DEVELOPMENT OF GENE MANIPULATION SYSTEMS FOR GREEN MICROALGA (Ankistrodesmus convolutus Corda.)

TRAN THANH

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DEVELOPMENT OF GENE MANIPULATION SYSTEMS FOR GREEN MICROALGA (*Ankistrodesmus convolutus* Corda.)

By

TRAN THANH

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

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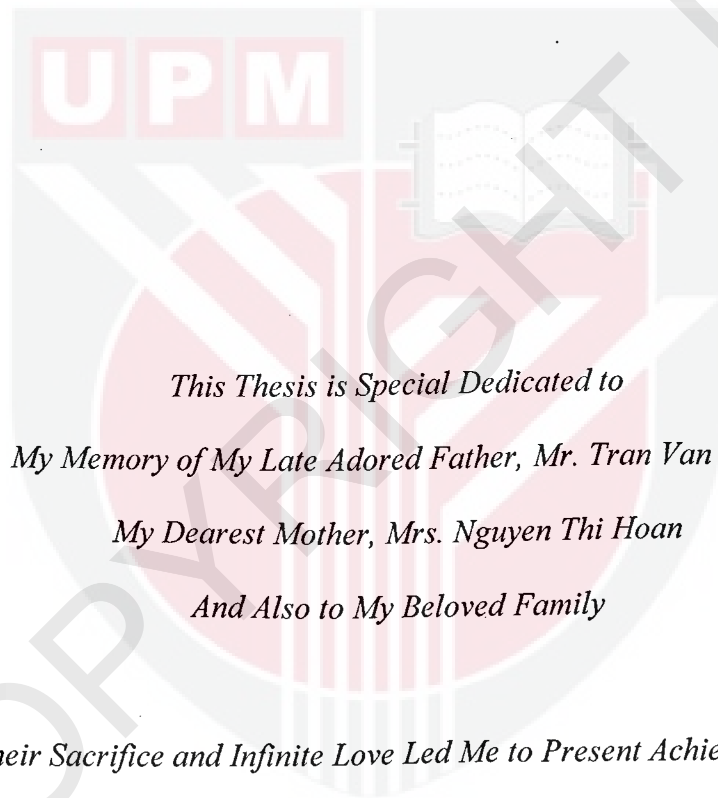

This Thesis is Special Dedicated to

My Memory of My Late Adored Father, Mr. Tran Van Chai

My Dearest Mother, Mrs. Nguyen Thi Hoan

And Also to My Beloved Family

Their Sacrifice and Infinite Love Led Me to Present Achievements

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

DEVELOPMENT OF GENE MANIPULATION SYSTEMS FOR GREEN MICROALGA (*Ankistrodesmus convolutus* Corda.)

By

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January2012

Chairman: **Associate Professor Suhaimi Napis, Ph.D**

Faculty : **Biotechnology and Biomolecular Sciences**

Green microalga *Ankistrodesmus convolutus* is a fast growing alga which produces appreciable amount of carotenoids and polyunsaturated fatty acids. In addition of the ease and cost-effectiveness of culture, the ability of *A. convolutus* to form floating aggregates during its normal growth facilitate harvesting and other beneficiary attributes making it an interesting candidate for many biotechnological applications such as production of natural products or expression of recombinant proteins. However, the lack of efficient genetic transformation systems has been a major limitation in the manipulation of green microalgae. The present study provides, for the first time, information on molecular manipulation in *A. convolutus*, which covered the isolation of nucleic acids, construction of cDNA library and generation of ESTs, cloning and characterization of a highly-expressive cDNA and its promoter towards the establishment of an alternative expression system using *A. convolutus*.

Green microalga *Ankistrodesmus convolutus* was collected from axenic freshwater, grown and maintained in bold's basal medium. *A. convolutus* is typically rich in

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lipids and polysaccharides, which make it difficult to get the intact RNA of high quality and quantity suitable for molecular research on this alga. In an effort to develop a suitable RNA extraction procedure for *A. convolutus*, a rapid, relatively non-toxic and effective method was finalized. This procedure was able to produce high quality and intact RNA which was of sufficient quality and suitable for downstream application such as RT-PCR, northern hybridization and cDNA library construction. The developed procedure may also have wider applicability for total RNA isolation from other green microalgae species. In order to provide a robust sequence resource that can be exploited for gene discovery, genome annotation and comparative genomics, a cDNA library of *A. convolutus* was constructed and preliminarily analysed. A total of 415 randomly selected clones were isolated from the primary cDNA library from which 201 high quality ESTs were produced after the sequencing of 337 clones. Among these ESTs, 21.4% of them were found to be related to gene expression, 14.4% to photosynthesis, 10.9% to metabolism, 5.5% to miscellaneous, 2.0% to stress response, and the remaining 45.8% were classified as novel genes. Due to the relatively high abundance, the full-length cDNA of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit (*RbcS*) which was classified into photosynthetic cDNA clone category was selected for further studies. Rubisco is currently the target enzyme for improving the efficiency of photosynthesis in the hope of increasing the yield and growth of crops and henceforth green algae. In this study, the full-length of *A. convolutus RbcS* cDNA (*AcRbcS*) contained an open reading frame of 165 amino acids. This cDNA encoded a protein with expected molecular weight of ~21 kD sharing high homology with corresponding protein from other microalgae. Southern hybridization analysis revealed that *AcRbcS* is a member of a small multigene family comprising of two to

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six members in *A. convolutus* genome. Under different illumination conditions, RT-PCR analysis showed that *AcRbcS* transcription was reduced in the dark, and drastically recovered in the light condition. As a primary step to investigate the use of *A. convolutus* as an alternative expression system for the production of recombinant proteins, the *AcRbcS* promoter region was isolated through PCR-based methods including ligation-mediated PCR and thermal asymmetric interlaced (TAIL)-PCR. In comparison with the ligation-mediated PCR technique, TAIL-PCR has proven to be an economic, efficient and rapid method to isolate *AcRbcS* promoter region. The *AcRbcS* promoter sequence was then analyzed and later used to construct an expression vector for expression of heterologous genes in *A. convolutus*. The transcription start site (TSS), consensus TATA-box and several putative *cis*-acting elements of representative light-regulatory as well as conserved motifs involved in light responsiveness were found in *AcRbcS* promoter region. The expression vector containing *AcRbcS* promoter and β-glucuronidase (*gusA*) reporter gene were constructed and introduced into *A. convolutus* cells by electroporation. As a result, transgenic *A. convolutus* lines was successfully generated and shown to contain the transgenes by PCR and southern hybridization analysis. It was also demonstrated that the *AcRbcS* promoter could function as a light-regulated promoter in transgenic *A. convolutus* as shown in northern hybridization analysis.

The results presented here represent the initial success on molecular manipulation of green microalgae *A. convolutus*, which has great potentials to become an interesting and excellent candidate for many biotechnological applications. The achievements therefore advance the development of *A. convolutus* as an alternative expression system, and it is now feasible that this alga can be examined as an alternative host for the expression of recombinant proteins.

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

**PEMBANGUNAN SISTEM MANIPULASI
GEN UNTUK MIKROALGA HIJAU
(*Ankistrodesmus convolutus* Corda.)**

Oleh

TRAN THANH

Januari 2012

Pengerusi : Profesor Madya Suhaimi Napis, Ph.D

Fakulti: Bioteknologi and Sains Biomolekul

Mikroalga hijau *Ankistrodesmus convolutus* adalah sejenis alga yang mampu tumbuh dengan cepat dan menghasilkan jumlah karotenoid and asid lemak politaktepu yang tinggi. Tambahan pula ia senang dikultur dan kos pengkulturan yang rendah, keupayaan *A. convolutus* untuk membentuk agregat terapung semasa pertumbuhan yang normal memudahkan penuaian dan waris sifat-sifat yang lain menjadikannya calon yang menarik untuk pelbagai aplikasi bioteknologi seperti penghasilan produk semulajadi atau ekpresi protein rekombinan. Walaubagaimanapun, kekurangan sistem transformasi genetik yang cekap telah menjadi halangan utama dalam manipulasi mikroalga hijau. Kajian ini, untuk kali pertama menyediakan, informasi untuk manipulasi molekul dalam *A. convolutus* yang meliputi pengasingan asid nukleik, pembinaan perpustakaan cDNA dan generasi penanda jujukan terekspres (EST), pengklonan dan pencirian ekspresi cDNA tahap tinggi dan promoternya ke arah pembinaan sistem ekpresi alternatif menggunakan *A. convolutus*.

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Mikroalga hijau *Ankistrodesmus convolutus* telah dikutip daripada air tawar axenik, dikultur dan dikekalkan dalam medium bold's basal. *A. convolutus* biasanya kaya dalam lipid dan polisakarida, yang menyebabkan ia sukar mendapatkan RNA utuh yang berkualiti tinggi dan kuantiti yang bersesuaian bagi penyelidikan molekul dalam alga ini. Dalam usaha untuk membangunkan prosedur pengasingan RNA bagi *A. convolutus*, satu kaedah yang, relatifnya tidak beracun dan berkesan telah ditemui dan disahkan. Prosedur ini telah berjaya menghasilkan RNA utuh yang berkualiti tinggi dan sesuai untuk aplikasi hiliran seperti RT-PCR, hibridisasi utara dan pembinaan perpustakaan cDNA. Prosedur yang dimajukan ini mampu diguna pakai secara meluas bagi pengasingan jumlah RNA daripada spesies mikroalga hijau yang lain. Untuk menyediakan sumber urutan yang teguh yang dapat dieksploitasi bagi penemuan gen, penjelasan genom dan genomik perbandingan, perpustakaan cDNA untuk *A. convolutus* telah dibinakan dan dianalisakan pada peringkat awal. Sejumlah klon 415 yang dipilih secara rawak telah diasingkan daripada perpustakaan cDNA primer dari mana 201 EST yang bersihkan telah dihasilkan setelah menjujukan 337 klon. Antara EST ini, 21.4% daripadanya yang ditemui berkaitan dengan ekpresi gen, 14.4% dengan fotosintesis, 10.9% dengan metabolisma, 5.5% dengan kepelbagaian, 2.0% dengan tindak balas tekanan dan yang bakinya 45.8% telah diklasifikasikan sebagai gen yang baru. Disebabkan relatif kelimpahan yang tinggi, rantaian cDNA penuh yang lengkap bagi ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) subunit kecil (*RbcS*) yang telah diklasifikasikan ke dalam kategori cDNA klon fotosintesis telah dipilih untuk pengajian seterusnya. Rubisco kini adalah sasaran enzim untuk memperbaiki kecekapan fotosintesis dengan harapan meningkatkan penghasilan dan penumbuhan tanaman termasuklah alga hijau. Dalam kajian ini, barisan cDNA *A. convolutus* (*AcRbcS*) yang penuh

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mengandungi bacaan bingkaian terbuka sebanyak 165 asid amino. cDNA ini mengkodkan protein dengan jangkaan berat molekul ~21 kD berkongsi homologi yang tinggi dengan protein yang kesamaan daripada mikroalga yang berlainan. Analisa hibridisasi selatan mendedahkan *AcRbcS* adalah ahli keluarga pelbagai gen kecil terdiri daripada dua hingga 5 ahli dalam genome *A. convolutus*. Di dalam keadaan illuminasi yang berbeza, analisa RT-PCR menunjukkan transkrip *AcRbcS* telah berkurang dalam keadaan gelap dan kembali pulih secara ketara dalam keadaan cahaya. Sebagai langkah utama untuk mengkaji kegunaan *A. convolutus* sebagai sistem ekpresi alternatif bagi menghasilkan protein rekombinasi, rantau promoter *AcRbcS* telah diasingkan melalui kaedah PCR termasuk PCR pencantuman pengantaraan dan PCR haba simetri berjalin (TAIL). Berbanding dengan kaedah PCR pencantuman pengantaraan, TAIL-PCR telah menunjukkan sebagai kaedah yang ekonomi, cekap dan pantas dalam pengasingan rantau promoter *AcRbcS*. Jujukan promoter *AcRbcS* kemudian dianalisa dan seterusnya digunakan untuk membina vektor ekpresi bagi pengekpresian gen heterologous di *A. convolutus*. Tapak permulaan transkripsi (TSS), konsensus kotak TATA dan beberapa unsur-unsur yang dianggap sebagai cis-bertindak yang mewakili pengawalseliaan cahaya serta motif diperlihat yang terlibat dalam responsif cahaya telah dijumpa dalam rantau promoter *AcRbcS*. Vektor ekpresi yang mengandungi promoter *AcRbcS* dan gen pelapor β-glucuronidase (*gusA*) telah dibina dan diperkenalkan ke dalam sel *A. convolutus* melalui elektroporasi. Keputusan mendapati, talian transgenik *A. convolutus* telah berjaya digenerasikan dan ditunjukkan mengandungi transgen melalui PCR dan analisa hibridisasi selatan. Ia juga menunjukkan bahawa promoter *AcRbcS* dapat berfungsi sebagai promoter pengawalselia cahaya dalam transgenik *A. convolutus* seperti yang ditunjukkan dalam analisa hibridisasi utara.

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Keputusan yang dibentangkan ini menunjukkan kejayaan awal dalam memanipulasi molekul untuk mikroalga hijau *A. convolutus*, yang berpotensi besar dan menjadi calon yang menarik dan cemerlang untuk pelbagai aplikasi bioteknologi. Penemuan dan pencapaian ini telah memajukan pembangunan *A. convolutus* sebagai sistem ekpresi alternatif dan kini alga ini dapat dikaji sebagai host alternatif bagi ekspresi protein rekombinan.

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
λ	Lambda
g	Gravitational acceleration
µg	Microgram
µl	Microliter
°C	Degree centigrade
%	Percentage
AD	Arbitrary degenerate
BBM	Bold's basal medium
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
Ca	Calcium
CaMV35S	Cauliflower mosaic virus 35S
cDNA	Complementary DNA
CI	Chloroform:isoamyl alcohol
Cl	Chloride
cm	Centimeter
C-terminal	Carboxyl terminal
CTAB	Cetyltrimethylammonium bromide
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate

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dGTP	2'-deoxy-guanosine-5'-triphosphate
DEPC	Diethyl pyrocarbonate
DMSO	Dimethylsulphonyl oxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotides
DTT	Dithiothreitol
dTTP	Thymidine-5'-tryphosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
EST	Expressed sequence tag
g	Gram
gusA	ß-glucuronidase
h	Hour(s)
HCl	Hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid
hpt	Hygromycin phosphotransferase
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
K	Potassium
KOAc	Potassium acetate
kb	Kilo base-pair
kV	Kilo volt
L	Liter
LB	Luria-bertani
LiCl	Lithium chloride

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M	Molar
Mb	Mega base-pair
MES	2-(N-Morpholino)ethanesulfonic acid
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
MOPS	3-(N-morpholino) propane-sulphonic acid
min	Minute(s)
mL	Milliliter
mM	Millimolar
mRNA	Messenger RNA
Na	Sodium
NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NCBI	National center for biotechnology information
ng	Nanogram
Nos	Nopaline synthase gene terminator
N-terminal	Amino-terminal
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCI	Phenol:chloroform:isoamyl alcohol
PCR	Polymerase chain reactions
pfu	Plaque forming units

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pI	Isoelectric point
PLACE	Plant *cis*-acting regulatory DNA elements
PlantCARE	Plant *cis*-acting regulatory elements
Poly $(A)^+$	Polyadenylated (mRNA)
ppm	Part per million
PVP	Polyvinylpyrrolidone
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SA-AP	Streptavidine alkaline-AP
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	Saline sodium citrate
TAE	Tris acetate EDTA
TAIL-PCR	Thermal asymmetric interlaced polymerase chain reaction
T-DNA	Transferred-DNA
TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethylethylene diamine
Tm	Melting temperature
TSS	Transcription start site
U	Unit

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UTR	Untranslated region
UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
X	Times
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER 1

INTRODUCTION

Algae are a remarkably diverse and fascinating group of organisms that are of fundamental ecological importance as primary producers and as basic components of the food chain. They are accountable for the net primary production of ~52 billion tons of organic carbon per year, which is ~50% of the total organic carbon produced on earth each year (Field et al., 1998). Algae are also of commercial importance in the food industry and in aquaculture and they are considered natural sources of high-value products such as carotenoids, long-chain polyunsaturated fatty acids, and phycocolloids (Apt and Behrens, 1999; Tseng, 2001). The diversified traits and living conditions of algae make them extremely attractive for commercial utilization particularly if the desired candidate alga is accessible to genetic manipulation.

The feasibility of green algae to be genetically modified and express heterologous genes opens up the possibility of enhancing the productivity of traditional algae compounds and producing new bioactive products for industrial and pharmaceutical applications through metabolic engineering (León-Bañares et al., 2004). Indeed, as eukaryotes, algae possess the chaperones and cellular machinery required to fold complex human proteins that bacteria and yeast may not process properly (Franklin and Mayfield, 2004). They can be cultured easily, rapidly and economically, and many of them are considered as safe food because they are free from human pathogens and endotoxins (Griesbeck et al., 2006). As a consequence of increasing global demand for recombinant proteins in a variety of industrial, algae have become the interesting species for novel pharmaceuticals, production of large quantities of

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proteins particularly in cases where traditional sources are limited due to cost and/or availability (Moore, 1999; Skulberg, 2000; Specht et al., 2010). As photoautotrophs, transgenic algae, particularly green microalgae, can be exploited as cell factories for the production of valuable recombinant products such as vaccines, specialty oils, and novel carotenoids (León-Bañares et al., 2004).

Such fundamental and applied research requires the development of molecular genetic techniques for each algal species that allow the *in vivo* analysis of gene function and regulation, the manipulation of endogenous genes, and the introduction and expression of foreign genes (Walker et al., 2005a). Over the last few years, several genomic studies have been carried out for some important species of green microalgae such as *Chlamydomonas reinhardtii* (Shrager et al., 2003), *Dunaliella salina* (Li et al., 2004), *Acetabularia acetabulum* and *Ostreococcus tauri* (Henry et al., 2004; Derelle et al., 2006). This has resulted in providing a wealth of information and a strong foundation for targeted manipulation. Also, the molecular characterization of these green algae may provide useful DNA elements for genetic engineering of the host alga such as strong promoters which are necessarily used for green algae efficient transformation systems. Moreover, there has been a concerted effort to develop and improve the molecular tools for several important algae species. For instance, several transformation systems have been developed for producing high-value recombinant proteins in *C. reinhardtii* (Mayfield et al., 2003; Sun et al., 2003), *Dunaliella salina* (Geng et al., 2003), and various *Chlorella* species (Hawkins and Nakamura, 1999; Kim et al., 2002; Borovsky, 2003). It has led to the possibility of using such transgenic algae as vectors to deliver either vaccines or toxins to animals that are fed with algae. In short, green algae have proven their utility and tractability as a production system for commercial importance compounds as well as

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for therapeutic or industrial proteins and peptides, and algae now seem poised to become the alternative expression system to other protein production systems.

Currently, the increasing importance of molecular research including generation of expressed sequence tags (ESTs), isolation and characterization of interested genes and its promoters, establishment of transformation systems in green algae, can be seen by the recent research reports and patents from numerous laboratories, the funding bodies as well as the establishment of several algal biotechnology companies. Even though the importance of algae biotechnology and genetic engineering has speedily increased, there are still difficulties, inconveniences and problems to be solved. Among these limitations, extensive research on molecular studies as well as optimal transformation constructs are necessary to be implemented. It is reviewed that although some common heterologous promoters (e.g., CaMV35S and the SV40 promoters) have been demonstrated to work in some green algae transformation constructs, inadequate recognition of the heterologous promoter region and lack of adequate regulation are major hurdles (Hallmann, 2007). Therefore, as a major step to optimize the transformation constructs, it is necessary to use a strong homologous promoter, which normally isolated from the high-expressive genes, for the efficient expression of heterologous proteins in green algae transformation.

As a fast growing alga producing appreciable amount of carotenoids and polyunsaturated fatty acids (Chu et al., 1992), freshwater green microalga *Ankistrodesmus convolutus* Corda has great potentials to become an interesting candidate for many biotechnological applications. The ability of *A. convolutus* to form floating aggregates during its normal growth to facilitate harvesting as well as

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other beneficiary attributes make it potential as an alternative expression system for production of natural products and expression of therapeutic proteins. At the time the present study was initiated, however, there was no report of any genetic or molecular studies on this green microalga species. Thus, the aims of this study were to isolate and characterize a highly expressed cDNA clone and its promoter for application on the expression of a heterologous gene in green microalga *Ankistrodesmus convolutus* towards the establishment of an alternative expression system using this microalga. The specific objectives of this study were as follows:

1. To develop a suitable procedure for the isolation of high quality and quantity of total RNA from *A. convolutus*.
2. To construct a cDNA library from *A. convolutus*.
3. To isolate and characterize a highly expressed cDNA clone and its promoter region from *A. convolutus*.
4. To use the isolated promoter to drive the expression of a heterologous gene in *A. convolutus*.

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