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MlWRKY12, a novel *Miscanthus* transcription factor, participates in pith secondary cell wall formation and promotes flowering

Yanchong Yu, Ruibo Hu, Huamei Wang, Yingping Cao, Guo He, Chunxiang Fu*, Gongke Zhou*

Key Laboratory of Biofuels, Chinese Academy of Sciences, Qingdao Institute of BioEnergy and Bioprocess Technology, Chinese Academy of Sciences (QIBEBT-CAS), Qingdao, Shandong, China

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ABSTRACT

WRKY proteins play crucial roles in various plant processes. An AtWRKY12 homologous gene, named *MlWRKY12*, was isolated from *Miscanthus lutarioriparius*. The *MlWRKY12* gene encodes a WRKY transcription factor belonging to the group IIc subfamily. MlWRKY12 is a nuclear protein. Gene expression pattern analysis revealed a relatively high *MlWRKY12* expression level in rhizomes, stems and leaf sheaths. *In situ* hybridization analysis further demonstrated that *MlWRKY12* was expressed in vascular bundle sheath, sclerenchyma and parenchyma tissues. The heterologous expression of *MlWRKY12* in an *atwrky12* background mutant successfully rescued the phenotype of pith cell walls caused by the defect of AtWRKY12. Most strikingly, the transgenic Arabidopsis plants overexpressing *MlWRKY12* exhibited early flowering. The transcript abundance of flowering related genes was measured by quantitative RT-PCR analysis, suggesting that overexpression of *MlWRKY12* in Arabidopsis had a significant impact on the expression level of CONSTANS (CO). Moreover, the expression levels of FLOWERING LOCUS T (FT), LFY (LEAFY), APETALA1 (AP1), CAULIFLOWER (CAL) and FRUITFULL (FUL) were upregulated in transgenic plants. These results demonstrated the conserved function of MlWRKY12 existing in secondary cell wall formation of monocotyledonous species and implied a possible impact of MlWRKY12 on flowering control.

1. Introduction

Miscanthus is a C_4 perennial rhizomatous grass native to eastern Asia. The genus *Miscanthus* comprises around 17 species, such as *M. sinensis*, *M.* × *giganteus*, *M. sacchariflorus* and *M. lutarioriparius*. *M. lutarioriparius* is a riparian grass endemic to the central area of China [1–4]. *Miscanthus* has been studied as an important bioenergy crop because of high biomass yield and remarkable adaptability to different environments [5]. Current studies of *Miscanthus* focus on areas such as genetic diversity analysis, identification of quantitative trait loci (QTLs) and cultivation. However, the lack of entire genome sequences and gene atlas information eliminates the possibility of genetic improvement of *Miscanthus* [6–9]. It has been suggested that the partial *Miscanthus* genome sequences possess high identity to that of *Sorghum bicolor* [10,11]. Therefore, it is an efficient approach to isolate genes from *Miscanthus* based on sorghum genome sequences.

Plant secondary cell walls are the source of lignocellulosic biomass which can be used to produce second generation biofuels [12–14]. Secondary cell wall formation is regulated by numerous transcription factors (TFs) [15]. Several NAM, ATAF1/2, CUC2 (NAC) and MYB transcription factors function as master switches in the process of secondary cell wall biosynthesis, and most of them are involved in secondary wall thickening in fibers [16]. Recently, a WRKY transcription factor, AtWRKY12, has been identified as a negative regulator of secondary cell wall formation in pith parenchyma cells. Loss-of-function mutants of AtWRKY12 with secondary cell wall thickening in pith cells exhibit ectopic deposition of lignin, xylan and cellulose, which consequently leads to about a 50% increase in biomass density [14].

WRKY proteins are a superfamily of transcription factors in plants. WRKY family TFs contain at least one conserved DNA-binding region, the WRKY domain (WRKYGQK) that is followed by a C_2H_2 or C_2HC zinc-finger motif. The DNA element in the promoter regions bound by the WRKY domain is called a W-box (TTGACT/C) [17]. A number of WRKY TFs have been elucidated to be involved in various developmental and physiological processes,

* Corresponding authors at: Key Laboratory of Biofuels, Chinese Academy of Sciences, Qingdao Institute of BioEnergy and Bioprocess Technology, Chinese Academy of Sciences (QIBEBT-CAS), Qingdao 266101, Shandong, China.
Tel.: +86 532 80662731; fax: +86 532 80662778.
E-mail addresses: fucx@qibebt.ac.cn (C. Fu), zhougk@qibebt.ac.cn (G. Zhou).

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such as pathogen defense [18,19], senescence [20,21], development [22–24], and abiotic stress response [25,26]. Ectopic overexpression of WRKY TFs can affect flowering time in *Arabidopsis* [20,27,28]. However, the mechanism is far less known.

Flowering is a crucial developmental process in the life cycle of plants. It is essential for achieving reproductive success. Moreover, flowering time is an important trait in improvement of bioenergy crops because delayed flowering time has the potential to increase biomass yield. The time of flower initiation is plastic, which is affected by environmental and endogenous cues [29–31]. Previous molecular genetic studies elucidated that there are four major pathways controlling flowering in *Arabidopsis* including autonomous, vernalization, gibberellic acid (GA) and photoperiod pathways [32–35]. These pathways mediate environmental and endogenous signals and finally converge to regulate the expression of floral pathway integrators such as *FLOWERING LOCUS T* (*FT*), *LFY* (*LEAFY*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) [36–39]. They activate downstream floral meristem identity (FMI) genes, *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*) and *FRUITFULL* (*FUL*), which in turn initiate the transition from vegetative to reproductive phase [40–42].

In order to identify the function of the WRKY family TFs involved in secondary cell wall formation in bioenergy crops, we isolated *MlWRKY12* from *M. lutarioriparius* and conducted motif recognition and subcellular localization. Furthermore, heterologous expression of *MlWRKY12* in an *atwrky12* mutant rescued the mutated phenotype resulting from the defect of *AtWRKY12*. Overexpression of *MlWRKY12* promoted flowering of transgenic *Arabidopsis* plants which were grown in either short day (SD) or long day (LD) condition, or treated by low temperature or GA3. We conclude that MlWRKY12 is a functional ortholog of AtWRKY12 and shows negative regulation of secondary cell wall formation in pith cells. Moreover, overexpression of *MlWRKY12* has the capacity to induce flowering related gene expression and, consequently, promotes flowering transition in *Arabidopsis*.

2. Materials and methods

2.1. Plant materials and growth conditions

M. lutarioriparius plants were grown in a greenhouse programmed for 14 h light and 10 h dark photocycle at 25 °C with 60% relative humidity. For gene cloning, stems were harvested from 4-week-old plants. For tissue-specific expression analysis, root, rhizome, basal stem, upper stem, leaf, leaf sheath and inflorescence were collected when plants bloomed. All collected samples were immediately frozen in liquid nitrogen and stored at −80 °C. Primers used in this study are listed in supplementary Table 1.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.07.010.

Arabidopsis seeds were incubated at 4 °C for 3 days before being sown into soil. *Arabidopsis* plants were grown in the greenhouse programmed for LD (16 h light and 8 h dark) or SD (8 light and 16 h dark) condition at 20 ± 2 °C with 60% relative humidity. The *Arabidopsis atwrky12* mutant was obtained from *Arabidopsis* Biological Resource Center (ABRC). For gene transcription level analysis, *Arabidopsis* seeds were surface-sterilized before plating on 1/2 MS agar and held at 4 °C for 72 h. They were then transferred into the specified green house under LD or SD conditions. The 14-day-old whole plants were harvested at zeitgeber time (ZT) 16 or at indicated time points and stored at −80 °C.

2.2. RNA isolation and amplification of MlWRKY12

Total RNA was extracted from plant tissues using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol, and then treated with RNase-free DNaseI (Promega, USA) to remove genomic DNA contamination. First-strand cDNA was synthesized using M-MLV reverse transcriptase (TaKaRa, Japan) according to the manufacturer's protocol.

M. lutarioriparius cDNA was used as the template to amplify *MlWRKY12*. The total volume of the PCR reaction mixture was 50 μl, which contained 2.5 μl cDNA, 0.5 μM of each primer, 1× PCR buffer, 0.3 mM dNTP and 2 units of KOD-Plus- enzyme (TOYOBO, Japan). The reaction was performed as follows: 94 °C for 5 min, 36 cycles of 94 °C for 30 s, 52 °C for 40 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The product was purified and cloned into pMD18-T vector (TaKaRa, Japan), transformed into *E.coli* strain DH5α and sequenced by BGI (China).

2.3. Sequence analysis

The DNAman 6.0 and BLAST software online (http://www.ncbi.nlm.nih.gov/) were used to analyze the DNA and protein sequences. The sequences of other WRKY proteins were obtained from website (http://www.phytozome.com). MEGA 5.10 was used to construct the phylogenetic tree of MlWRKY12 and other WRKY proteins. The online tool WoLF PSORT program (http://wolfpsort.org/) was used to predict the localization of the MlWRKY12 protein.

2.4. Subcellular localization of MlWRKY12 protein

The complete ORF of *MlWRKY12* without stop codon was amplified and cloned into the pB7FWG2 vector (Invitrogen, USA). The *35S::MlWRKY12-GFP* fusion construct was introduced into *Arabidopsis via Agrobacterium*-mediated transformation [43]. Transgenic plants were selected by spraying T1 progeny with Basta. The roots of 7-day-old transgenic plants were examined under a confocal laser scanning microscope (Fluo View FV1000, Olympus, Japan). Propidium iodide (PI, Solarbio, China) was used to stain the membranes and nuclei of dead cells.

2.5. In situ hybridization

The upper second node of 8-week-old *M. lutarioriparius* was fixed in 70% (v/v) ethanol, 5% (v/v) formaldehydeand 5% (v/v) glacial acetic acid and then dehydrated by passing through an ethanol and a xylene series. The xylene was gradually replaced by paraplast plus (Sigma, USA) chips at 60 °C, and, finally, the stems were embedded in paraplast plus boxes and stored at 4 °C before sectioning. The 201 bp fragment of MlWRKY12 was cloned into the pGM-T vector (Tiangen, China). Probes were generated *in vitro* with digoxigenin-UTP using the SP6 or T7 RNA polymerase transcription kit (Roche, Switzerland). The stems were sectioned into 8 μm slices, mounted on lysine treated slides and then dried for two days at 42 °C. Sections were de-waxed with xylene and hydrated through an ethanol series (100% to 0%). The *in situ* hybridization procedure followed Mayer [44]. Images were captured with the OLYMPUS DX51 (Olympus, Japan).

2.6. Immunohistochemistry and toluidine blue O (TBO) staining

Stems of different lines were harvested at 7 weeks. The materials for the section were prepared according to the method described for *in situ* hybridization. The stems were sectioned into 8 μm slices and mounted on slides, and then dried at 42 °C for two days. Sections were de-waxed with xylene and hydrated through