DNA FREE TRANSCRIPTIONAL ACTIVATION USING CRISPR/DCAS9 NUCLEOPROTEINS TO ENHANCE THE BIOSYNTHESIS OF STEVIOL GLYCOSIDES IN STEVIA (*Stevia rebaudiana* Bertoni)

By

ASISH KUMAR GHOSE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

Dedicated to My Heavenly Father

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Professor Datin Siti Nor Akmar binti Abdullah, PhD
Institute : Plantation Studies

Steviol glycosides (SGs) are responsible for the sweetness of stevia (*Stevia rebaudian* Bertoni) which are 100–300 folds sweeter than sucrose. Stevioside and rebaudioside A present in stevia are the most prominent and desirable SGs as natural sweetening agents endowed with various medicinal properties. *In vitro* regeneration offers great potential to meet commercial demand for expanding stevia cultivation. This study aims in developing an efficient *in vitro* propagation protocol for stevia through callus induction and evaluates the effect of nutrient content in growth media on SGs biosynthetic gene expression and production of stevioside and rebaudioside A in the leaves. In order to enhance the production of the high-value rebaudioside A, the CRISPR/dCas9 a gene activation system was investigated as a novel way of improving the production of this metabolite. The efficacy of Clorox and ethanol as surface sterilizing agents for explant (leaf segments) was investigated. The highest percentage of survivability (88.90 ± 5.55) of explants was found at 15 and 30 days after inoculation (DAI) on Murashige and Skoog (MS) media by sterilization with 30% Clorox for 5 min and 10% Clorox for 10 min, respectively. Addition of 2, 4-D (0.00 to 2.00 mg/L) and Zeatin (0.1 mg/L) was evaluated for callus induction from the leaf explants. The MS media containing 0.50 mg/L 2, 4-D and 0.1 mg/L zeatin stimulated 50% of explants to develop callus at 15 DAI while 1.50 mg/L 2, 4-D and 0.1 mg/L zeatin resulted in 76.67% callus at 30 DAI. The effectiveness of adding BAP (0.0 to 10.0 mg/L) and NAA (0.0 to 1.0 mg/L) for initiation of shoots from stevia calli was investigated. The highest shoot proliferation per callus was achieved with 10.0 mg/L 6-benzyl amino purine (BAP) in MS at 15 DAI (5.8) and 30 DAI (12.33). The highest average length of shoots was achieved with BAP (10.0 mg/L) and 1.0 mg/L naphthalene acetic acid of 4.31 cm and 6.04 cm at 15 and 30 DAI, respectively. The different strengths of MS media were utilized as rooting media. MS media (0.50 strength) induced 2.86 and 6.20 roots per shoot and produced 3.25 cm and 7.82 cm long roots at 15 and 30 DAI, respectively. The highest concentration of rebaudioside A (6.53%) accumulated in the leaves of stevia grown on 0.25 MS and this was correlated with its biosynthetic gene uridine-diphosphate-dependent (UDP)-

glycosyltransferase *76G1* (*UGT76G1*) expression level. The dCas9 fused with VP64 as transcriptional activation domain (TAD) was produced and purified for the formation of ribonucleoproteins (RNPs) by mixing with four *in vitro* transcribed sgRNAs designed by online based tool, benchling. The protocol for efficient protoplasts isolation was optimized by utilizing the combinations of different cell wall degrading enzymes (cellulase R-10 and macerozyme R-10) at different concentrations. The highest protoplast yield was from leaf mesophyll of *in vitro* grown stevia plantlets (3.16×10^6/g of FW) using ES5 (1.25 % cellulase R-10 and 0.75% macerozyme R-10). The transcriptional activation efficiencies were evaluated from the transfected protoplasts with different RNPs through Polyethylene glycol (PEG)-mediated transfection. The highest endogenous activation of *UGT76G1* gene expression was detected at 27.51-fold after 24 h of transfection with RNP30 consisting of CRISPR/dCas9-TAD with sgRNA30 and similar activation level was obtained using RNP18, RNP33, and RNP34, produced using sgRNA18, sgRNA33, and sgRNA34, respectively. Activation of *UGT76G1* by RNP18 led to significant increase in the expression of the rate limiting enzyme *UGT85C2* by 2.37-fold and there was an increasing trend in the expression of *UGT85C2* using RNP30, RNP33 and RNP34. The results obtained from *in vitro* regeneration of stevia provided a protocol for high quality planting materials production for commercial cultivation. The expression of *UGT76G1* can be a universal biomarker for monitoring the biosynthesis of rebaudioside A, the most desirable SGs in efforts to improve its production through growth media manipulation. Successful application of CRISPR/dCas9-TAD RNP in activating specific genes in stevia protoplasts provided a platform for gene functional studies in stevia while paving the way for production of DNA-free genetically modified crops that can improve public acceptance.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGAKTIFAN TRANSKRIPSI BEBAS DNA MENGGUNAKAN NUKLEOPROTEIN CRISPR/DCAS9 UNTUK MENINGKATKAN BIOSINTESIS STEVIOL GLIKOSIDA DALAM STEVIA (*Stevia rebaudiana* Bertoni)

Oleh

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Steviol glikosida (SGs) bertanggungjawab terhadap kemanisan stevia (*Stevia rebaudian* Bertoni) yang mana 100–300 kali ganda lebih manis daripada sukrosa. Steviosida dan rebaudiosida A yang terdapat dalam stevia adalah SG yang paling utama dan diperlukan sebagai agen pemanis semulajadi yang dikurniakan pelbagai khasiat perubatan. Penjanaan semula *in vitro* menawarkan potensi yang besar untuk memenuhi permintaan komersial untuk mengembangkan penanaman stevia. Kajian ini bertujuan untuk membangunkan protokol pembiakan *in vitro* yang cekap untuk stevia melalui induksi kalus dan menilai kesan kandungan nutrien dalam media pertumbuhan ke atas ekspresi gen biosintetik SG dan pengeluaran steviosida dan rebaudiosida A dalam daun. Untuk meningkatkan pengeluaran rebaudiosida A bernilai tinggi, sistem pengaktifan gen CRISPR/dCas9 A telah disiasat sebagai cara baru untuk menambahbaik pengeluaran metabolit ini. Keberkesanan Clorox (15% natrium hipoklorida) dan etanol sebagai agen pensterilan permukaan untuk eksplan (segmen daun) telah disiasat. Peratusan kemandirian tertinggi (88.90 ± 5.55) eksplan didapati pada 15 dan 30 hari selepas inokulasi (DAI) pada media Murashige dan Skoog (MS) secara pensterilan masing-masing dengan 30% Clorox selama 5 minit dan 10% Clorox selama 10 minit. Penambahan 2, 4-D (0.00 hingga 2.00 mg/L) dan zeatin (0.1 mg/L) telah dinilai untuk aruhan kalus daripada eksplan daun. Media MS yang mengandungi 0.50 mg/L 2, 4-D dan 0.1 mg/L zeatin menghasilkan 50% kalus pada 15 DAI manakala 1.50 mg/L 2, 4-D dan 0.1 mg/L zeatin menghasilkan 76.67% kalus pada 30 DAI. Keberkesanan penambahan BAP (0.0 hingga 10.0 mg/L) dan NAA (0.0 hingga 1.0 mg/L) untuk permulaan pucuk daripada kalus stevia telah disiasat. Percambahan pucuk tertinggi bagi setiap kalus dicapai dengan 10.0 mg/L 6-benzyl amino purine (BAP) dalam MS pada 15 DAI (5.8) dan 30 DAI (12.33). Purata panjang pucuk tertinggi dicapai dengan BAP (10.0 mg/L) dan 1.0 mg/L asid asetik naftalena dengan 4.31 cm dan 6.04 cm masing-masing pada 15 dan 30 DAI. Kekuatan media MS yang berbeza telah digunakan sebagai media pengakaran. Media MS (kekuatan 0.50) menginduksi 2.86 dan 6.20 akar bagi setiap

pucuk dan menghasilkan 3.25 cm dan 7.82 cm akar panjang masing-masing pada 15 dan 30 DAI. Kepekatan tertinggi rebaudiosida A (6.53%) terkumpul dalam daun stevia yang ditanam pada 0.25 MS yang dikaitkan dengan tahap ekspresi gen biosintetik uridine-diphosphate-dependent (UDP)-glycosyltransferase *76G1* (*UGT76G1*). dCas9 yang digabungkan dengan VP64 sebagai domain pengaktifan transkrip (TAD) telah dihasilkan dan ditulenkan untuk pembentukan ribonukleoprotein (RNPs) dengan mencampurkan dengan empat sgRNA yang ditranskripsi secara *in vitro* yang direka oleh alat berasaskan dalam talian, iaitu benchling. Protokol untuk pengasingan protoplas yang cekap telah dioptimumkan dengan menggunakan gabungan enzim pengurai dinding sel yang berbeza (selulase R-10 dan macerozyme R-10) pada kepekatan yang berbeza. Hasil protoplas tertinggi adalah daripada mesofil daun anak benih stevia yang dibiak secara *in vitro* (3.16×10^6/g FW) menggunakan ES5 (1.25 % selulase R-10 dan 0.75% macerozyme R-10). Kecekapan pengaktifan transkrip dinilai daripada protoplas yang ditransfeksi dengan RNP yang berbeza melalui transfeksi PEG-perantara. Pengaktifan endogenus tertinggi bagi gen *UGT76G1* dikesan pada 27.51-kali ganda selepas 24 jam transfeksi dengan RNP30 yang terdiri daripada CRISPR/dCas9-TAD dengan sgRNA30 dan tahap pengaktifan yang serupa diperoleh menggunakan RNP18, RNP33, dan RNP34, masing-masing dihasilkan menggunakan sgRNA18, sgRNA33, dan sgRNA34. Pengaktifan *UGT76G1* oleh RNP18 membawa kepada peningkatan ketara dalam ekspresi enzim pengehad kadar *UGT85C2* sebanyak 2.37-kali ganda dan terdapat trend peningkatan dalam ekspresi *UGT85C2* menggunakan RNP30, RNP33 dan RNP34. Keputusan yang diperoleh daripada penjanaan semula *in vitro* stevia menyediakan protokol untuk pengeluaran bahan tanaman berkualiti tinggi untuk penanaman komersial. Ekspresi *UGT76G1* boleh menjadi penanda bio universal untuk memantau biosintesis rebaudiosida A, SG yang paling diingini dalam usaha menambahbaik pengeluarannya melalui manipulasi media pertumbuhan. Aplikasi CRISPR/dCas9-TAD RNP yang berjaya dalam mengaktifkan gen tertentu dalam protoplas stevia menyediakan platform untuk kajian fungsi gen dalam stevia disamping membuka jalan untuk penghasilan tanaman diubah suai genetik bebas DNA yang boleh menambahbaik penerimaan awam.

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ASISH KUMAR GHOSE, 2023

I certify that a thesis examination committee has met to conduct the final examination of name on his Doctor of Philosophy thesis entitled in accordance with Universiti Pertanian Malaysia (Higher Degree) act 1980 and Universiti Pertanian Malaysia (Higher Degree) regulations 1981. The committee recommends that the candidate be awarded the relevant degree.

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Declaration by Members of the Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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ATP	Adenosine Triphosphate
bp	base pair
Cas9	CRISPR-associated protein 9
cDNA	Complementary Deoxyribonucleic Acid
CRISPR	Clustered Regularly Spaced Palindromic Repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
crRNA	CRISPR RNA
DAI	Days After Inoculation
dCas9	deactivated Cas9
DNA	Deoxyribo Nucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotide Triphosphate
DSB	Double-Stranded Break
dsRNA	Double-Stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
GFP	Green Fluorescent Protein
GMO	Genetically Modified Organism
GOI	Gene of Interest
gRNA	Guide RNA
GUS	b-glucuronidase
HDR	Homology Directed Repair
HR	Homologous Recombination

HRMA	High Resolution Melting Analysis
kb	Kilobase
MEP	Methylerythriol-4-Phosphate
miRNA	microRNA
mRNA	messenger RNA
NHEJ	Non-Homologous End Joining
OD	Optical Density
ORF	Open Reading Frame
PAM	Protospacer Adjacent Motif
PCR	Polymerase Chain Reaction
PEG	Poly Ethylene Glycol
qPCR	Real-Time qPCR
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
RPM	Revolutions Per Min
RT	Reverse Transcription
RT-PCR	Reverse Transcriptase-PCR
scRNA	Scaffold RNA
SDS	Sodium Dodecylsulfate
sgRNA	Single Guide RNA
SGs	Steviol Glycosides
TAD	Transcription Activation Domain
TALE	Transcription Activator-Like Effectors
TALEN	Transcription Activator-Like Effector Nucleases

TBE	Tris/Borate/EDTA Buffer
tracrRNA	transactivating CRISPR RNA
TSS	Transcription Start Site
UGTs	Uridine-Diphosphate-Dependent Glycosyltransferases
VIGS	Virus Induced Gene Silencing
VP16	Viral Protein 16
VP64	Four Repeats Of VP16
DW	Dry Weight
FW	Fresh Weight
ROS	Reactive Oxygen Species

CHAPTER 1

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a member of the Asteraceae family and considered as the most promising plant having natural sweetening and medicinal values. It bears sweet steviol glycosides (SGs) which are 250 times sweeter than table sugar (Lemus-Mondaca et al., 2012) and can be used as a sucrose replacement (Talevi, 2018; Michael, 2017). Stevia is extensively utilized as a substitute for table sugar in beverages, foods, and medication in several countries, and many commercial products containing the derivatives of stevia have been developed (Momtazi-Borojeni et al., 2016). It is currently grown for food and pharmaceutical applications in Japan, China, Korea, Brazil, Thailand, Taiwan, the Philippines, Hawaii, Malaysia, and across South America (Noranida et al., 2015). China is the largest market of the manufactured products in the world. The Global Stevia Market has an estimated market value in 2021 of $539.68 million and in 2028, it is projected to reach US$ 965.82 million with a growing Compound Annual Growth Rate (CAGR) of 8.7% from 2021 to 2028 (Global Stevia Market Forecast Report 2021-2028).

According to recent studies, stevia derivatives might also be employed for various medicinal uses such as anti-diabetic (Kurek and Krejpcio, 2019), anti-microbial, anti-oxidant, and anti-inflammatory (Lemus-Mondaca et al., 2018), anti-carcinogenic (Panagiotou et al., 2018), and anti-hyperglycemic (Kamath, 2016). The SGs does not break down in the human body due to them having zero-caloric potential but rather they just pass through the digestive tract making them safe for diabetic patients (Zaidan et al., 2019; Kurek and Krejpcio, 2019).

SGs are a complex combination of related chemicals, with some SG forms imparting a sweet flavour and others imparting a bitter or metallic flavour (Ceunen and Geuns, 2013a). Out of the eight SGs, stevioside is the major constituent with sweetening potential and the rebaudioside A is the most desirable sweetening ingredient having appealing flavour without bitter taste after consumption (Yadav et al., 2011). The biosynthesis of SGs has been well researched and studied (Brandle and Telmer, 2007). Cytosolic UDP-dependent glycosyltransferases catalyse the majority of the critical processes in SGs production (UGTs). *UGT85C2*, *UGT74G1*, and *UGT76G1* were identified as being crucial in the production of rebaudioside A, the most highly valued SGs based on the expression of these genes that encode UGTs in the biosynthetic pathway of SGs (Yadav and Guleria, 2012). The protein UGT85C2 converts steviol to steviolmonoside, UGT74G1 is responsible for the conversion of steviolbioside to stevioside and in the final step of the MEP-pathway, UGT76G1 converts the stevioside to rebaudioside A. The accumulation of rebaudioside A directly correlated with the expression level of *UGT76G1* (Behroozi et al., 2017).

In vitro regeneration of plants is the only tool for rapid and efficient development of stevia, which is needed to leverage the industrial application of stevia (Pande and Gupta,

2013; Yadav et al., 2011). Plant tissue culture techniques offer tremendous ability to improve consistency and abundance in the supply of stevia planting materials, as well as the bioactive compounds. The variety of explants used, the category and amount of plant growth hormones, and the *in vitro* growth environment all impact the formation of tissue culture plantlets from stevia (Kazmi et al., 2019), and need to be optimized for commercial production. For the development of disease-free genotypes and enhanced production of active compounds, advancements in plant tissue culture technologies like refinement of growing media for *in vitro* production of plant are widely utilized. Furthermore, significant amounts of steviol glycosides have been extracted from *in vitro* regenerated plant cells compared to field-grown stevia (Golkar et al., 2019; Kazmi et al., 2019). Under *in vitro* conditions, the plant tissue culture can be used to generate steviol glycosides on a long-term basis.

The "Clustered Regularly Interspaced Short Palindromic Repeats" (CRISPR)/Cas9 system with dysfunctional Cas9 endonuclease offers a powerful targeted genetic modification apparatus for gene functional studies and ultimately heritable trait improvement. Despite the fact that this dead variant of Cas9 is unable to break DNA, the dCas9 still can locate and attach to DNA with the same specificity as functional Cas9. CRISPR/dCas9 system has been recently proven to be a versatile tool that can recruit various modifying enzymes and transcriptional activators to the targeted genomic site through fusion with dCas9 (Moradpour and Abdullah 2020). Artificial transcriptional activators provide a useful means for gene activation by administering a transcription activation domain (TAD) to a particular gene promoter at the native genomic locus via a programmable DNA-binding module (Li et al., 2017). Recruitment of these regulators to promoters adjacent to transcription start site (TSS) through the CRISPR/dCas9 system can modify the level of expression of the targeted genes (Lowder et al., 2018; Li et al., 2017). A few efficient dCas9-based gene activation systems for plant cells have recently been reported (Li et al., 2017; Lowder et al., 2015; Piatek et al., 2015; Vazquez-Vilar et al., 2016). In terms of precision, efficiency, and versatility, it outperforms other methods such as utilization of transcription activator-like effectors (TALEN) and zinc-finger proteins (Qi et al., 2013).

Although the modifications caused by the CRISPR/dCas9 systems are similar to naturally occurring mutations, the employment of transgenic systems during the creation of specific varieties needs to follow GMO laws in nations that rely on process-based regulations (Murovec et al., 2018). Furthermore, insertional mutagenesis that occurs as a consequence of the stable integration of DNA coding regions into plant genomes utilising CRISPR techniques, may result in mutations at off-target sites. These disadvantages can be avoided by delivering ribonucleoproteins (RNPs) complexes containing purified recombinant enzyme dCas9 and single guide RNA (sgRNA) produced *in vitro*. dCas9 binding competes with the natural transcription process at the targeted site, enabling reversible gene activation or suppression, rather than irrevocably changing the genome (Moradpour and Abdulah, 2020).

This study focused on two strategies for enhancing SG production in stevia. The first was through media manipulation in tissue culture. The second was through transcriptional gene activation via a CRISPR/dCas9 platform of a key SG biosynthetic

gene, *UGT76G1*. UGT76G1 converts stevioside to rebaudioside A, which increases the organoleptic characteristics of SGs (Yoneda et al., 2017; Moon et al., 2020). Selection of the perfect transcriptional regulators, including the intended gene, particular target sites, and delivering the CRISPR/dCas9 and sgRNA complex is all critical to the application's success. The objectives of this study were :

1) To develop an efficient *in vitro* propagation protocol for stevia and to evaluate the effect of nutrient content of growth media on SGs biosynthetic gene expression and production of stevioside and rebaudioside A;
2) To design, and synthesize single guide RNAs (sgRNAs) through *in vitro* transcription to be used for direct formation of ribonucleoprotein (RNPs) complexes by preassembling of purified dCas9-TAD protein;
3) To develop protoplast isolation and transfection methods for stevia; and
4) To detect and evaluate the transcriptional activation efficiencies of the RNPs complexes composed of dCas9-TAD and different sgRNAs targeting key genes for rebaudioside A production in transfected stevia protoplasts.

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