SOMATIC EMBRYOGENESIS DETECTION IN CARNIVOROUS PLANTS

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ABSTRACT

Carnivorous plants are generally uncommon and some of the species are endangered. These plants have the most unusual adaptation to survive in environment that is nutrient deficient by capturing preys. Hence, propagation is required to prevent the extinction of these species. *In vitro* propagation could mass propagate the species to address the problem. Somatic embryogenesis (SE) could produce higher number of propagates. Besides, SE is studied to understand similar process of zygotic embryogenesis. The purpose of this research was to look for cellular and molecular markers during the early events of SE. Explants of *D. burmannii* and *D. tokaiensis* were subcultured on a medium supplemented with thidiazuron to induce SE. Explants were sampled based on different incubation period where day-0 sampling served as negative control. Major events that occurred from the transition of induced cells into somatic embryo, and up to globular structure were investigated using histology. Specific primers targeting EF1 and GAPDH were designed from previous isolated sequences from *D. tokaiensis*. Degenerate primers were also designed from downloaded database sequences. RT-PCR was performed using the designed specific and degenerate primers followed by sequence analysis. Isolation of SE induced cells from their surrounding was observed. Early events leading to the formation of globular structures in epidermis cells reconfirmed a previous study, which was documented alongside some new findings elaborated in the Results section. SE from induced mesophyll cells proposed previously was documented in this study. Specific primers targeting EF1 and GAPDH genes successfully amplified their target sequence and could be used for quantitative PCR (qPCR) in future. ACT and SERK1 sequences were isolated and could be used for designing qPCR specific primers for detection of SE in *D. tokaiensis*. SERK1 gene could be used for early detection of SE while ACT, EF1 and GAPDH could be used as a normalisation in qPCR. Findings obtained in this research facilitate deeper understanding of early development of SE and SE regeneration pathway could serve as a reference to provide understanding in other plant species.

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LIST OF ABBREVIATIONS

°C	degree Celsius
ΔG	Gibbs free energy
μM	Micromolar
2,4-D	2,4-dichlorephenoxyacetic acid
aa-tRNA	Aminoacylated-tRNA
ACT	Actin
ADC	Anticlinally divided cell
bp	base pair
$CaCl_2 \cdot 2H_2O$	calcium chloride dehydrate
Cm	Centimetre
$CoCl_2 \cdot 6H_2O$	cobalt (II) chloride hexahydrate
$CuSO_4 \cdot 5H_2O$	copper (II) sulphate pentahydrate
D. burmannii	*Drosera burmannii* Vahl
DSE	Direct Somatic Embryogenesis
D. tokaiensis	*Drosera tokaiensis* (Komiya & C. Shibata) T. Nakam. & K. Ueda
DWS	dish washing solution
ECC	embryogenic competent cell
EC	embryogenic cell
EF-1α	elongation factor 1α
$FeSO_4 \cdot 7H_2O$	iron (II) sulphate heptahydrate
GAPDH	glyceraldehyde-3-phosphodehydrogenase
H_2O_2	hydrogen peroxide
H_3BO_3	boric acid
HCl	hydrochloric acid
Hr	Hour

IDC	Isolated Divided Cell
IKI	iodine-potassium-iodine
ISE	Indirect Somatic Embryogenesis
$KAl(SO_4)_2$	aluminium potassium sulphate
Kb	kilo base
Kg	Kilogram
KH_2PO_4	monopotassium phosphate
KI	potassium iodine
KNO_3	potassium nitrate
MΩ	Megaohm
$MgSO_4$	magnesium sulphate
Min	Minutes
MS medium	Murashige & Skoog (1962) medium
$MgSO_4 \cdot 7H_2O$	magnesium sulphate heptahrydrate
$MnSO_4 \cdot 4H_2O$	manganese sulphate
MSA	multiple sequence alignment
$Na_2EDTA \cdot 2H_2O$	ethylenediaminetetraacetic acid, disodium dehydrate
Na_2HPO_4	disodium phosphate
$Na_2MoO_4 \cdot 2H_2O$	sodium molybdate dehydrate
$Na_2S_2O_5$	sodium metabisulfite
$Na_2S_2O_3 \cdot 5H_2O$	sodium thiosulfate pentahydrate
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	monosodium phosphate
NaOH	sodium hydroxide
NBF	neutral buffered formalin

NCBI	National Center for Biotechnology Information
NH_4NO_3	ammonium nitrate
PAS	Periodic-Acid Schiff
PDC	Periclinal Divided Cell
PCR	Polymerase Chain Reaction
PP	Polypropylene
Pyridoxine-HCl	pyridoxine hydrochloride
qPCR	Quantitative Polymerase Chain Reaction
RT–PCR	reverse transcription Polymerase Chain Reaction
S	Seconds
SE	somatic embryogenesis
SERK	Somatic Embryogenesis Receptor-like Kinase
SO_2	sulphur dioxide
T_m	melting temperature
TBA	tert-butyl alcohol
TBO	toluidine blue O
TDZ	Thidiazuron
TWS	Scott's tap water substitute
v/v	volume per volume concentration
w/v	weight per volume concentration
$ZnSO_4 \cdot 7H_2O$	zinc sulphate heptahydrate

CHAPTER 1

INTRODUCTION

Conservation is paramount to conserve endangered or soon to be endangered species. A wide range of wildlife species are included in the CITES Appendices, lists of species compiled based on how threatened the species are, extending to many prominent species which include carnivorous plants (CITES, 2013). Carnivorous plants have the most unusual adaptation to survive in an environment that is nutrient deficient. Most plants obtain their nutrients from soil whereas carnivorous plants obtain extra nutrient supply through carnivory. Their leaves have the ability to trap, digest and absorb the nutrients from their preys therefore decreasing their dependence on photosynthesis (Król et al., 2011). Carnivorous plant is generally uncommon, therefore illegal poaching and destruction of its natural habitat through urbanisation, agriculture, and forestry are threatening the population of carnivorous plants (Botanical Society of America, 2012; IUCN, 2015). Hence, carnivorous plants should be conserved to prevent extinction of these wonderful species and one of the approaches is though plant propagation.

Plant propagation is a cultivation technique which involves in production of plants using different initial materials such as leaves, roots, stems, or other explants. Propagation techniques include *in vitro* propagation and conventional propagation (Bareja, 2010). *In vitro* propagation is able to produce large number of plantlets in a sterile and disease-free environment and provides essential nutrients for the plantlets to grow (Fay, 1994). In addition, *in vitro* propagation is preferred as the propagation can be controlled with the use of growth regulator and favourable environment (Bhojwani & Dantu, 2013).

There are two regeneration modes in *in vitro* propagation, namely somatic embryogenesis and organogenesis. Somatic embryogenesis is a development process to generate somatic embryos from somatic cells under appropriate conditions. Meanwhile organogenesis in plant produces plant organs, mainly shoots and roots, induced by plant growth regulators. Somatic embryogenesis enables production of

large number of plantlets when induced and the process does not require high labour force compared to organogenesis (Bhojwani & Dantu, 2013). In addition, plantlets derived from somatic embryogenesis are lesser in variation among each other; in contrast, organogenesis are more prone to undergo mutations and epigenetic changes (Tsukaya & Beemster, 2006). However, problem faced by many plant biologists was that growth and development of cells and tissues that occurred in a short span of time was unable to be observed with naked eyes especially during certain embryogenesis stages (Sia, 2015). Somatic embryogenesis can be recognised if specific cellular or molecular cues are detected.

Plant anatomy is important in this research to recognise and determine the developmental events which occurred inside the plant at different stages and to provide a better understanding of relationship between the expression of gene and biological events during the developmental process of somatic embryogenesis (Yang, & Zhang, 2010). Developmental events can be determined by comparing cellular structure and morphology with cellular marker (Arnold, Sabala, Bozhkov, & Filonova, 2002). These developmental events provide an in-depth understanding of mechanisms required during the transition into embryos in term of biochemical, morphological and cellular structure (Kurczynska et al., 2012). Hence, histology approach plays a role in this research to observe the difference between the explants at cellular level sampled at specific intervals.

During the early stage of embryogenesis, it was proposed that there were two possible pathways of embryogenesis (Sia, 2015). One of the pathways was described in detail with evidences but the other pathway lacked sufficient information to describe the transition of somatic cells into somatic embryos. More samplings should be done to reconfirm these previous observations and discover development stages not reported previously. The more complete the regeneration model of selected carnivorous plants, the better it can be used for somatic embryogenesis detection.

Another approach which complements histological approach is via molecular detection of somatic embryogenesis-related gene expression in developing explants and embryos (Chugh & Khurana, 2002). Previous study by Nor Farrah Wahidah (2015) found that elongation factor gene expression was the highest at day 28 where