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

Li Gao¹, Ye Tian, Meng-Ci Chen¹, Li Wei, Tian-Ge Gao, Hong-Ju Yin, Jin-Lin Zhang, Tanweer Kumar, Lin-Bo Liu, Suo-Min Wang*

State Key Laboratory of Grassland Agro-ecosystems, Key Laboratory of Grassland Livestock Industry Innovation, Ministry of Agriculture and Rural Affairs, College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou, 730020, P.R. China

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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; Roupahel 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 SSGa 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 WX1 driven by constitutive promoter CaMV

*Corresponding author.
E-mail address: smwang@lzu.edu.cn (S.-M. Wang).
¹These authors contributed equally to this work.

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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 tabacum 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-GFP accumulated specifically in the epidermis, both in the SAMs and leaves (Xu et al., 2011). So far, most researches on 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 monococcum) (Vickers et al., 2006), maize (Zea mays) (Hamilton et al., 1992), rice (Oryza sativa) (Liu et al., 2010) and ornamental plant like petunia (Petunia hybridra) (Delange et al., 1993) and cattail (Typha orientalis) (Nandakumar et al., 2004). Furthermore, fusion of entire ChlC2 promoter with γ-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, BPI Clonase and LRI 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 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 200μmol 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 with 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 MtML1F-P GGGGACAAGTCTTTGACAAAGGAGGCTTA AAGAAAAGTATTTGTAC and MtML1P-R GGGGACCATTGTGTA C AAGAACTGTTGTTGTTTTGTAC were synthesized with attB1 and attB2 linker sequence (attB1: GGGGACAAGTCTTTGACAA AGGAGGCTTA, attB2: GGGGACCATTGTGTAACAAAACTGTT). 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 μL/μL) prime star, and 32.5 μL sterilized ddH2O, 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.
2.7. Vectors construction

The purified PCR products were ligated into the pDNOR/Zeo vector by BP reaction in a total volume of 10 $\mu$L which containing 0.5 $\mu$L PⅡClonase, 1 $\mu$L PCR product, 1 $\mu$L pDONR/Zeo vector, 7.5 $\mu$L EB buffer. The LR reaction was performed by using 1 $\mu$L pDONR/Zeo vector along with target promoter, 0.5 $\mu$L PⅡClonase, 1 $\mu$L pBIB-BASTA-GUS-GWR vector, 7.5 $\mu$L EB buffer. Then, the ligated product was transformed into E. coli DH5α cells and sequenced successfully. Finally, we obtained plant expression vectors named pMtML1::GUS, pATML1::GUS and pAtCER6::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 p35S::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 pMtML1::GUS, pATML1::GUS and pAtCER6::GUS vectors was inoculated in YEB solution, and then diluted 100 times with fresh YEB solution harboring 10 mM MES, 20 $\mu$M acetosyringone (AS), gentamicin and kanamycin (50 mg/L), shaken at 28 °C at 180 rpm. To collect cells of GV3101, the OD600 value was adjusted to 0.6−0.8 with YEB supplemented with 10 mM MgCl2, 10 mM MES, 150 $\mu$M 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 cm² were later stained at 37 °C for 7–8 h in GUS buffer (50 mM sodium phosphate buffer (pH 7), 0.1 M K3Fe(CN)6, 0.1 M K4Fe(CN)6, 10 mM Na2EDTA, 0.001% Triton X-100, 20% Methanol, 0.5 mg/ml X-Gluc). The stained leaves were decolorized in 70% ethanol and viewed under the stereo light microscope.

2.9. Agrobacterium-mediated transformation of A. Thaliana

The vectors containing pMtML1::GUS and pAtCER6::GUS were
Subsequently, the leaves and siliques of the homozygous plants were pooled by PCR and RT-PCR detection to get homozygous transgenic lines. Subsequently, the leaves and siliques of the homozygous plants were sprayed once every 4 days for three times and foliar interval. Screening of seedlings with two green cotyledons was performed. Agrobacterium inoculation with the method as reported previously (Clough and Bent, 1998). Every inoculation was repeated twice in a week in order to get transgenic plants.

The material transferred into A. thaliana by the Agrobacterium-mediated floral dip method as reported previously (Clough and Bent, 1998). Every inoculation was repeated twice in a week in order to get transgenic plants. Screening of seedlings with two green cotyledons was performed. Agrobacterium inoculation with the method as reported previously (Clough and Bent, 1998). Every inoculation was repeated twice in a week in order to get transgenic plants.

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

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). In addition, several cis-regulatory elements that respond to wound, pathogen, salicylic acid, gibberellin, and MeJA (Methyl jasmonate) -responsiveness and known to upregulate the gene expression during wound stress were validated in MtML1 promoter (Fig. 1a). 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 were stained at 37 °C for 7–8 h in GUS buffer cleared in 70% ethanol, and viewed under the stereo light microscope.

Table 2

The differences of MtML1, ATML1 and AtCER6 promoter.

<table>
<thead>
<tr>
<th>MtML1 promoter</th>
<th>ATML1 promoter</th>
<th>AtCER6 promoter</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ UTR Py-rich stretch</td>
<td>5’ UTR Py-rich stretch</td>
<td>——</td>
<td>cis-acting element conferring high transcription levels</td>
</tr>
<tr>
<td>Box III</td>
<td>Box III</td>
<td>——</td>
<td>Protein binding site</td>
</tr>
<tr>
<td>LTR</td>
<td>LTR</td>
<td>——</td>
<td>cis-acting element involved in low-temperature responsiveness</td>
</tr>
<tr>
<td>HSE</td>
<td>HSE</td>
<td>——</td>
<td>cis-acting element involved in heat stress responsiveness</td>
</tr>
<tr>
<td>MBS</td>
<td>MBS</td>
<td>——</td>
<td>cis-acting element involved in defense and stress responsiveness</td>
</tr>
<tr>
<td>TC-rich repeats</td>
<td>TC-rich repeats</td>
<td>——</td>
<td>fungal elicitor responsive element</td>
</tr>
<tr>
<td>Box-W1</td>
<td>Box-W1</td>
<td>——</td>
<td>gibberellin-responsive element</td>
</tr>
<tr>
<td>GARE-motif</td>
<td>GARE-motif, TATC-box</td>
<td>——</td>
<td>MYB binding site involved in flavonoid biosynthetic genes regulation</td>
</tr>
<tr>
<td>TCA-element</td>
<td>TCA-element</td>
<td>——</td>
<td>cis-acting element involved in salicylic acid responsiveness</td>
</tr>
<tr>
<td>as-2-box</td>
<td>TGA-element</td>
<td>——</td>
<td>auxin-responsive element</td>
</tr>
</tbody>
</table>

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.
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 pBB-BASTA-GUS-GWR by BP and LR reaction to drive GUS gene, respectively (Fig. 2). Ultimately, we obtained three plant expression vectors, pMtML1 :: GUS, pATML1 :: GUS, and pAtCER6 :: GUS.

3.4. Tobacco transient assays

Tobacco leaves were infected by A. tumefaciens contained pMtML1 :: GUS, pATML1 :: GUS and pAtCER6 :: GUS vector respectively, to determine transient GUS expression activity of MtML1 promoter. Meanwhile, GV3101 strain was used as negative control, p35S :: 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 pMtML1 :: GUS and pAtCER6 :: 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 pMtML1 :: GUS and pAtCER6 :: 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 pMtML1 :: GUS lines. But in pAtCER6 :: GUS lines, the GUS staining also showed in the vascular bundle (Fig. 5g–h) and leaf veins (Fig. 5d). It’s worth interesting to note that pMtML1 :: 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 ltp1 gene and contained many CTAGCTAG motifs. Abe (2001) detected that L1-box (TAAATGCA) plays a crucial role in the regulation of PROTOTERMANAL FACTOR1 (PDF1) expression in L1 cells. It was found that PDF1 promoter contained L1-box (TAAATGCA) by aligning with promoter sequences of 11 PDF1 (Abe et al., 1999, 2001) and MERISTEM LAYER1 (ATML1) (Lu et al., 1996; Sessions et al., 1999), FIDDLEHEAD (FDH) (Yeremiev 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 et al., 1999).
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 (Coffee 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 AdDHS 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 (Jacarpha curcas) CP2 promoter could drive GUS gene expression under salicylic acid, absicacid 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 CxCER7 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 absicacidic 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, absicacid 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 (Hoeker 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 CaIRI promoter was unable to drive GUS expression in non-wounded leaves of transgenic tobacco plants, in contrast to the normal level of CaIRI 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 ofessential 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 pMtML1::GUS and pAtCER6::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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