UNIVERSITI PUTRA MALAYSIA

FUNCTIONAL ANALYSIS OF BARLEY (HORDEUM VULGARE L.) CELLULOSE SYNTHASE-LIKE F6 PROMOTER THROUGH TRANSGENE EXPRESSION IN RICE (ORYZA SATIVA L.)

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By

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The knowledge of the functional aspects of the promoters are necessary prior to the application of the interested promoters to overexpress transgene in transgenic plants for gene study, improvement of quality traits and biofortification. Currently, there are lacking of characterised endosperm-specific promoters to produce strong transgene expression in the endosperm tissue of cereal plants at a specific grain development or maturation period. The Cellulose synthase-like F6 (*CslF6*) gene is majorly responsible for the production of beta-glucan in the cereal plants, including barley, oat, wheat and rice. Beta-glucan can be found ubiquitously in the endosperm tissues of barley grains. The *HvCslF6* promoter is predicted to drive strong endosperm-specific expression at mid to late grain development stage in transgenic rice, based on previous *HvCslF6* gene expression studies. The present study characterised the functional length of *HvCslF6* promoter and its tissue-specificity expression pattern through transgene expression in rice. The 2771 bp putative promoter of *HvCslF6* gene from *Sloop* barley was isolated and analysed *in-silico*. Multiple endosperm-specific elements were identified along the promoter region, suggesting that the promoter may drive endosperm-specific expression pattern. Two transformation vectors, F6Prom1 (2771 bp *HvCslF6*prom::G*US* gene) and F6Prom3 (1257 bp *HvCslF6*prom::*GUS* gene) were successfully constructed and permanently transformed into the *Nipponbare* rice. The *HvCslF6* promoter was functional in transgenic rice as the GUS blue staining was observed in all tested body part of mature T0 plants and remained in the T1 seedlings. The promoter also drove selectively strong expression activity in the endosperm tissue and embryo of the rice grain in comparison to other plant body parts regardless of the promoter lengths. Both GUS histochemical staining and quantitative GUS activity analysis revealed that the expression of the *GUS* gene driven by 1257 bp *HvCslF6* promoter was more potent than that of 2771 bp. The data suggested that the 1257 bp *HvCslF6* promoter was sufficient to direct strong transgene expression specifically in the endosperm

tissue of the transgenic rice. This verified endosperm-specific promoter will be useful to drive the expression of transgene in rice grain, including beta-glucan synthase, for generating high beta-glucan content rice in the future.

Keywords: Endosperm-specific promoter, *CslF6* gene, barley, transgenic rice, permanent plant transformation, *GUS* reporter gene

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ANALISIS FUNGSI PROMOTER "BARLEY (*Hordeum vulgare* L.) CELLULOSE SYNTHASE-LIKE F6" MELALUI EKSPRESI TRANSGEN DALAM PADI (*Oryza sativa* L.)

Oleh

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Maklumat tentang fungsi sesebuah promoter adalah sangat penting sebelum promoter tersebut boleh digunakan untuk mengkaji fungsi gen, penambahbaikan kualiti ciri tumbuhan dan biofortifikasi. Kini, promoter khusus endosperma untuk mengarahkan ekspresi transgen yang tinggi dalam tisu endosperma tanaman bijirin pada waktu tertentu masih diperlukan. Gen Sintase selulosa-seperti F6 (*CslF6*) bertanggungjawab untuk menghasilkan sejumlah besar beta-glukan dalam tanaman bijirin termasuk barli, oat, gandum dan padi. Beta-glukan boleh didapati dalam kuantiti yang besar dalam tisu endosperma biji barli. Berdasarkan hasil penyelidikan sebelumnya, promoter *HvCslF6* dijangka dapat mendorong ekspresi yang kuat pada tisu endosperma padi transgenik pada pertengahan sehingga hujung fasa permatangan biji benih. Tujuan kajian ini adalah untuk mengenalpasti saiz *HvCslF6* promoter yang berfungsi dan corak ekspresinya melalu ekspresi transgen di dalam padi. Promoter *HvCslF6* bersaiz 2771 bp dari barli jenis *Sloop* telah diasingkan dan elemen pengatur-*cis* juga telah dikenal pasti. Terdapat beberapa elemen yang berkaitan dengan tisu endosperma telah ditemui dalam promoter tersebut yang mencadangkan bahawa promoter ini boleh mendorong corak ekspresi yang khusus di dalam tisu endosperma sahaja. Dua vektor transformasi, F6Prom1 (2771 bp Prom*HvCslF6*::gen *GUS*) dan F6Prom3 (1257 bp Prom*HvCslF6*::gen *GUS*) telah dibina dan dimasukkan secara kekal ke dalam padi jenis *Nipponbare*. Promoter *HvCslF6* berfungsi di dalam pada transgenik apabila perwarnaan biru GUS kelihatan di semua bahagian tumbuhan yang diuji pada matang pokok generasi T0 dan aktivitinya masih kekal pada anak pokok generasi T1. Promoter *HvCslF6* juga dapat mendorong ekpresi yang kuat pada tisu endosperma dan embrio biji padi tanpa menghiraukan saiz promoter yang digunakan. Analisis perwarnaan GUS dan kuantitatif aktiviti GUS protein menunjukkan bahawa ekspresi gen *GUS* yang didorong oleh 1257 bp promoter *HvCslF6* lebih kuat berbanding dengan 2771 bp. Oleh itu, 1257 bp promoter

HvCslF6 berpotensi untuk dipilih sebagai promoter khusus endosperma untuk menyatakan ekspresi transgen dalam bijipadi. Promoter khusus endosperma yang telah dikenalpasti ini sangat berguna untuk mendorong ekspresi transgen termasuk gen yang bertanggungjawab untuk membuat beta-glukan di dalam beras untuk mencipta beras yang mengandungi kandungan beta-glukan yang tinggi di masa yang akan datang.

Kata kunci: Promoter pengkhususan endosperm, gen *CslF6*, barli, beras transgenik, transformasi tumbuhan kekal, *GUS* gen lapor

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I certify that a Thesis Examination Committee has met on 5 November 2020 to conduct the final examination of Azreena binti Jamahari on her thesis entitled "Functional Analysis of Barley (*Hordeum vulgare* L.) Cellulose Synthase-Like F6 Promoter through Transgene Expression in Rice (*Oryza sativa* L.)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

HvCslF6	*Hordeum vulgare* Cellulose synthase-like F6
CslF	Cellulose synthase-like F
CslH1	Cellulose synthase-like H1
CslJ	Cellulose synthase-like J
SCFA	short chain fatty acid
CamV35s	cauliflower mosaic virus 35s
Asglo1	*Avena sativa* globulin 1
TSS	transcription start site
TFIIB	transcription factor II B binding protein
P-Box	Prolamin Box
Dof	DNA-binding with one finger
GUS	β-glucuronidase
DAP	day after pollination
DSB	double stranded breaks
SSG	single stranded gap
T-DNA	transfer deoxyribonucleic acid
LB	Luria Bertani
$CaCl_2$	calcium chloride
$MgCl_2$	magnesium chloride
dNTP	deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
PEG-8000	Polyethethylene glycol 8000
SDS	Sodium dodecyl sulfate

MS	Murashige and skoog
NAA	Naphthylacetic acid
ABA	Abscisic acid
6-BA	6-Benzylaminopurine
CTAB	Cetyl Trimethyl Ammonium Bromide
NaH_2PO_4	Monosodium phosphate
EDTA	Ethylenediaminetetraacetic acid
BSA	Bovine serum albumin
Abs	Absorbance
ul	Microlitre
T0	transgenic plant generation 0
T1	transgenic plant generation 1
SNP	single nucleotide polymorphism
bp	base pair
kb	kilo base pair
4-MU	4-methylumbelliferone
OD	optical density

CHAPTER 1

INTRODUCTION

Mixed-linkage 1,3;1,4-β-D-glucan or also known as beta-glucan is a functional food component in barley that able to reduce the blood cholesterol level upon direct consumption. It is a randomly arranged linear polymer of glucose linked by 1,3- or 1,4- glycosidic bonds. The irregular insertion of 1,3-linkage in between 1,4-linkaged beta-glucan chain forming non-rigid conformation of beta-glucan, making it soluble in water. The beta-glucan is not digested by enzymes in human gastrointestinal tract. Thus, it acts as a source of dietary fibre, increases digesta viscosity as well as act as a food source for gut microbes to improve good gut microbes which help to improve human health.

Beta-glucan is mainly found in the *Poaceae* family, including barley, oat, wheat, rye and others. In cereal plants, beta-glucan presents mostly in the endosperm tissues of the grains with some also being detected transiently in the vegetative tissue with limited quantity (Burton et al., 2008; Coon, 2012; Wong et al., 2015). Beta-glucan is thought to act as energy storage in the grains where it will be consumed for germination. It is a simpler energy source in comparison to starch as the beta-glucan can be broken down by 1,3;1,4-beta-glucan endohydrolase and beta-glucan glucohydrolase enzymes to release the glucose monomers (Bulone et al., 2019).

The Cellulose synthase-like sub-family genes (*CslF*, *CslH* and *CslJ*) were found to produce beta-glucan through transgene expression in the monocotyledonous and dicotyledonous plants (Burton et al., 2011; Doblin et al., 2009; Little et al., 2018; Vega-Sánchez et al., 2015). Among them, barley *CslF6* (*HvCslF6*) was the most potent beta-glucan synthesis gene and highly expressed in the barley endosperm tissues (Burton et al., 2011). Other *CslF* and *CslH* genes, despite expressed relatively low level in other body tissues, were shown to synthesise beta-glucan with distinctive ratio of cellotriose:cellotetraose residues (DP3:DP4) (Little et al., 2018). The DP3:DP4 ratio is assumed to affect the water solubility of the beta-glucan. High water solubility beta-glucan is preferred as an energy source in plants as it improves the surface area for enzyme digestion in aqueous solution (Burton et al., 2010). Meanwhile, beta-glucan in less soluble form is preferable when it is in a plant structural support role (Burton et al., 2010). Based on the previous result, it is concluded that each beta-glucan synthase genes produce beta-glucan with different DP3:DP4 ratio.

However, it is currently not possible to further confirm the assumption without proper transgene expression study in a clean background. Previously, researches were conducted to induce transgene expression in barley grain using constitutive promoters. The resulting grains were malformed, and the plant growth was not normal since the high amount of beta-glucan in all plant tissues negatively affected the plant growth (Burton et al., 2011). Meanwhile,

expression of the *HvCslF6* gene in *Nicotiana benthamiana* leaves produced minute amount of beta-glucan, which was insufficient for further analysis (Wong et al., 2015).

It is essential to produce transgene expressions in large quantities, and in spatiotemporal-specific manner before we can identify the role of each *CslF* and *CslH* genes in beta-glucan synthesis and fine structure determination. Given this, the best option is by using the *HvCslF6* promoter to drive transgene expression in cereal grain with almost non-existence beta-glucan content. The attempt to overexpress *Luciferase* gene driven by the 3 kb *HvCslF6* promoter was shown to be not reactive in *Nicotiana benthamiana* leaves (Dimitroff, 2016). In contrast, relatively strong expression of the reporter gene was observed when driven by 1.75 kb *HvCslF6* promoter in the transiently expressed barley coleoptile, root and first base leaf (Dimitroff, 2016). An alternative for studying the role and expression pattern of the *HvCslF6* promoter is by using rice as the host candidate since it is lacking beta-glucan in their grain and belongs to the cereal plant group. Hence, current project is designed to identify the functional *HvCslF6* promoter region that drives the expression of reporter gene when integrated into rice host plants. The objectives of this project are:

1. To identify the *cis*-regulatory elements within the putative promoter of *HvCslF6* gene that drive the endosperm-specific expression of the transgene
2. To characterise the tissue-specific expression of *HvCslF6* promoter through transgene expression in rice

Scope and Limitation

The study covers the fundamental information on the *HvCslF6* promoter activity and expression pattern in the transgenic rice. The isolation of *HvCslF6* promoter from *Sloop* barley cultivar and identification of the endosperm-specific *cis*-regulatory elements within the promoter region were performed following the protocol by Dimitroff (2016). The promoter was fused with β-Glucuronidase (*GUS*) reporter gene using Hot Fusion cloning to develop the interested plant expression construct (Fu et al., 2014). The permanent rice transformation procedure was performed using *Nipponbare* rice cultivar according to the protocol established by Liu et al. (1998). The quantitative and qualitative analysis of GUS expression pattern was conducted in accordance to the GUS fluorometry and histochemical staining analyses directed by Jefferson (1990) and Alotaibi et al. (2018). The *HvCslF6* promoter activity was analysed from 35 day after pollination (DAP) of the mature transgenic rice plant as well as 3 weeks old T1 generation rice seedlings, based on the *HvCslF6* gene expression profile in barley (Burton et al. 2008). All the chemicals used were purchased from Merck (Germany) otherwise stated.

Research works related to the generation of transgenic rice as well as the analysis of GUS expression pattern and activity was completed during eight

months research attachment at Yangzhou University, China. This includes permanent rice transformation using *Agrobacterium* infection on rice callus, cultivation of transgenic rice until they mature, selection of positive transgenic rice, GUS histochemical staining and GUS fluorometry procedures.

Thesis layout

There are six main chapters outlined in this thesis. The fundamental information of this study was mentioned in Chapter 1. The subsequent chapter shed lights on the detailed knowledge gained about the plant promoter, the importance of studying endosperm-specific promoter to overexpress transgene in plants that lack beta-glucan content and the potential of endogenous promoter of *HvCslF6* gene as an endosperm-specific promoter. In the next section, the methods used to construct the plant expression vector, develop transgenic rice and analysis of the reporter gene activity were outlined. Thereafter, the findings on the *cis*-regulatory elements that may affect the functionality of the *HvCslF6* promoter and its tissue-specificity expression pattern in transgenic rice were evaluated experimentally. The findings were further discussed while limitations of this study were highlighted in Chapter 5. Finally, the conclusion and future research recommendations were also outlined to further improve the knowledge of the *HvCslF6* promoter activity and its applications to express strong transgene expression in rice grain.

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