MOLECULAR DETECTION OF SOMATIC EMBRYOGENESIS IN *Drosera tokaiensis*

VIMUKTHI BANDARA JAYASUNDARA

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Vimukthi Bandara Jayasundara	Dr. Choong Chieh Wean
I15007791	(SUPERVISOR)
13/12/2017	

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ABSTRACT

Drosera tokaiensis is a carnivorous plant with many potential uses in the fields of medicine. Hence potential demand for *D. tokaiensis* may cause endangerment to the species. A rapid need for plant production which can be achieved by somatic embryogenesis (SE) which produces higher propagates with less variation compared to organogenesis. Detection of SE is important because it is similar in structure to organogenesis and callogenesis. SE detection by observation of cellular structures is time consuming. Thus, a molecular detection of SE induction using elongation factor α (EF1) and somatic embryogenesis receptor-like kinase (SERK1) was proposed using quantitative polymerase chain reaction (qPCR) approach. RNA samples were available from *D. tokaiensis* leaves induced for SE using thiadiazuron from day (D)-0 to D28, RNA integrity of these samples was confirmed by agarose gel electrophoresis. Then cDNA was synthesised and qPCR was carried out using specific primers designed for SERK1 and EF1 along with house-keeping genes, ACT and GAPDH. The experiment was reliable with PCR efficiently measured, and amplification specificity proven by database comparison and local alignment to a known sequence. EF1, ACT and GAPDH could be accurately quantified where R-squared value of the generated standard curve was more than 0.99. Expression of EF1 and SERK1, after normalization with ACT and GAPDH as references, was shown to be upregulated during SE induction. In conclusion, this research was able to confirm that EF1 and SERK1 is upregulated during induction stages of SE induction and could be used as genetic markers for detection of SE induction for production lines and also tissue culture experiments involving SE induction.

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LIST OF ABBREVIATIONS

µL	microlitre
ΔG	Gibbs free energy
°C	degrees Celsius
ACT	actin
bp	base pairs
C	cytosine
D. tokaiensis	*Drosera tokaiensis*
DNA	deoxyribonucleic acid
EF1	elongation factor 1
g	gram
G	guanine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
hr	hour
IDT	Integrated DNA Technologies
kb	kilo base
LAF	laminar air flow
min	minute
mL	millilitres
mg/mL	milligrams per millilitre
NCBI	National Centre for Biotechnology Information
PCR	Polymerase Chain Reaction
PTC	plant tissue culture
qPCR	quantitative Polymerase Chain Reaction
RNA	ribonucleic acid
SE	somatic embryogenesis

SERK1	Somatic Embryogenesis Receptor-Like Kinase
TDZ	thiadiazuron
T_m	melting temperature
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Green plants began to colonise land about 510 million years ago (Bennici, 2008). An ancestry algal species had evolved to produce different species of plants adapted to survive on different terrestrial conditions present on Earth over millions of years. Out of these, carnivorous plants evolved distinctly from other plant species (Bennici, 2008). Carnivorous plant, as the name suggests, possess the ability to trap small organisms such as insects and amphibians, and digest them using hydrolytic enzymes in order to obtain nutrients such as nitrates. Carnivorous plants are also able to photosynthesise but mainly rely on prey in order to obtain sufficient nutrients. Carnivorous plants have the ability to grow on soil in environments with low nutrient content such as peat bogs (Clapa, Fira, Pacurar, & Sotropa, 2010) or habitats with low light condition which reduces the ability of these plants to photosynthesise efficiently (McPherson, 2010).

There are 194 recorded species of *Drosera* (McPherson, 2010) and throughout the world and only a few species are considered threatened or endangered. Plant extract obtained from *Drosera indica* was shown to have anti-cancer activity (Asirvatham, Christina & Murali, 2013) while *Drosera binata* extracts have antimicrobial activity when applied to burnt wounds (Krychowiak, 2014). Plant extracts derived from *Drosera rotundifolia* and its hybrid, *D. tokaiensis* has shown medical properties which make it a prime candidate for potential antimicrobial drug development (Kačániová et al., 2014) and for respiratory disorders such as bronchitis and asthma (Banasiuk et al., 2012; Jayaram & Prasad, 2006). Large scale medical application of these plants may pose threat of extinction to these species (Banasiuk, Kawiak, & Krölicka, 2012). In the US, several species which belong to the *Drosera* genus including *Drosera anglica* and five other *Drosera* species are considered threatened and at risk of being endangered (USDA, n.d.). Apart from some rare cases, most *Drosera* species are not listed at risk of extinction or endangerment.

Most endangered *drosera* plant species produce fertile but recalcitrant seeds, which have short viability, and cannot be desiccated and stored at low temperature for

preservation in seedbanks (Jiménez, 2001), thus conventional conservation strategy through seed storage is ineffective. By the use of somatic embryogenesis (SE) endangered plant species could be propagated in large scale producing disease free clones (Jiménez, 2001). Unlike conventional propagation methods, plant tissue culture is less labour intensive and more economical than conventional propagation strategies because large number of explants could be produced within a short time from a single meristem obtained from a parental plant (Kamle, Bajpai, Chandra, Kalim, & Kumar, 2011).

There are two possible strategies for an explant to be regenerated to produce complete plants. These two mechanisms are organogenesis and SE. In organogenesis, shoot and root development depend on plant growth regulators present in the culture medium and one of the main characteristic of organogenesis is the presence of vascular linkage between the parental tissue and the regenerating section (Jiménez, 2001). In contrast, SE is a process by which either haploid or diploid somatic cells develop into zygotic embryo-like structures without the requirement of gamete fusion and the presence of bipolar structures without vascular connections with parental tissue (Bhojwani,& Dantu, 2013). Key benefits of utilising SE is that it permits the production of large number of plantlets (Jiménez, 2001) complete with both shoots and roots, easy to scale-up transfers with minimum labour, ability to produce pure cultures as somatic embryos originate from single cells and plants derived from SE is less variable than those from organogenesis (Bhojwani, & Dantu, 2013).

It is crucial to detect SE, and distinguish it from organogenesis, as both SE and organogenesis can occur when plant growth regulators are present in media (Nolan, Kurdyukov, & Rose, 2009). In order to confirm the occurrence of SE, different stages of SE could be detected using a histological approach which is time consuming. A more current and time effective method for detection of somatic embryogenesis is through molecular approach which detects specific genes' expression in developing cells of explants.

In a previous research on SE molecular detection on *D. tokaiensis,* actin (ACT) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were isolated to serve as housekeeping genes while Somatic Embryogenesis Receptor-like Kinase (SERK1) and

elongation factor 1 (EF1) gene could be used as a molecular marker for early and late stages SE induction respectively (Wurtele, Wang, Durgerian, Nikolau, & Ulrich, 1993). Nor Farrah Wahidah (2015) found that EF1 was preferentially expressed in explants induced with 1 mg/L thiadiazuron (TDZ) at day-28, which coincided with the highest amount of globular structure observed histologically (Sia, 2015). However, she used degenerate primers instead of specific primers for the quantitative PCR (qPCR) gene expression study, and her data relied on only one housekeeping gene, i.e. GAPDH. Later, Kok (2016) designed specific primers for EF1 and GAPDH to be used for qPCR. In addition, he isolated two more genes, ACT which is the additional housekeeping gene (Turabelidze, Guo, & DiPietro, 2010), and SERK1 which is a gene specific to the early stage of SE induction. There were no specific primers for qPCR targeting ACT and SERK1 designed yet. Therefore, the aim of this study was to design specific primers for qPCR from ACT and SERK1 sequences of D. *tokaiensis* isolated by Kok (2016). Subsequently, specific primers targeting GAPDH, ACT, EF1 and SERK1 was used for qPCR gene expression study on *D. tokaiensis* explants, which were treated with 1 mg/L TDZ and sampled at different time frame based on RNA extracted by Nor Farrah Wahidah (2015). This was done to establish a molecular detection technique of SE in *D. tokaiensis* for early stages of SE induction.

CHAPTER 2

LITERATURE REVIEW

2.1 GENUS *Drosera*

One of the largest genera of carnivorous plants is *Drosera* which is commonly known as sundew. *Drosera* genus consists of more than 194 recorded species and can be found on all the continents except Antarctica (McPherson, 2010). Anatomically sundews possess leaves that consist of structures such as calyx and petiole with photosynthetic pigment chlorophyll, but with the absence of leaf blade. The most distinguishable feature of the *Drosera* species is the presence of sticky glandular tentacles, on its leaf-like structures, which secrete sweet mucilage to attract insects (Bennici, 2008) with chemo attractants (Kreuzwieser et al., 2014). When an insect comes in contact with the tentacles, the sticky mucilage limits the movement of the insect. At the same time, stimulation by the movement of the insect causes the leaf to coil around the insect, trapping the insect. Hydrolytic enzymes such as proteases, nucleases and others, are secreted onto the insect and products of the digestion are absorbed by the sundew while the undigested parts of the insect fall off by eventual uncoiling of the leaves. Thus, sundews are able to use their leaves to trap new prey while their leaves photosynthesise.

2.1.1 *Drosera tokaiensis*

D. tokaiensis is a natural hybrid of *Drosera rotundifolia* and *Drosera spatulate* native to Japan. *D. tokaiensis* like its ancestry parents *Drosera rotundifolia* possess medical properties and is used as an herb by indigenous people. Plant extracts derived from *Drosera rotundifolia* and its hybrid, *D. tokaiensis* has shown medical properties which make it a prime candidate for potential antimicrobial drug development (Kačániová et al., 2014) and for respiratory disorders such as bronchitis and asthma (Banasiuk et al., 2012; Jayaram & Prasad, 2006). *Drosera rotundifolia* is currently endangered in the US due to exploitation of its medical benefits and due to habitat loss, *D. tokaiensis* however is not at risk of going extinct due to its success at surviving harsh environmental conditions when compared to its parental origins, thus studying the