Plant Physiology and Biochemistry 47 (2009) 351–358

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Characterization of expression of the *OsPUL* gene encoding a pullulanase-type debranching enzyme during seed development and germination in rice

Qian-Feng Li [a,b], Gui-Yun Zhang [a], Zhi-Wei Dong [a], Heng-Xiu Yu [a], Ming-Hong Gu [a], Samuel S.M. Sun [b], Qiao-Quan Liu [a,*]

[a] *Key Laboratory of Plant Functional Genomics of Ministry of Education, Jiangsu Key Laboratory of Crop Genetics and Physiology, Agricultural College, Yangzhou University, 12 Wenhui East Road, Yangzhou, Jiangsu 225009, China*

[b] *Institute of Plant Molecular Biology and Agri-Biotechnology, Department of Biology, The Chinese University of Hong Kong, Shatin, Hong Kong, China*

ARTICLE INFO

Article history:
Received 11 August 2008
Accepted 5 February 2009
Available online 12 February 2009

Keywords:
Rice (*Oryza sativa* L.)
Pullulanase-type starch-debranching enzyme
Promoter analysis
Endosperm-specific expression
Real-time PCR
GUS activity

ABSTRACT

Starch-debranching enzymes (DBEs) are key enzymes involved in starch metabolism in cereals, having a dual function, in both starch synthesis and degradation. However, their precise roles in this pathway, particularly their expression profiles, remain unclear. In the present study, we performed a quantitative real-time PCR (Q-PCR) analysis of the expression pattern of the *OsPUL* gene encoding a pullulanase-type DBE in different tissues as well as in seeds at different developmental stages. The results showed that this gene was expressed only in seeds. In addition, the 1177-bp *OsPUL* promoter sequence was cloned, and some endosperm-specific motifs such as the GCN4 and AACA motifs were observed to exist in this region. The promoter was then fused with the *GUS* reporter gene and its expression was carefully investigated in transgenic rice. The data from both histochemical and fluorometric analyses showed that the *OsPUL* promoter was capable of driving the target gene to have a high level of endosperm-specific expression. The *OsPUL* gene maintained a relatively high expression level during the entire period of seed development, and peaked in the middle and late stages. This observation was very consistent with that of the endogenous transcription analysis by Q-PCR. Furthermore, the seed germination experiment showed that the *OsPUL* promoter actively functions in the late stage of seed germination. The expression of the *OsPUL* gene was maintained at a significant level during the entire grain filling period and in the late stage of seed germination, which coincided with its involvement in starch anabolism and catabolism.

1. Introduction

Starch serves as the most important dietary source of energy for humans and is also an inexpensive, natural and renewable raw material that can be utilized for the manufacture of different foodstuffs. It is also important for various other industrial applications depending on its composition and structure. Therefore, a comprehensive understanding of the starch biosynthetic pathway in plants can help us in regulating starch synthesis and to generate novel types of starch with specific compositions and/or structures [24].

In the starch biosynthetic pathway, starch-debranching enzymes (DBEs) play an important role in the direct hydrolysis of the α-1,6-glucosidic linkages of alpha-polyglucans [31]. According to their sequence similarity and substrate specificity, DBEs in higher plants can be divided into two types: isoamylase (ISA, which mediates the debranching of glycogen and amylopectin) and pullulanase (PUL, also known as limit dextrinase or R-enzyme that mediates the debranching of pullulan and amylopectin) [22]. Thus far, the essential roles of DBEs, particularly of isoamylase, in starch metabolism in plants have been widely investigated using DBE-deficient mutants such as *sugary*-type mutants in maize [16], rice [20,27], barley [3], and *Arabidopsis* [36]. The mutant that affects pullulanase expression in maize was first identified by Dinges et al. [7]. Their data showed that pullulanase-type DBE has multiple functions during both starch biosynthesis and catabolism. Wattebled et al. [36] demonstrated that in *Arabidopsis*, pullulanase can partially overcome the lack of isoamylase, although this effect remains dormant when isoamylase is present within the plant cell. In the *sugary-1* mutant of rice, the lack of isoamylase 1 (ISA1) has been shown to exert a pleiotropic effect on other enzymes, particularly on pullulanase [26], and the same phenomena has also

Abbreviations: GUS, beta-glucuronidase; DBE, starch-debranching enzyme; ISA, isoamylase; PUL, pullulanase; DAF, days after following; DAS, days after soaking; Q-PCR, quantitative real-time PCR.

* Corresponding author. Fax: +86 514 87996817.
E-mail address: qqliu@yzu.edu.cn (Q.-Q. Liu).

0981-9428/$ – see front matter © 2009 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.plaphy.2009.02.001

been observed to occur with the *sugary* mutant of maize [7], implying that both types of DBEs might be functioning together, for example, for the production of a larger enzyme complex, during the synthesis of the amylopectin fine structure [20].

In rice, there are three distinct ISA isoforms that are encoded by *OsISA1*, *OsISA2*, and *OsISA3* [21], in contrast to only one copy, namely, *OsPUL*, for pullulanase [10]. Numerous studies have examined the role of isoamylases in starch biosynthesis through biochemical and genetic analyses of ISA-deficient mutants; however, there have been limited studies on the regulation of the expression of these genes, which, therefore, remains unclear. The mRNA level of the maize *PUL* gene (*Zpu1*) in the endosperm was shown to be abundant throughout starch biosynthesis; however, it was not detected in leaves or roots [1]. Recently, Chen et al. [5] fused the maize *Zpu1* promoter with the *GUS* reporter gene and introduced it into tobacco plants. The result on the investigation of GUS expression suggests that the *Zpu1* promoter is active in both anabolism and catabolism of starch. In rice, the expression profile of the *OsPUL* gene using real-time RT-PCR showed that the level of the *OsPUL* transcript in the seeds was significantly higher than that in the leaf [28]. However, no other tissues were examined, and the regulation of gene expression was not investigated.

In the present study, with the aim of obtaining a clear comprehension of *OsPUL* gene expression and its regulation, we characterized the expression of the promoter of this target gene in transgenic rice using the *GUS* reporter gene and performed a detailed analysis. We also performed an RT-PCR analysis of the expression pattern of the endogenous *OsPUL* gene in different parts of the plant body as well as in different developmental stages of the seeds of the rice plant. The present observations, revealed from both promoter and transcriptional expression analyses, showed that the *OsPUL* gene has a precisely specific expression pattern during seed development and germination, indicating its essential role in starch anabolism and catabolism.

2. Materials and methods

2.1. Plant materials and tissue preparation

Two rice varieties, *Oryza sativa* ssp. *indica* cv. 9311 and *O. sativa* ssp. *japonica* cv. Wuxiangjing 9 (WX9), were used in this study. The DNA and RNA from 9311 were used for promoter isolation and transcriptional analysis, respectively, of the endogenous *OsPUL* gene, while the callus from the seeds of WX9 was used for *Agrobacterium*-mediated transformation. Rice plants were grown in the field under normal cultural conditions, and seeds were sampled at different developmental stages and collected at 3, 6, 10, 15, and 20 d after flowering (DAF). Other organs or tissues, such as leaves, leaf sheaths, stems, and roots, were harvested during grain filling. All of the collected samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Total RNA extraction and quantification

Total RNA was extracted from the stored rice samples by the cold-phenol method described by [38]. It was then treated with DNase I (QIAGEN), and was purified by using the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. The quality and quantity of the total RNA were determined by denatured agarose gel electrophoresis as well as by imaging densitometry.

2.3. Two-step real-time RT-PCR

Four housekeeping genes, i.e., those encoding the eukaryotic elongation factor 1-alpha (*eEF-1a*), eukaryotic initiation factor 4a (*eIF-4a*), a-tubulin (*TBL*), and actin 1 (*ACT 1*), were selected as an internal control for normalization of the real-time PCR data [6,30,39]. The primer sequences of the *eEF-1a* and *eEF-4a* genes as described by Jain et al. [15] were used. The primer sequences of the *ACT 1* gene were CTTCATAGGAATGGAAGCTGCGGGTA (forward) and CGACCACCTTGATCTTCATGCTGCTA (reverse), and those of the *TBL* gene were GGAAATACATGGCTTGCTGCTT (forward), and TCTCTTCGTCTTGATGGTTGCA (reverse). The lengths of the amplicons obtained using the above four primer sets were 103, 76, 196, and 86 bp, respectively. The primers for real-time PCR analysis of the *OsPUL* gene (forward, GCTGTCGCTTCTTATGATGCTC and reverse, AAGTGGTCCA GTATAAGCAAACAT) were designed according to the sequence reported by Francisco et al. [10], and the length of the amplicon obtained was 100 bp.

For real-time RT-PCR analysis, 2 μg of each RNA sample was used for reverse transcription performed by using the SuperScript™ First-Strand Synthesis System (Invitrogen) with random hexamers according to the manufacturer's instructions. PCR amplification was performed in a 20-μL volume containing 10 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems, USA), 150 nmol of each specific forward and reverse primer, and 10 ng of the synthesized cDNA. Real-time PCR was performed using an ABI PRISM™ 7700 Sequence Detection System. The amplification protocol was as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The amplification product was validated by melting curve analysis and was checked on a 3% agarose gel.

2.4. Construction of the OsPUL promoter expression vector

Genomic DNA was extracted from the leaf of 9311 by the CTAB method [25]. According to the available whole-genome sequences of 9311 (www.genomics.ac.cn), a set of primers (forward, 5′-CAAGCTTCTAGATTGGACGGAACCTA-3′ and reverse, 5′-CAGGATCCTTGAGAGGGTTTGGATTCGGG-3′) were designed to isolate the 5′-flanking sequence of the *OsPUL* gene (length, 1177 bp) by PCR amplification. The restriction enzyme recognition site of HindIII or Bam HI (underlined within the primers) was added to the 5′-end of the designed primer for the convenience of further vector construction. The cloned DNA fragment was sequenced, and the correct sequence was then fused with the *GUS* coding region and the *NOS* terminator on the binary vector pCAMBIA1300 (www.cambia.com.au). The resultant expression vector p13PUL-GUS was transferred into *Agrobacterium tumefacien* strain EHA105, and was used for rice transformation. In addition, another GUS binary vector expressing *GUS*, in which the *GUS* reporter gene was controlled by the maize *Ubi* promoter, was also used for rice transformation as a positive control with a constitutive expression pattern in the rice plant. The confirmed sequence of the cloned *OsPUL* promoter was searched for known *cis*-regulatory DNA elements using the PLACE database [11].

2.5. Rice transformation and identification of the transformants

Calli derived from the immature embryos of the rice cultivar WX9 were used as explants for *Agrobacterium*-mediated transformation. Tissue culture, transformation, selection, and regeneration of transgenic plants were performed according to the procedure described by [23]. Both PCR and Southern blot analyses were carried out to confirm the integration of the *GUS* fusion gene into the transgenic plants. The primers used in PCR analysis (GUS-F,