic expression of specific functional genes such as the cuticle-related genes. Previous studies indicated that both *ECERIFERUM 6* (*AtCER6*) and *MERISTEM L1 LAYER* (*ATML1*) promoters from *Arabidopsis thaliana* can drive gene expression specifically in the epidermis of shoot apical meristems (SAMs) and leaves. However, the epidermis-specific promoters from legume plants have not been reported. Here, we cloned a 5′ flanking sequence from the upstream -2150 bp to the translational start ATG codon of *MtML1* gene of legume model plant *Medicago truncatula*. PlantCARE analysis indicated that this sequence matches the characteristics of a promoter, having TATA box and CAAT box, as well as contains some conserved elements of epidermis-specific promoters like *AtCER6* and *ATML1* promoters. The β-glucuronidase (GUS) histochemical analysis showed that *MtML1* promoter can drive *GUS* gene expression in transiently transformed *Nicotiana tabacum* leaves under non-inducing condition. Furthermore, it can also control GUS expression in leaves and siliques rather than roots of the stably transformed Arabidopsis. More importantly, the leaf cross-section observations indicated that *MtML1* exclusively expressed in the epidermis of leaves. These results suggested that *MtML1* promoter performed the epidermis-specific in plant shoot. Our study establishes the foundation for driving the cuticle-related gene to express in epidermis, which may be very useful in genetic engineering of legume plants.

1. Introduction

Adverse environmental conditions such as drought, salt, heat, frost and pathogens are major limiting factors for agriculture and livestock production worldwide (Farooq et al., 2009; Li et al., 2009; Feng et al., 2014; Rouphael et al., 2015). In recent years, high-frequency and long persistence of drought seriously affected water cycling leads to arid and semi-arid area expansion (Fereres and Soriano, 2007; Durack et al., 2012). Many valuable crops are utmost needed to maintain high yield stability under stress conditions and to minimize the environmental impacts toward crop production (Zhang et al., 2018; Watson et al., 2018). Exploring stress-resistant genetic resources for crop improvement via genetic engineering is one of the most effective way to solve these problems. Promoter is 5′ flanking DNA sequence of structural gene, which activates RNA polymerase for binding the template DNA precisely. Thus, promoter could endow the specificity of transcription initiation. In the light of facts, promoter selection is an essential factor to ensure proper spatio-temporal expression patterns of the target genes. Promoters are classified into constitutive, inducible and tissue-specific, of which, some constitutive and tissue-specific promoters that were specifically expressed in vascular bundles, roots and floral organs which have been widely used for the genetic improvement of important legume forages (Annicchiarico et al., 2015). However, transcriptional control of constitutive promoters often causes undesirable phenotype, higher metabolic costs as well as lower activity compared to the tissue-specific promoters (Kasuga et al., 1999; Hsieh et al., 2002; Chen et al., 2014; Jeong and Jung, 2015). Several researches showed that seed-specific promoter *8SGα* from *Vigna radiata* has higher activity than Cauliflower Mosaic Virus (CaMV) *35S* promoter (Chen et al., 2014). The promoter deletion study indicated that oil palm (*Elaeis guineensis*) stearoyl-acyl-carrier-protein desaturase (*Des*) promoters were able to drive higher expression of downstream genes in seed and fruit than CaMV *35S* (Leong et al., 2013). Furthermore, overexpression of cuticle-related gene *CER6* and *WXP1* driven by constitutive promoter CaMV

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35S cause plant growth and development retardation and fail to enhance wax deposition (Millar et al., 1999; Zhang et al., 2005). The epidermis-specific *CER6* promoter can drive *CER6/CUT1* gene expression in leaves and stems of *A. thaliana* and *Nicotiana tobacum* respectively, which can improve their drought tolerance (Hooker et al., 2002). Therefore, tissue-specific promoters might be considered a better choice for ectopic expression of tissue-specific functional genes in cuticle-related genes.

Plant leaf, as the main site of stomatal and non-stomatal water loss, comprises epidermal cells, mesophyll cells and bundle sheath (Leegood, 2008). Of note, epidermal cells have the huge commitment to protect plants from adverse stresses. In higher plants, SAMs are divided into the epidermal and internal layers. The epidermis has a single sheet of cells derived from the L1 (the superficial layer of shoot apical meristem cell layers) of meristem (Satina et al., 1940). Lu et al. (1996) first discovered *MERISTEM LAYER 1* (*ATML1*) gene from *A. thaliana*, that encodes for homeodomain protein and reappeared only in L1 layer of meristem from the earliest stages of meristem patterning and throughout shoot development. Later, Sessions et al. (1999) cloned the *ATML1* promoter from Arabidopsis and first described it could drive *GUS* gene expression in the meristems of flowers, buds, and roots. Subsequently, *ATML1* promoter was used to drive *CCT8* gene expression and the results showed that *CCT8-iGFP* accumulated specifically in the epidermis, both in the SAMs and leaves (Xu et al., 2011). So far, most researches about the *ML1* epidermis-specific promoters mainly focus on *A. thaliana* whereas the meaningful but still poorly researched epidermis-specific promoter is contained in the model legume *Medicago truncatula*, which may be a better driver for genetic improvement of legume forages according to host specificity (Brandalise et al., 2009). Mandaci and Dobres (1997) cloned a 2.8 kb promoter fragment from *Blec4* gene of pea (*Pisum sativum*) and proved that it could drive epidermal-specific gene expression in alfalfa (*Medicago sativa*), which has become an evidence of exogenous epidermal-specific promoters in legume forages. For epidermis-specific expression demonstration, the transient expression assay has been widely applied to cereal crops such as wheat (*Triticum monococum*) (Vickers et al., 2006), maize (*Zea mays*) (Hamilton et al. 1992), rice (*Oryza sativa*) (Liu et al., 2010) and ornamental plant like petunia (*Petunia hubrida*) (Delange et al., 1993) and cattail (*Typha orientalis*) (Nandakumar et al., 2004). Furthermore, fusion of entire *ChtC2* promoter with β-glucuronidase (GUS) reporter gene confirmed that it exclusively expressed in the epidermis of transgenic potato (*Solanum tuberosum*) (Ancillo et al., 2003) and the promoter of *AtCUT1* gene was sufficient to drive epidermal expression of *GUS* gene in stems, leaves and siliques in Arabidopsis (Kunst et al., 2000). Taken together all these evidences, provide a solid theoretical basis for our functional characterization of epidermis-specific promoter *MtML1* through transient expression analysis.

In the present study, we isolated the upstream regulatory region of *MtML1* gene and performed bioinformatics analysis via comparing the *MtML1* promoter sequence with the epidermis-specific promoters *ATML1* and *AtCER6*. Some conserved elements were validated and might contribute to the epidermis-specificity of *MtML1* promoter. Then, we confirmed its epidermis-specific expression pattern via GUS histochemical analysis in *N. tabacum* and *A. thaliana.* Our finding suggests that *MtML1* promoter can be used for ectopic expression of epidermal-related genes such as trichome and waxes-related genes involved in stress-tolerance to achieve desired traits.

2. Materials and methods

2.1. Bacterial strains and plasmids

The vectors containing pBIB-BASTA-GUS-GWR and pDONR/Zeo and *Escherichia coli* strain DB3.1 were generously provided by Professor Jia Li from School of Life Sciences, Lanzhou University, China. *Agrobacterium tumefaciens* strain GV3101 and *E. coli* strain DH5α competent cells were purchased from TransGen Biotech.

2.2. Antibiotics and enzymes

The antibiotics stocks such as kanamycin was acquired from TransGen Biotech. Gentamicin, rifampicin and herbicide BASTA were purchased from Takara (Shanghai, China). Zeocin, BPII Clonase and LRII Clonase were ordered from Invitrogen (Beijing, China). PrimeSTAR® HS DNA Polymerase was obtained from Takara (Dalian, China).

2.3. Plant materials and growth conditions

Seeds of *M. truncatula* were surface sterilized with concentrated sulfuric acid, and then were planted in the mixture of peat and vermiculite (1:1). High-quality genomic DNA was extracted from 3-week-old seedlings and stored at 4 °C until use.

Surface sterilized seeds of *A. thaliana* (Col-0) were cultured on 1/2 Murashige and Skoog (MS) medium supplemented with 0.5% (w/v) sucrose and 0.8% (w/v) agar. One week young seedlings were transferred into the mixture of soil (PINDSTRUP SUBSTRATE) and vermiculite (5:1). Flowering plants and one-week-old seedlings plants were used for the extraction of DNA and genetic transformation, respectively. Subsequently, surface sterilized seeds of *Nicotiana tabacum* were cultured in soil for 4 weeks for the transient infection.

All these plants were grown under the following conditions: 22 ± 2 °C/20 ± 2 °C (day/night) in a 16-h-light/8-h-dark photoperiod with 200 μmol m^{-2}·s^{-1} light intensity and 60 ± 5% ambient humidity.

2.4. Total DNA extraction

Total DNA was extracted from leaves of three-week-old seedlings of *M. truncatula* and one-week-old seedlings of *A. thaliana* by the cetyl trimethyl ammonium bromide (CTAB) method (Rogers et al., 1985).

2.5. Isolation of MtML1 promoter fragment

According to the sequence of *MtML1* gene provided by Dr. Rujin Chen from Plant Biology Division, Samuel Roberts Noble Foundation, USA, primers *MtML1*p-F GGGGACAAGTTTGTACAAAAAAGCAGGCTA AAGAAAGGATAATTGTGATTC and *MtML1*p-R GGGGACCACTTTGTAC AAGAAAGCTGGGTGTTGCTTGTTTCTTTGTTACTC were synthesized with *att*B1 and *att*B2 linker sequence (*att*B1: GGGGACAAGTTTGTACA AAAAAGCAGGCT, *att*B2: GGGGACCACTTTGTACAAGAAAGCTGGGT). Polymerase chain reaction amplification (PCR) was performed in a 50 μL reaction volume containing 10 μL buffer, 4 μL (2.5 mM) dNTP mixture, 1 μL (10 μM) primer F, 1 μL (10 μM) primer R, 1 μL (100 ng) template DNA, 0.5 μL (2 U/μL) prime star, and 32.5 μL sterilized ddH_2O, and followed by 30 cycles: 98 °C for 10 s, 50 °C for 30 s, 72 °C for 2 min 30 s. The amplified products were electrophoresed in a 1% agarose gel at 150 V for 10 min and purified by using StarPrep Gel Extraction Kit StarPrep (GenStar BioSolutions Co., Ltd), then sequenced. Meanwhile, for comparison, the upstream fragment of *ATML1* and *AtCER6* were also isolated as described previously (Mehrotra et al., 2000).

2.6. Bioinformatics analysis

The promoter region of *MtML1*, *ATML1* and *AtCER6* were analyzed to identify the promoter motifs and speculate potential functions through online available web tools PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The transcription start sites were identified generally based on the position of the predicted TATA box. The *cis*-elements were marked using PlantCARE and then a comparative analysis was performed to identify their structure motifs.

Table 1
The similarities of *MtML1*, *AtML1* and *AtCER6* promoter.

MtML1 promoter	*AtML1* promoter	*AtCER6* promoter	Functions
TATA-box	TATA-box	TATA-box	core promoter element around -30 of transcription start
CAAT-box	CAAT-box	CAAT-box	common *cis*-acting element in promoter and enhancer regions
TCT-motif, AE-box, GATA-motif, Box I, GAG-motif, Sp1, ATCT-motif, Box 4,	ACE, Box 4, ATCT-motif, CATT-motif, G-Box, G-box, GATA-motif, GT1-motif, TCT-motif, AE-box	Box 4, G-Box, G-box, AE-box, GATA-motif, GT1-motif, I-box, L-box, TCT-motif,	part of a light responsive element
ABRE	ABRE	ABRE	*cis*-acting element involved in the abscisic acid responsiveness
ARE	ARE	ARE	*cis*-acting regulatory element essential for the anaerobic induction
CGTCA-motif, TGACG-motif	CGTCA-motif, TGACG-motif	CGTCA-motif, TGACG-motif	*cis*-acting regulatory element involved in the MeJA-responsiveness
GCN4_motif, Skn-1_motif	Skn-1_motif	GCN4_motif, Skn-1_motif	*cis*-regulatory element involved in endosperm expression
circadian	circadian	circadian	*cis*-acting regulatory element involved in circadian control

Fig. 1. Sequence analysis of various promoters. **(a)** Sequence analysis of *MtML1* promoter from *M. truncatula.* **(b)** Sequence analysis of *ATML1* promoter from *A. thaliana.* **(c)** Sequence analysis of *AtCER6* promoter from *A. thaliana.*

2.7. Vectors construction

The purified PCR products were ligated into the pDNOR/Zeo vector by BP reaction in a total volume of 10 μL which containing 0.5 μL BPII Clonase, 1 μL PCR product, 1 μL pDONR/Zeo vector, 7.5 μL TE Buffer. The LR reaction was performed by using 1 μL pDONR/Zeo vector along with target promoter, 0.5 μL LRII Clonase, 1 μL pBIB-BASTA-GUS-GWR vector, 7.5 μL TE Buffer. Then, the ligated product was transformed into *E. coli* DH5α cells and sequenced successfully. Finally, we obtained plant expression vectors named p*MtML1* :: *GUS*, p*ATML1* :: *GUS* and p*AtCER6* :: *GUS*. The plant expression vectors were further transformed into *A. tumefaciens* GV3101 with a freeze-thaw method and verified by colony PCR (Mehrotra et al., 2000).

2.8. Transient expression in N. Tabacum

The experiment was carried out by a GV3101 empty strain regarded as a negative control and the p*35S* :: *GUS* as a positive control. The four-week-old plants of *N. tabacum* leaves were incised and immersed into bacterium suspension (the single colony of *A. tumefaciens* strain GV3101 containing p*MtML1* :: *GUS*, p*ATML1* :: *GUS* and p*AtCER6* :: *GUS* vectors was inoculated in YEB solution, and then diluted 100 times with fresh YEB solution harboring 10 mM MES, 20 μM acetosyringone (AS), gentamicin and kanamycin (50 mg/L), shaken at 28 °C at 180 rpm. To collect cells of GV3101, the OD_{600} value was adjusted to 0.6 – 0.8 with YEB supplemented with 10 mM $MgCl_2$, 10 mM MES, 150 mM AS, and finally incubated at room temperature for 2 h and executed vacuum filtration for 30 min at -0.85 MPa). Subsequently, the explants were incubated at 20–22 °C for 3–4 d, and leaf segment of size about 1 cm^2 were later stained at 37 °C for 7–8 h in GUS buffer (50 mM sodium phosphate buffer (pH 7), 0.1 M $K_3Fe(CN)_6$, 0.1 M $K_4Fe(CN)_6$, 10 mM Na_2EDTA, 0.001% Triton X-100, 20% Methanol, 0.5 mg/ml X-Gluc). The stained leaves were decolored in 70% ethanol and viewed under the stereo light microscope.

2.9. Agrobacterium-mediated transformation of A. Thaliana

The vectors containing p*MtML1* :: *GUS* and p*AtCER6* :: *GUS* were

Table 2
The differences of *MtML1*, *AtML1* and *AtCER6* promoter.

MtML1 promoter	*AtML1* promoter	*AtCER6* promoter	Function
5′ UTR Py-rich stretch	5′ UTR Py-rich stretch	——	*cis*-acting element conferring high transcription levels
Box III	Box III	——	Protein binding site
LTR	LTR	——	*cis*-acting element involved in low-temperature responsiveness
——	HSE	HSE	*cis*-acting element involved in heat stress responsiveness
MBS	——	MBS	MYB binding site involved in drought-inducibility
TC-rich repeats	TC-rich repeats	——	*cis*-acting element involved in defense and stress responsiveness
——	Box-W1	——	fungal elicitor responsive element
GARE-motif	GARE-motif, TATC-box	——	gibberellin-responsive element
MBSII	——	——	MYB binding site involved in flavonoid biosynthetic genes regulation
TCA-element	TCA-element	——	*cis*-acting element involved in salicylic acid responsiveness
——	——	TGA-element	auxin-responsive element
——	as-2-box	——	involved in shoot-specific expression and light responsiveness

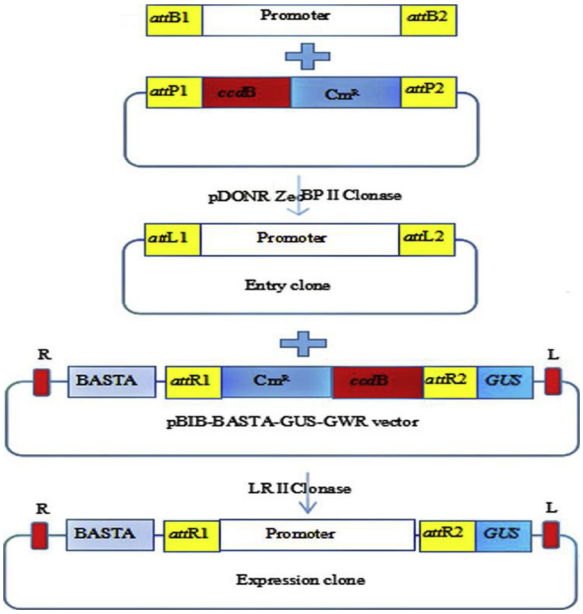

Fig. 2. Schematic representation of Gateway vector construction of *MtML1*, *AtCER6*, and *ATML1* promoters.

transferred into *A. thaliana* by the *Agrobacterium*-mediated floral dip method as reported previously (Clough and Bent, 1998). Every inflorescence was infected with suspension cultures of *A. tumefaciens* (50 µL of target bacterium solution was inoculated into 100 mL LB medium supplemented with kanamycin and 50 mg/L rifampicin, shaken overnight at 28 °C at 200 rpm, and cells were re-suspended with 5% sucrose and 0.03% silwet-77), and incubated in dark for 7–12 h. Inoculation with the *Agrobacterium* was repeated twice in a week interval. Screening of seedlings with two green cotyledons was performed on 0.01% BASTA sprayed once every 4 days for three times and followed by PCR and RT-PCR detection to get homozygous transgenic lines. Subsequently, the leaves and siliques of the homozygous plants were stained at 37 °C for 7–8 h in GUS buffer cleared in 70% ethanol, and viewed under the stereo light microscope.

3. Results

3.1. Isolation of MtML1 promoter

Using specific primers and adapter-templates, a 5′ flanking sequence from the upstream -2150 bp to the start ATG codon of *MtML1* gene were isolated from *M. truncatula*. Meanwhile, the promoter region of *ATML1* and *AtCER6* genes, with a length of 2162 bp and 1209 bp, respectively, were also cloned successfully.

3.2. Sequence analysis of MtML1 promoter and the comparison with ATML1 and AtCER6 promoters

The PlantCARE analysis of motifs in *MtML1* promoter indicated that the presence of TATA and CAAT boxes, as well as elements involved in light responsiveness like AE-box, ATCT-motif, G-box, TCT-motif (Table. 1, Fig. 1a). Likewise, CGTCA-motif, TGACG-motif involved in the MeJA (Methyl jasmonate) -responsiveness and known to upregulate the gene expression during wound stress were validated in *MtML1* promoter (Table 1). In addition, the *cis*-regulatory element involved in low-temperature, drought-inducibility, defense and stress responsiveness were also found in *MtML1* promoter (Fig. 1a).

Comparative analysis of *MtML1*, *ATML1* and *AtCER6* promoters showed that a plurality of 5′ UTR Py-rich stretch conferred high transcription levels, which exhibited a positive effect on the overall expression level existed in *MtML1* (Fig. 1a) and *ATML1* promoter (Bolle et al., 1994; Daraselia et al., 1996) (Fig. 1b) rather than *AtCER6* promoter (Fig. 1c). Similar as *AtCER6* promoter, the MBS motif involved in drought-inducibility was discovered in *MtML1* promoter (Fig. 1a and c). In addition, abiotic stress responsive motifs are predominant in *MtML1* promoter and *ATML1* promoter (Fig. 1a and b). Furthermore, several biotic stress related *cis*-elements that respond to wound, pathogen, salicylic acid, gibberellin, and MeJA were found in these three regulatory regions. It demonstrated that the structural elements *MtML1*

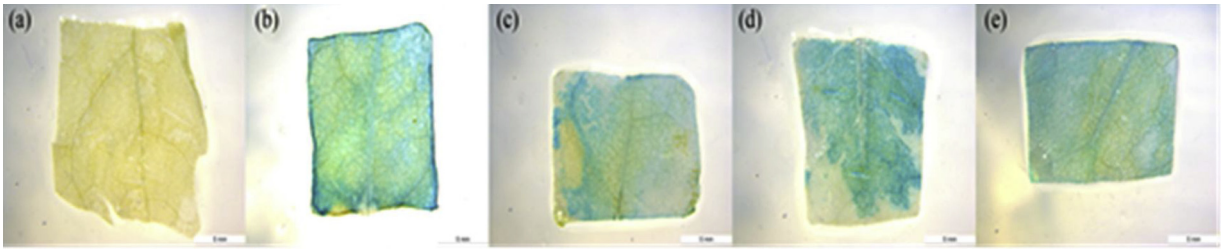

Fig. 3. Histochemical GUS assay in tobacco leaf in transient expression. **(a)** Negative control. **(b)** Positive control. **(c)** The material transferred *MtML1* promoter. **(d)** The material transferred *ATML1* promoter. **(e)** The material transferred *AtCER6* promoter.

Fig. 4. Screening of BASTA herbicides in transgenic strains. **(a), (b)** Screening of *AtCER6* promoters of transgenic Arabidopsis. **(c)**, **(d)** Screening of *MtML1* promoter of transgenic Arabidopsis. (a), (c)Bar = 2 cm; (b), (d)Bar = 5 cm.

promoter resemble the other two promoters and these three promoters may perform similar functions (Tables 1 and 2, Fig.1a–c).

3.3. Construction of gateway-compatible vectors

The *ATML1*, *MtML1* and *AtCER6* promoters were successfully constructed in pBIB-BASTA-GUS-GWR by BP and LR reaction to drive *GUS* gene, respectively (Fig. 2). Ultimately, we obtained three plant expression vectors, p*MtML1* :: *GUS*, p*ATML1* :: *GUS*, and p*AtCER6* :: *GUS*.

3.4. Tobacco transient assays

Tobacco leaves were infected by *A. tumefaciens* contained p*MtML1* :: *GUS*, p*ATML1* :: *GUS* and p*AtCER6* :: *GUS* vector respectively, to determine transient GUS expression activity of *MtML1* promoter. Meanwhile, GV3101 strain was used as negative control, p*35S* :: *GUS* was regarded as positive control. The results showed that *MtML1* promoter could drive *GUS* gene expression transiently in *N. tabacum* leaves with similar pattern of *AtCER6* promoter (Fig. 3a–e).

3.5. Tissue-specific assay in A. Thaliana

To investigate tissue-specific expression pattern of the *MtML1* promoter, we transformed p*MtML1* :: *GUS* and p*AtCER6* :: *GUS* vectors into Arabidopsis and obtained six and four transgenic lines by screening with BASTA, respectively (Fig. 4a–d). All the transgenic independent lines were used for the GUS staining. The results showed that both p*MtML1* :: *GUS* and p*AtCER6* :: *GUS* expressed in leaves, stem and siliques but were not identified in the roots (Fig. 5a–f). Moreover, the leaf cross-section analysis showed that the GUS staining of both *MtML1* promoter and *AtCER6* promoter detected in epidermal cells. The GUS activity was not obviously detected in the internal tissues of p*MtML1* :: *GUS* lines. But in p*AtCER6* :: *GUS* lines, the GUS staining also exhibited in the vascular bundle (Fig. 5g–h) and leaf veins (Fig. 5d). It's worth interesting to note that p*MtML1* :: *GUS* positive plants showed less GUS activity compared with *AtCER6* plants in leaf, stem and siliques respectively (Fig.5a–f).

4. Discussion

Promoters have been divided into three major types, on the basis of function and mode of actions including constitutive, inducible and tissue-specific promoters. As important choice for gene drivers, plenty of exogenous promoters have been widely used for genetic transformation in plants. However, it is accepted that constitutive expression of genes might cause energy consumption, metabolic disturbance and some unexpected traits (Kasuga et al., 1999; Hsieh et al., 2002). To maximize the advantages of genetically modified plants, it's better to use tissue-specific promoters that contain specific motifs for regulating exogenous gene expression in specific organs and tissues (Josefsson et al., 1987). In recent years, investigations on tissue-specific promoters were mainly focused on the vascular bundles, roots, seeds and floral organs, while few studies have been conducted on epidermis-specific promoters (He et al., 2014; Porto et al., 2014). In current investigations, we isolated the putative promoter region of *MtML1* gene with a length of 2150 bp. Similar with other promoters, its core promoter is a minimal stretch of DNA sequences (e.g., the TATA box, initiator, and downstream core promoter element) surrounding the transcription start site that directly interacts with the components of basal transcription machinery (Smale and Kadonaga, 2003). The TATA box had positive effect on the regulated expression of the *Sea urchin H2A* gene, deletion of this region induced 15 to 20- fold decrease in *H2A* gene expression. (Grosschedl and Birnstiel, 1980; Benoist and Chambon, 1981). Here, we found that *MtML1* promoter fragment also contained promoter TATA box and CAAT box (Fig.1a), which were regarded as core promoter elements around -30 of transcription start and known as enhancer regions that activate or enhance gene transcription frequency (Maniatis et al., 1987). Another regulatory element identified in *MtML1* promoter is TGACG-motif involved in the MeJA-responsiveness and enhanced root and leaf activity (Kumar et al., 2012); beyond that, elements involved in the abscisic acid responsiveness, endosperm expression and circadian control were also found (Table 1). Interestingly, the epidermis-specific promoters of *MtML1* and *ATML1* compared with *AtCER6* promoter had a plurality of 5′ UTR Py-rich stretch motif, which positively influences the overall expression level (Table 2). Canevascini et al. (1996) had analyzed the promoter sequence of the tobacco epidermis-specific *Itp1* gene and contained many CTAGCTAG motifs. Abe (2001) detected that L1-box (TAAATGCA) plays a crucial role in the regulation of *PROTODERMAL FACTOR1* (*PDF1*) expression in L1 cells. It was found that *PDF1* promoter contained L1-box (TAAATGCA) by aligning with promoter sequences of *PDF1* (Abe et al., 1999, 2001) and *MERISTEM LAYER1* (*ATML1*) (Lu et al., 1996; Sessions et al., 1999), *FIDDLEHEAD* (*FDH*) (Yephremov et al., 1999), *SCARECROW* (*SCR*) (Wysocka-Diller et al., 2000) and *LIPID TRANSFER PROTEIN1* (*LTP1*) (Thoma et al., 1994). These investigations suggested that CTAGCTAG motif and L1-box play important roles in controlling gene expression in the plant epidermis. We identified these two motifs existed in *ATML1* promoter, while not exhibited in *MtML1* and *AtCER6* promoters. The differences in cis-elements among different promoters may induce the different expression patterns. For instance, Sessions et al (1999) identified *ATML1* promoter is active in the root tip in Arabidopsis, however in our experiment the *MtML1* promoter didn't drive GUS expression in root. In addition, previous studies showed that *ML1* and *CER6* genes were expressed in plant epidermal cells (Millar et al., 1999; Sessions

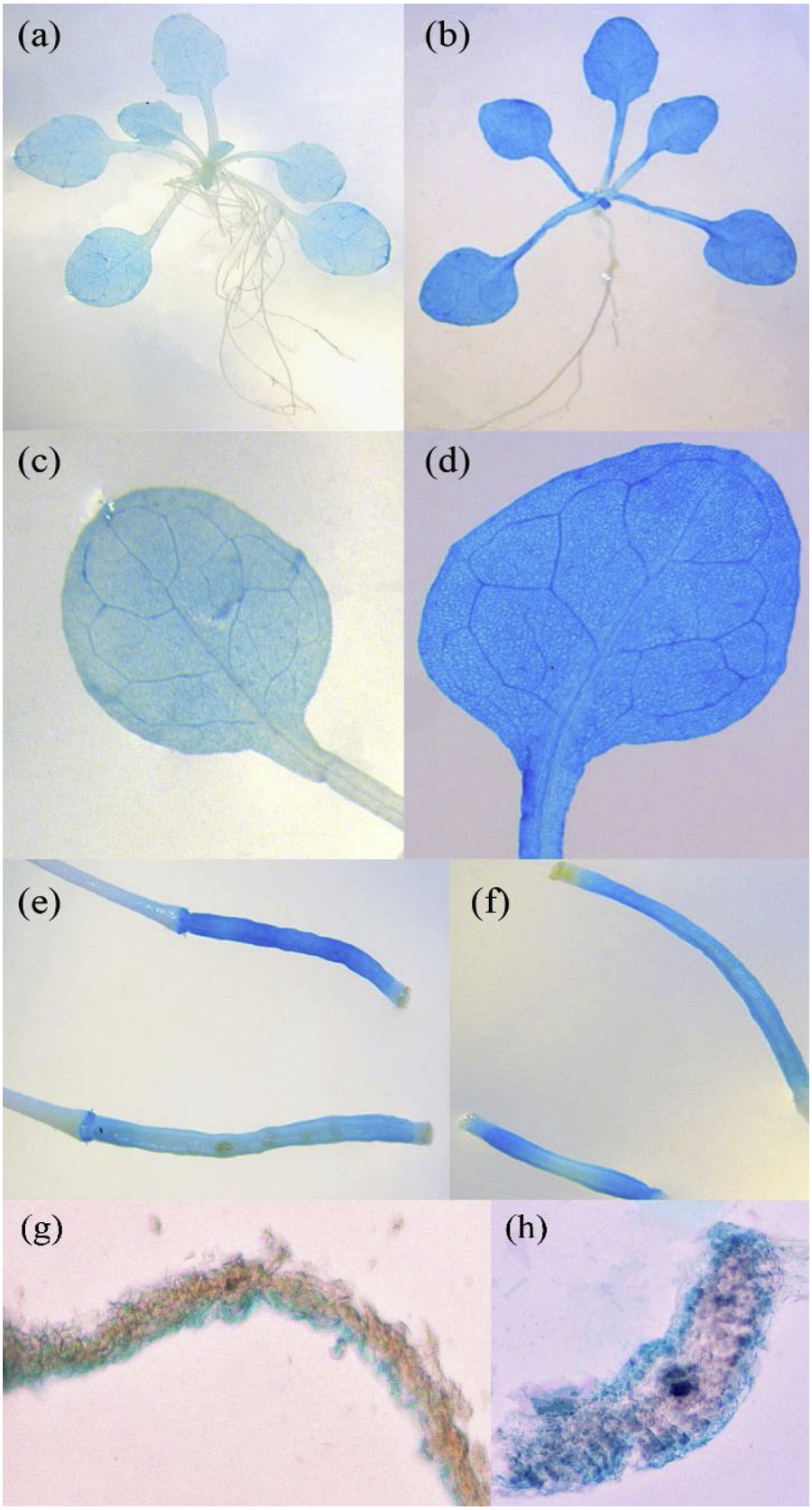

Fig. 5. Histochemical GUS assay of the *AtCER6* promoter and *MtML1* promoter in transgenic *A. thaliana*. Histochemical GUS assay of 2 weeks seedlings **(a)**, leaf **(c)**, pod **(e)**, leaf cross section **(g)** transformed with the *MtML1* promoter. Histochemical GUS assay of 2 weeks seedlings **(b)**, leaf **(d)**, pod **(f)**, leaf cross section **(h)** transformed with the *AtCER6* promoter.

et al., 1999; Xu et al., 2011) and mutations in *CER6* gene decreased lipid content on the surface of fruits, pollen and stems (Millar et al., 1999; Fiebig et al., 2000; Vogg et al., 2004; Leide et al., 2007). Presumably, *MtML1* and *AtCER6* promoters may have other specific regulatory motifs to control gene expression in the plant epidermis.

Moreover, previous studies showed that successful manipulation of the epidermis-promoters is affected by many factors. The coffee (*Coffea arabica*) leaf-specific *RBCS1* promoter contained the *cis*-acting light responsive elements that were important components for transcriptional control of gene expression by light (Marraccini et al., 2003). Song et al. (2007) revealed that rice chlorophyll *a*/b binding protein promoter could be used for driving leaf-specific expression of related genes. The *AtDHS* promoter could drive *GUS* gene temporal and spatial expression, especially, when it was expressed not only in rosette leaves, but also in the anthers of developing flowers (Duguay et al., 2007). Qin et al. (2009) reported that manioca (*Jatropha curcas*) *CP2* promoter could drive *GUS* gene expression under salicylic acid, abscisic acid and adverse stresses such as drought, cold, high temperature and UV, respectively. Liu et al. (2014) reported that the promoter of wax biosynthesis-related gene *CsCER7* from cucumber (*Cucumis sativus*) included several well-characterized elements such as ABREs responding to ABA; and the expression of *CsCER7* increased the accumulation of fruit cuticular wax under ABA treatment. Similar results were obtained when sequence analysis was performed by PlantCARE, i.e., the promoter region of *MtML1* gene contained a variety of *cis*-acting elements involved in light, circadian control, salicylic acid, the abscisic acid, defense and abiotic stress responses, such as heat, low-temperature and drought, as well as *ATML1* and *AtCER6* promoters contain analogous motifs (Tables 1,2). We speculated that the activity of *MtML1* and *AtCER6* promoters might be affected by light, adverse stress, abscisic acid and salicylic acid. Therefore, following the above-obtained results, it was preliminarily confirmed that we have obtained the functional promoter sequence of *MtML1* gene.

To further investigate its expression sites and functional activity, we constructed three fusion vectors and transiently infected *N. tabacum,* consistent with *AtCER6* and *ATML1* promoters, *MtML1* promoter could drive *GUS* gene transiently in *N. tabacum* (Fig. 3a–e), indicating that *MtML1* promoter has the function of transcription initiation. Additionally, *MtML1* and *AtCER6* promoters were transformed into *A. thaliana* and followed by GUS histochemical analysis and found that *GUS* gene driven by *AtCER6* and *MtML1* promoter were expressed mainly in plant leaves and siliques rather than in roots (Fig. 5a–f). In previous studies, CCT8-iGFP driven by the *ATML1* promoter, accumulated specifically in the epidermis of both SAM and leaves (Xu et al., 2011). Similarly, the *AtCER6* promoter was highly effective in driving the epidermis-specific expressions of *GUS* and *WXP1* genes in Arabidopsis, tobacco, transgenic alfalfa, and showed increased wax accumulation (Hooker et al., 2002; Jiang et al., 2009). Our finding was consistent with previous research on *AtCER6* promoter and *ATML1* promoter (Sessions et al., 1999), exhibited that *MtML1* promoter specifically drove gene expression in epidermis of plants. Moreover, we found *AtCER6* promoter could drive *GUS* gene expression more significantly than *MtML1* promoter in tissue-specific assay of Arabidopsis, which was on account of host specificity. Sunilkumar et al. (2002) fused cotton α-globulin promoter with *GUS* gene transformed into cotton, Arabidopsis and tobacco and detected diverse GUS activity respectively, of which, Arabidopsis GUS activity was 16.7% of cotton while in tobacco was poorly less than 1%. Similar results have also been reported that the coffee *CaIRL* promoter was unable to drive GUS expression in non-wounded leaves of transgenic tobacco plants, in contrast to the normal level of *CaIRL* expression observed in undamaged coffee leaves (Brandalise et al., 2009). Our results could be attributed to the existence of different regulatory mechanisms between different plants, including the lack of essential regulatory elements within the cloned promoter region, or the presence of host-specific trans-acting factors. Thus, *MtML1* promoter cloned from the model legume *M. truncatula* assumed more probably effective for driving the exogenous gene expression in legume plants.

5. Conclusion

In conclusion, we cloned *MtML1* promoter from *M. truncatula* and constructed *MtML1* :: *GUS* expression vector then infected tobacco leaves, the tobacco transient assays illustrated that *MtML1* promoter could drive *GUS* gene expression. Furthermore, tissue-specific assay in *A. thaliana*, transformed with p*MtML1* :: *GUS* and p*AtCER6* :: *GUS* vectors. It highlighted that *MtML1* promoter is epidermal-specific promoter that could drive *GUS* gene expression only in epidermis of Arabidopsis. The transformation of *AtCER6* demonstrated higher expression than *MtML1* in Arabidopsis, similarly, *MtML1* promoter may possess better driving capacity for legume plants due to host specificity. Our study revealed that *MtML1* promoter play a pivotal role in driving downstream gene expression located in the plant epidermis, as well as provide better tissue-specific promoter selection for legumes genetic engineering in future, especially for driving the cuticle-related gene to express in the epidermis of genetically related forage species like alfalfa.

Author contributions

S.M.W designed the experiments; Y.T, M.C.C and L.W carried out the experiments; Y.T, M.C.C, J.L.Z, T.K, L.B.L and S.M.W analyzed data; Y.T, L.G, T. G. G, H.J.Y and S.M.W wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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