MOLECULAR CLONING OF *Zat12* PROMOTER FROM *Arabidopsis thaliana*

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MOLECULAR CLONING OF *Zat12* PROMOTER

FROM *Arabidopsis thaliana*

By

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ABSTRACT

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Reactive oxygen species (ROS), despite of its toxicity effects on plant cells' survival, act as signaling molecules that regulate and control various physiological and biochemical processes in plants. The ROS-responsive *Zat12* gene encodes zinc finger protein Zat12, which is expressed within minutes during oxidative burst, plays an important role in ROS signaling pathways. Hence, *Zat12* promoter (*proZat12*) is a potential intracellular ROS sensor. *Zat12* promoter was amplified by PCR using genomic DNA from *Arabidopsis thaliana*. The amplified fragment was cloned into the intermediate vector pENTR™/ D-TOPO® and used to transform competent *Escherichia coli* cells. Screening of transformants using colony PCR showed presence of positive clones carried recombinant pENTR™/ D-TOPO® with *proZat12* in the desired orientation. The orientation of the insert was verified by PCR and the cloned *proZat12* fragment was verified by DNA sequencing. Using GATEWAY® Recombination Cloning Technology, the cloned *proZat12* insert in pENTR™/ D-TOPO® vector was transferred into the GATEWAY®

binary vector pGWB4, thereby place *proZat12* immediately upstream of the *sGFP* gene, which encodes for green fluorescent protein with the S65T mutation. Screening of transformants using colony PCR showed 12 out of 21 colonies carried recombinant pGWB4 with *proZat12*. Hence, the result suggested that *proZat12* was amplified from *Arabidopsis thaliana* and cloned into cloning site of pGWB4. Confirmation of the orientation of insert (*proZat12*) in pGWB4 by colony PCR should be conducted in the future. Recombinant pGWB4 with *proZat12* in the desired orientation can be extracted and transformed into plants through *Agrobacterium*-mediated transformation. The ROS-sensing function of *proZat12*::*sGFP* construct can be performed through co-infiltration with ROS-inducing plasmid carrying *OsrbohB* gene. Direct application of hydrogen peroxide at the infiltration site can also be used to test the ROS-sensing function of *proZat12*::*sGFP* in plants.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

Wong Say Chong

APPROVAL SHEET

This project report entitled "**MOLECULAR CLONING OF *Zat12* PROMOTER FROM *Arabidopsis thaliana*"** was prepared by WONG SAY CHONG and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biotechnology at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **WONG SAY CHONG** (ID No: **08ADB06351**) has completed this final year project entitled "**MOLECULAR CLONING OF *Zat12* PROMOTER FROM *Arabidopsis thaliana***" supervised by Assoc. Prof. Dr. Wong Hann Ling from the Department of Biological Science, Faculty of Science.

I hereby give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

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LIST OF ABBREVIATIONS

µs	microseconds
$^1O_2$	Singlet oxygen
AP	Action potential
APX	Ascorbate peroxide
Ca^{2+}	Calcium ion
cDNA	Complementary deoxyribonucleic acid
DanePy	2,2,6,6-tetramethyl-piperidine-dansyl
DanePyO	2,2,6,6-tetramethyl-piperidine-dansyl (oxidized form)
DCF	Dichlorofluorescin (oxidized form)
DCFH	Dichlorofluorescin (reduced form)
DNA	Deoxyribonucleic acid
DPI	Diphenyleneiodonium
EDTA	Disodium ethylene diamine tetraacetate
EtBr	Ethidium bromide
ETC	Electron transport chain
Fe^{2+}	Ferrous ion
Fe^{3+}	Ferric ion
GFP	Green fluorescent protein
GUS	B-glucuronidase

H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
hrs	hours
JA	Jasmonic acid
KI	Potassium iodide
Luc	Luciferase
M	Molar
MAPK	Mitogen-activated protein kinase
min	minutes
ms	miliseconds
N	Normal
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
O_2	Ground state oxygen molecules
$O_2\bullet^-$	Superoxide anion
O_3	Ozone
OD	Optical density
OH·	Hydroxyl radical
PCI	Phenol/chloroform/isoamly alcohol (25:24:1)
pGWB	GATEWAY® binary vector
PR	Pathogenesis-related

proZat12	Promoter of *Zat12* gene
*proZat12*F	Forward primer for *Zat12* promoter amplification
*proZat12*R	Reverse primer for *Zat12* promoter amplification
PSI	Photosystem I
psi	Pounds per square inch
PSII	Photosystem II
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RuBisCo	Ribulose-1,5-bisphosphate carboxylase oxygenase
SA	Salicylic acid
SDS	Sodium dodecyl sulphate
sec	seconds
SOD	Superoxide dismutase
SWP	Slow wave potential
TBE buffer	Tris/borate/EDTA buffer
TE buffer	Tris/EDTA buffer
UV	Ultra violet
VP	Variation potential
XTT	T (sodium 3´-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate
Zat12	Zinc Finger Protein Zat12

CHAPTER 1

INTRODUCTION

1.1 Objective

This project focused on directionally cloning of *Zat12* promoter (*proZat12*) from *Arabidopsis thaliana* into binary vector pGWB4 and placed immediately upstream of the *sGFP* gene, using GATEWAY® Recombination Cloning Technology by Invitrogen. This *proZat12*::*sGFP* construct can be used to detect intracellular accumulation of ROS through sensing of ROS by *proZat12* which subsequently drives expression of green fluorescent protein (GFP).

1.2 Reactive Oxygen Species (ROS)

Oxygen (O_2) molecules were introduced into our atmosphere since the first appearance of O_2-evolving photosynthetic organism, specifically due to evolution of photosynthetic capability of cyanobacteria at approximately 2.2 billion years ago. Ground-state O_2 molecules have two unpaired electrons at the π^* antibonding orbitals which have the same spin quantum number, or simply parallel spins. This spin restriction makes O_2 to accept its electrons one at a time, which eventually leads to formation of reactive oxygen species (ROS) (refer to Appendix A) (Halliwell, 2006). Non-reactive

ground state O_2 can be converted into much more reactive oxygen intermediates or reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) either through energy transfer or through electron reduction reactions (refer to Appendix B) (Apel & Hirt, 2004).

1.3 ROS as Signalling Molecules

During oxidative stress, accumulation of ROS is followed by cellular damage that may eventually lead to cell death. Biological processes, such as respiratory mechanism which involving mitochondrial electron transport chain that contains electrons with sufficient free energy to generate ROS, impose unavoidable damage to eukaryotic cells. In addition to that, photosynthetic organisms such as higher plants encounter higher chance of oxidative damage due to the oxidizing condition and the abundance of photosensitizer and polyunsaturated fatty acids in the chloroplast envelope (Gill & Tuteja, 2010).

However, during the course of evolution, plants have adapted and evolved regulatory mechanism to control over ROS toxicity and used it as signalling molecules (Mittler, Vanderauwera, Gollery, & Breusagem, 2004). ROS producing mechanism and ROS scavenging mechanism must be equilibrated to determine the functions of ROS as either damaging molecules that cause DNA damage, lipid peroxidation, and protein oxidation, or signalling molecules that regulate specialized processes such as plant growth and defence mechanism. Circumstances such as UV radiation, drought, high light

intensity, temperature extremes, and threats of herbivores and pathogens disturb the equilibrium and leads to oxidative burst that eventually leads to cellular damage. Activation of ROS defence mechanism will then lower the production rate of ROS and maintain low steady-state levels of ROS. Furthermore, tightly-regulated interplay between ROS producing and ROS scavenging mechanism also determined the intensity, duration, and localization of different ROS signals (Harir & Mittler, 2009; Gill & Tuteja, 2010).

Water-soluble H_2O_2 has relatively long half-life (1 ms) and high permeability across membrane (Gill & Tuteja, 2010). Besides, "auto-propagating" characteristic of ROS signals has been described in which the generated H_2O_2 diffuses to the neighbouring cells and reacts with intracellular target that cause activation of NADPH oxidase. NADPH-dependent $O_2^{\bullet -}$ production takes place and signal is propagated to distal sites (Miller et al., 2009; Wong & Shimamoto, 2009). All these unique characteristics of H_2O_2 contribute to its potential as signalling molecules. Studies have also shown the role of $O_2^{\bullet -}$ and $^1O_2$ in signalling physiological processes (Mittler et al., 2004).

1.4 Zinc Finger Protein Zat12 from *Arabidopsis thaliana*

Arabidopsis thaliana, a flowering plant in the family of Brassicaceae, has gained increasing popularity as model system for the study of plant biology (Goodman, Eckers, & Dean, 1995). It contains five chromosomes with small genome of 125 million base pairs in total (as sequenced in year 2000) which gives the advantage in the study of cellular and molecular biology of flowering plants (The Arabidopsis Information

Resource, 2011). With the completion in Arabidopsis sequencing project, 25,500 genes has been sequenced (The Multinational Coordinated *Arabidopsis thaliana* Functional Genomics Project, 2009). *Zat12* gene (At5g59820) is one of the ROS-responsive genes which first described by Iida, Kazuoka, Torikai, Kikuchi, and Oeda (2000).

As mentioned by Miller et al. (2009), *Zat12* gene is expressed within minutes in response to wounding or to accumulation of ROS. Davletova et al. (2005a) proposed that expression of Zat12 proteins is highly responsive to intracellular accumulation of ROS. Davletova et al. (2005a) proposed that cytosolic ascorbate peroxidase (APX1) serves as a central component of reactive oxygen gene network of *Arabidopsis*, which was proposed by Mittler et al. (2004). Zat12 is required in up-regulation for expression of ROS-scavenging enzymes (APX1) during oxidative stress and abiotic stresses (Rizhsky, Davletova, Liang, & Mittler, 2004). Hence, zinc finger protein Zat12 is a potential indicator for the intracellular accumulation of ROS. In other words, *Zat12* promoter (*proZat12*) which drives transcription of *Zat12* gene is important in detecting ROS, which can be used as intracellular ROS sensor.

Zat12 promoter (*proZat12*) that was used in this study contains 1,000 base pairs in total, which contains 5'UTR of *Zat12* gene that consists of 309 base pairs. The promoter also includes 691 base pairs upstream of *Zat12* transcription start site. Davletova, Schlauch, Coutu, and Mittler (2005b) reported the fusion of this *Zat12* promoter and luciferase (Luc) reporter gene in analysis of Zat12 expression.

1.5 GATEWAY® Recombination Cloning Technology

Without the use of ligase and restriction enzymes, blunt-end PCR product can be clone directionally into multiple binary vectors system using GATEWAY® Recombination Cloning Technology. Blunt-end PCR product is directionally cloned into one of the pENTR™TOPO® vectors such as pENTR™/D-TOPO® to generate an entry clone. Using LR recombination reaction which is catalyzed by GATEWAY® LR Clonase™ II enzyme mix, the blund-end PCR product is inserted into GATEWAY® destination vector of choice. The expression construct can be introduced into expression host (Invitrogen, 2006).

Various GATEWAY® binary vectors (pGWBs) were constructed for GATEWAY® cloning, such as pGWB4. Generally, pGWBs contain both kanamycin and hygromycin resistance gene. Different pGWBs can be used for different purposes. For example, pGWB2 can be used for overexpression due to the presence of CaMV35S promoter and pGWB3 for promoter::GUS analysis (Nakagawa et al., 2007).

Binary vector pGWB4 was used in this study as a GATEWAY® destination vector of choice. Binary vector pGWB4 is used for C-terminal fusion with independent promoter. It contains *sGFP* reporter gene, encodes for green fluorescent protein with S65T mutation, with no promoter upstream of the gene. Upstream of the *sGFP* gene is a region flanked by *attR1* and *attR2*. These two short sequences have few nucleotides that are similar to *attL1* and *attL2* in the entry vector pENTR™/D-TOPO® (Nakagawa et al., 2007). During LR recombination reaction, blunt-end PCR product (*proZat12*) will be

cloned directionally from pENTR™/D-TOPO® into the region flanked by *attB1* and *attB2* in the pGWB4 (refer to Appendix C).

Binary vector pGWB4 was used in this study as a GATEWAY® destination vector of choice because a GATEWAY® cassette is located upstream of the sGFP gene making it suitable for the study of on promoter. Green fluorescent protein sGFP fluoresces at 509 nm (visible spectrum) with excitation at 489 nm (Patterson, Knobel, Sharif, Kain, & Piston, 1997). When the promoter (*proZat12*) is activated, sGFP proteins will be expressed and detected in vivo through excitation at 489 nm. There is no involvement of expensive substrate, such as luciferin in the luciferase reporter system, as well as other synthetic fluorescent chemicals. Furthermore, the activity of promoter can be study without harvesting samples from plant and series of isolation and purification processes.

1.6 Overview

The overall idea for this study is to clone *proZat12* into pGWB4 in the correct orientation. *Zat12* promoter from *Arabidopsis thaliana* was obtained using polymerase chain reaction (PCR). The blunt-end PCR product (*proZat12*) was directionally cloned into entry vector pENTR™/D-TOPO® and sequenced. Using LR recombination reaction which is catalyzed by GATEWAY® LR Clonase™ II enzyme mix, *proZat12* was transferred into pGWB4 which fused at C-terminal of *sGFP* gene (refer to Appendix C).

CHAPTER 2

LITERATURE REVIEW

2.1 Signalling in Plants

In response to environmental variability such as variation in light intensity, temperature, water and nutrient content, wind strength and direction, herbivores and pathogenic threats, plants evolved sophisticated mechanism which involved information storage and acquisition system (Brenner et al. 2006). It involved intercellular communication or signal transduction between plant cells which requires electrical and chemical signals.

2.1.1 Electrical Signalling

Shepherd (1999) stated that Jagadis Chandran Bose was the first to recognize the importance of intercellular electrical signalling between plant cells in response to variation in environment. Bose studied the leaf movements of *Mimosa* and *Desmodium* and showed plants produce systemic electrical pulses for long distance electrical signaling. There are two types of electrical signals in plants which are action potentials (APs) and slow wave potentials (SWPs) or variation potentials (VPs). Rapid cooling or mechanically touching the apical tip of a leaf pinna resulted in initiation of rapid moving AP which transmitted in the rhachis at 20 – 30 $mm.s^{-1}$ and stopped at the base of the

pinna. Leaf movement can be observed as a result of this AP transmission (Fromm & Lautner, 2007). Flaming the leafs or another parts of plants initiate hydraulic signals, followed by propagating electrical signals that resemble slow re-polarization characteristic of SWPs, which resulted in rapid increase of xylem pressure, turgor pressure, growth rate, and leaf and stem thickness (Stahlberg, Cleland, & Van Volkenburgh, 2006).

2.1.2 Chemical Signalling

Plants use various chemical signals such as auxins, cytokinins, abscisic acid, gibberelin, ethylene, systemin, jasmonic acid, and salicylic acid to coordinate physiological processes in response to environmental variation (Mulligan, Chory, & Ecker, 1997).

Auxins, with its main production site at young apical leaves, are well known for its long-distance signalling in development of organs such as lateral roots. Auxins are further characterized by its role in regulating patterns of cell division and differentiation in root meristematic cells (Friml, 2003).

High level of ethylene production during the ripening of climacteric fruits, such as tomato, signals the acceleration of ripening process (Alexander & Grierson, 2003). Exposure of exogenous ethylene to dark-grown seedlings leads to inhibition of root and hypocotyls elongation, radial swelling of the hypocotyls, and horizontal growth habit of seedlings, which are described as “triple response” (Mulligan et al., 1997).

Pathogenic infection on plants leads to elevation of salicylic acid (SA) at the site of infection which signals the resistance mechanism toward second infection in systemic organs, known as systemic acquired resistance (Heil & Ton, 2008). Mechanical-induced wound on leaf of *Nicotiana sylvestris* resulted in approximately ten-fold increase in jasmonic acid (JA) concentration compared to undamaged leafs and correlated with enhanced nicotine biosynthesis in roots (Baldwin et al., 1997). Baldwin and Schultz (1983) described airborne signalling mechanisms which induced resistance or defence mechanism in neighbouring undamaged plants due to volatile chemical signalling molecules such as methylated salicylic acid and methylated jasmonic acid released by nearby damaged plants.

Many researches on chemical signals-induced physiological processes have been conducted. Over the years, researches have provide evidence that reactive oxygen species (ROS) function as signaling molecules in coordinating and regulating various physiological processes in plants, despite of their toxicity properties (Mittler et al. 2004).

2.2 ROS Production

Ground state O_2 can be converted into $O_2^{\bullet -}$ through addition of one electron into one of its vacant spaces in the π^* antibonding orbitals. Superoxide anion undergoes another single electron reduction which followed by protonation resulting in the generation of H_2O_2. Fenton reaction takes place in the presence of transition metal such as iron, which leads to generation of $OH^{\bullet}$. Despite of generation of ROS by univalent

reduction, energy transfer can also lead to formation of ROS, particularly in generation of $^1O_2$ (Gill & Tuteja, 2010).

2.2.1 Production of Superoxide Anion ($O_2^{\bullet -}$)

Univalent reduction of ground state O_2 molecules is the main route in the generation of superoxide anion ($O_2^{\bullet -}$), a reactive oxygen derivatives with approximately 2-4 μs of half-life (Gill & Tuteja, 2010). Unavoidably, cellular compartment such as chloroplasts, mitochondria, and peroxisomes, with high level of oxidizing metabolic activity and electron flow, are the major sites for $O_2^{\bullet -}$ production.

Presence of electron transport chain (ETC) in photosystem I (PSI) and photosystem II (PSII) makes chloroplast as an eligible candidates for $O_2^{\bullet -}$ production. Excited photosystem centers initiate electron flow which later reduces $NADP^+$ to NADPH. Under abiotic stresses such as limitation of CO_2 due to stomatal closure during drought or temperature stress, where electron surplus in ETC occurs, electron flow is diverted to photoreduction of O_2 to $O_2^{\bullet -}$ through Mehler reaction in PSI (Veljovic-Jovanovic, 1998). Peroxisomes are also regarded as one of major sites for intracellular ROS production. One of the $O_2^{\bullet -}$ production sites is in the organelle matrix which involves xanthine oxidase that generates $O_2^{\bullet -}$ as a by-products for oxidation of xanthine and hypoxanthine to uric acid (del Rio, Sandalio, Corpas, Palma, & Barroso, 2006). NAD(P)H-dependent $O_2^{\bullet -}$ production site , is a small ETC composed of flavoprotein NADH and cytochrome b that is located in the peroximal membrane, used O_2 as terminal electron acceptor and generates $O_2^{\bullet -}$ (Gill & Tuteja, 2010; Corpas, Barroso, & del Rio,

2001). Mitochondrial ETC, particularly in Complex I and Complex III, is also another major sites for $O_2^{\bullet -}$ production (Turrens, 1997).

2.2.2 Production of Hydrogen Peroxide (H_2O_2)

Superoxide anion serves as the precursor for the production of H_2O_2 through the enzymatic action of superoxide dismutase (SOD). A process called dismutation, which is catalyzed by SOD, involves single electron reduction of $O_2^{\bullet -}$ and followed by protonation resulting in H_2O_2 formation (Gill & Tuteja, 2010).

As temperature increases or intracellular CO_2 concentration declines during photorespiration, oxygenation of ribulose 1, 5- bisphosphate by RuBisCo produced glycolate, which is translocated from chloroplast to peroxisomes. Glycolate oxidase in peroxisomes catalyzed the oxidation of glycolate to produce glycoxylate. Hydrogen peroxide is also produced as by-product of this oxidation process (Apel & Hirt, 2004; Veljovic-Jovanovic, 1998).

2.2.3 Production of Hydroxyl Radical (OH·)

In the presence of transitional metals such as iron, OH· is produced from H_2O_2 and $O_2^{\bullet -}$ through Fenton reaction. Superoxide anion reduces ferric ion (Fe^{3+}) and produces ferrous ion (Fe^{2+}) that will reacts with H_2O_2 to produce highly reactive OH· (Gill & Tuteja, 2010). Horseradish peroxidase was described to participate in reducing H_2O_2 to OH· in the presence of reducing agents such as NADH (Chen &

Schopfer, 1999). This short-lived, highly reactive oxygen intermediate randomly react with all biological molecules located in close proximity with the sites of generation. Due to absence of enzymatic scavenging mechanism to remove reactive OH•, its generation normally leads to cell damage and even cell death (Gill & Tuteja, 2010).

2.2.4 Production of Singlet Oxygen ($^1O_2$)

Single oxygen ($^1O_2$) formation does not involve electron transfer, but due to energy transfer that causes rearrangement of electrons at π* antibonding orbital of ground state O_2 molecules. Insufficient energy dissipation during photosynthesis, especially at PSII, leads to formation of chlorophyll triplet state that causes $^1O_2$ formation by transferring excess excitation energy to O_2 molecules (Halliwell, 2006; Krieger-Liszkay, 2005). This reactive oxygen derivative, with its 3 μs of half-life and ability to diffuse over a considerable distance, is thought to be responsible for destruction of photosynthetic apparatus during photoinhibition (Gill & Tuteja, 2010).

2.3 ROS Signalling

Despite of its toxicity effect, studies of ROS have revealed the role of ROS as signalling molecules. Various researches have shown evidence regarding the role of ROS signalling on biochemical and physiological processes in plants such as defence mechanism, stomatal closure, gene expression regulation, programmed cell death, cross-

tolerance, plant growth regulator signalling and organ development (Mittler et al. 2004; Wong & Shimamoto, 2009).

2.3.1 ROS and Cell Death

Pathogen attacks trigger hypersensitive response (HR) that involved oxidative burst with production of $O_2^{\bullet -}$ and H_2O_2. Hypersensitive cell death as the result of HR caused restricted lesions formation, delimited from surrounding healthy cells that is thought to restrict further infection of pathogen to other parts. Study showed that $O_2^{\bullet -}$ did not directly involved the hypersensitive cell death. H_2O_2 which was formed from SOD-catalyzed dismutation of $O_2^{\bullet -}$, together with nitric oxide (NO) contributed to plant hypersensitive cell death. With absence of SOD, $O_2^{\bullet -}$ did not cause hypersensitive cell death but reacts with NO to form peroxynitrite ($ONOO^-$) (Delledonne, Zeier, Marocco, & Lamb, 2001). This showed the specificity of different ROS in signalling different physiological processes.

Role of ROS in signalling the activation of mitogen-activated protein kinase (MAPK) cascades has been well-documented. Developmental signals such as plant growth regulators led to accumulation of H_2O_2 that triggers physiological processes such as developmental programmed cell death (PCD) through activation of MAPK cascades (Gechev & Hille, 2005; Kuriyama & Fukuda, 2002). Oxidative stress-activated MAP triple-kinase 1 (OMTK1), a novel MAPK kinase kinase isolated from alfalfa (*Medicago sativa*), was found to be specifically activated by H_2O_2 that later specifically activated MMK3, one of the MAPKs, resulting in increased cell death rate (Nakagami, Kiegerl, &

Hirt, 2004). This study showed significant involvement of ROS in signalling cell death through activation of MAPK cascades.

2.3.2 ROS and Plant Development.

In a study of gravitropism of maize roots, redistribution of auxin, a plant growth regulator, by gravity induced an increase in the gravitropic curvature in maize root through the generation of ROS. The study showed the interaction between ROS and other chemical signals such as plant growth regulator in controlling plant development. Asymmetric appilication of H_2O_2 on maize roots induced curvature of vertical roots indicated that ROS plays role as downstream component in the auxin-mediated signalling pathways. Furthermore, application of antioxidant N-acetyl-cysteine at the site of H_2O_2 application led to impairment of root gravitropism (Joo, Bae, & Lee, 2001). This result agreed with the importance of equilibrium between ROS-producing and ROS-scavenging mechanism that maintains the basal level of ROS for signalling purposes.

ROS also involved in regulating the redox status for slow-dividing cells of quiescent centre and rapid dividing cells of peroximal meristem. Accumulation of ROS such as H_2O_2 and $O_2^{\bullet -}$ led to low level of ascorbate (5× lower) and high level of oxidized ascorbate (1000× higher) at quiescent centre compared to proximal meristem. Similar trend was reported in lower level of glutathione (10× lower) and higher level of oxidized glutathione (10× higher) at quiescent centre compared to proximal meristem. Depletion of glutathione in quiescent centre trapped cells in the G_1 phase. As a result of that, the cell division happened at much slower rate at quiescent centre compared to proximal

meristem. Shift in auxin maximum due to formation of root cap caused the changes of redox status in quiescent centre which activated and stimulated cell division at quiescent centre (Jiang, Meng, & Feldman, 2003). This study suggested the role of ROS in signalling the halt of cell growth at quiescent centre through maintaining the oxidized redox state, which in turn contributes to the organization of root meristems.

Study of role of ROS in elongation growth in the expanding zone of maize leaf blades showed the active ROS producing activity in the expanding region of elongation zone while almost undetectable in the expanded region. Application of KI and diphenyleneiodonium (DPI) on segment of elongation zone resulted in the decrease in DCF fluorescence, which was used to detect the presence of H_2O_2. Application of KI only did not modify the elongation growth indicated that H_2O_2 is not responsible for the growth, but maybe contributed by another ROS (Rodriguez, Grunberg, & Takeisnik, 2002). This suggested that different ROS may responsible for different physiological processes such as plant development.

Accumulation of ROS during lignifications process of plant xylem tracheary elements suggested the role in ROS in stiffening of the cells wall by promoting cross-linking between polymers. The study also proposed that cell wall lignifications may be regulated by H_2O_2 through the catalytic activation of peroxidase that causes polymerization of cinnamyl alcohols (Ros-Barcelo, Pomar, Lopez-Serrano, Martinez, & Pedreno, 2002). The study showed effect of ROS in signalling the plant development process through its interaction with enzymes.

2.3.3 ROS and Abiotic Stress

Role of ROS in temperature stress has been studied and well-documented (Suzuki & Mittler, 2006). Study of effects of H_2O_2, as well as other signalling molecules such as salicylic acid and abscisic acid, on the acquisition of thermotolerance of *Agrostis stolonifera* showed the involvement of ROS in protection of plant against subsequent heat-induced oxidative damage. Pre-treatment of H_2O_2 protected creeping bentgrass against heat damage which suggested the role of ROS in plant during heat acclimatization (Larkindale & Huang, 2004). In response to cold stress, Zat12, which is an ROS-response zinc-finger protein, participates in induction and repression of cold-induced genes which in turn increase in freezing tolerance of plants, known as cold acclimatization (Vogel, Zarka, Van Buskirik, Fowler, & Thomashow, 2005). These results suggested the role of ROS in signalling the acclimatization of plant in protection against repeated stress condition.

Depending on the concentration, stomatal behaviours are affected by H_2O_2. Application of low concentration H_2O_2 or ROS-inducing methyl viologen inhibited stomatal opening and promoted stomatal closure through increase in guard cell $[Ca^{2+}]$. This study showed that ROS generated during exposure of pollutant gas O_3 (abiotic stress) did not directly affect stomatal behaviour but through alteration of guard cell Ca^{2+} homeostasis (McAinsh, Clayton, Mansfield, & Hetherington, 1996). Under drought stress, abscisic acid, which is one of the major root-to-shoot stress signals, led to ROS production in plants which in turn mediated leaf stomatal closure through activating of plasma membrance calcium channels. Changes of intracellular $[Ca^{2+}]$ in guard cells led to stomatal closure to limit water loss through transpiration flux (de Carvalho, 2008).

2.3.4 ROS and Cross Tolerance

Abiotic treatment with pollutant gas ozone or UV radiation caused generation of ROS in the plant. ROS is believed to regulate the accumulation of SA which in turn caused induction of several defence-related genes that provide resistance of the treated plants against bacterial infection. Exposure of tobacco to UV radiation or ozone stimulates biosynthesis of SA, accumulation of pathogenesis-related proteins, and increased resistance against tobacco mosaic virus. It is well-documented that UV radiation and exposure of ozone cause oxidative burst in plants, which is similar to pathogen infection. Hence, ROS may participate in cross tolerance of plant against pathogen infection after treatment with mild abiotic stress, through accumulation of SA in plant (Sharma, Leon, Raskin, & Davis, 1996; Yalpani, Enyedi, Leon, & Raskin, 1994).

2.3.5 ROS and Gene Expression

Mittler et al. (2004) described reactive oxygen gene network of at least 152 genes in *Arabidopsis* involved in regulating steady-level baseline of ROS on which different signals can be registered. Using cDNA microarray analysis, expression of 175 genes were altered followed by H_2O_2 treatment. Among these ROS-responsive genes, 113 genes were upregulated while the remaining genes were repressed by exposure of H_2O_2. Functions of ROS-induced genes cover cellular organization and biogenesis, signal transduction, metabolism, energy transfer, transcription regulation, protein destination and transport, and cell rescue/defence. For example, H_2O_2 -induced genes such as genes encode heat shock protein and heat shock transcription factor lead to development of

tolerance against further temperature stress, in minimizing the deleterious effect of temperature stress toward survival of plant cells (Desikan, Mackerness, Hancock, & Neill, 2001). These studies suggested that intracellular ROS is regulated by reactive oxygen gene network which in turn affect physiological and biochemical process of plants through signalling the alteration of gene expressions.

2.4 Detection of ROS

Various researches have used different methods in quantification and detection of the presence of intracellular ROS in the study of oxidative stress which included usage of fluorescent chemicals that react with ROS specifically (Fryer, Oxborough, Mullineaux, & Baker, 2002). Besides, ROS was also detected through molecular technique such as Northern blot in detecting the presence of ROS-responsive gene such as *APX1*, a gene encodes an ROS scavenging enzyme ascorbate peroxide (Ridzsky et al., 2004). Reporter gene systems, such as luciferase reporter gene system, have also been used to detect the presence of intracellular ROS (Miller et al., 2009).

2.4.1 Detection of ROS using Fluorescent Compounds.

Allan and Fluhr (1997) used dichlorofluorescin (DCFH), a non fluorescent compound, in detecting the intracellular production of H_2O_2 in greenhouse grown tobacco plants. Upon treatment with rose bengal, oxidation of DCFH by H_2O_2 yielded highly fluorescent DCF (excitation at 488 nm; emission at 525 nm) which was detected by laser

scanning confocal microscopy. In the study of ROS signalling in affecting the root gravitropism of maize plants, DCF-DA, which is converted to DCF upon oxidation by H_2O_2, was used in detecting the distribution of ROS in the maize root (Joo et al., 2001).

Intracellular accumulation of H_2O_2 was detected using scopoletin (Sigma) through loss of fluorescence at 460 nm after excitation at 350 nm. Superoxide anion accumulation was detected using cytochrome *c* (Sigma) where shift in absorbance of the suspension cells added with cytochrome *c* from 540 nm to 550 nm was recorded. XTT was also used to detect $O_2^{\bullet -}$ through reduction of XTT by this ROS that cause absorbance of the medium at 470 nm (colorimetric assay) (Delledone et al., 2001). XTT assay in detecting $O_2^{\bullet -}$ was used in study of up-regulation of antioxidant enzymes upon water stress that generates ROS within maize leaves (Jiang & Zhang, 2002).

Reaction of highly fluorescent DanePy (excited at 345 nm) with $^1O_2$ yielded a non-fluorescent DanePyO. Hence, the presence of $^1O_2$ was detected by the loss of fluorescence upon excitation at 345 nm. Detection of $O_2^{\bullet -}$ can also be done through reaction of nitroblue tetrazolium with $O_2^{\bullet -}$ which yielded dark blue insoluble formazan compound. A compound named 3,3'-diaminobenzidine was used to detect hydrogen peroxide in leaves through formation of deep brown polymerization product upon reaction with H_2O_2 (Fryer et al., 2002).

DanePy, which is specific to $^1O_2$, and HO-1889NH, which has higher sensitivity in detecting $O_2^{\bullet -}$, were used in the study by Hideg et al. (2002). Production of ROS upon treatment of photosynthetically active radiation (PAR) or UV caused fluorescence

quenching of both dansyl-based ROS sensors, at different intensity depending on the intensity of ROS generated.

In the study of role of ROS in signalling the organization of maize roots, carboxy-H2DCFDA (C-400) dye (Molecular Probes, Eugene Oregon, catalogue no. C-6827), which is colourless when chemically reduced, but fluoresces green emission at 530 nm upon excited at 340 nm, after oxidized by ROS. This fluorescent product is reported to trap inside cell to facilitate long term observation (Jiang et al. 2003).

Hu et al. (2003) have used a H_2O_2-detecting reagent which contains 200 mm Tris (pH 7.0), 400 µM of 4-aminoantipyrine, 20 µL of *N*,*N*-dimethylanaline and 2 units of horseradish peroxidase (Sigma, St. Louis) in detecting and quantify H_2O_2. Mixture of H_2O_2-containing supernatant and H_2O_2-detecting reagent has absorbance at 550 nm. This showed an example of *in vitro* detection of intracellular produced ROS.

Detection of ROS using fluorescence-emitting compound has the advantage of high sensitivity and specificity toward ROS. For example, DCFH is selective against H_2O_2 compared to other ROS (Allan & Fluhr, 1997). However, these fluorescent compounds can be expensive. Furthermore, the effectiveness of these compounds may vary depending on the condition of the dye before usage. For example carboxy-H2DCFDA (C-400) dye (Molecular Probes, Eugene Oregon, catalogue no. C-6827) must be prepared in water pH 6.8 freshly prior to experiment because it can easily oxidized by a wide variety of oxidants (Jiang et al. 2003).

2.4.2 Detection of ROS using RNA and Protein Blot

Increase in levels of intracellular ROS was detected by elevation of expression of ROS-responsive gene using protein blot. Upon treatment of light stress, APX proteins such as thylakoid ascorbate peroxide (tylAPX), stromal/mitochondrial ascorbate peroxide (s/mAPX), and cytosolic ascorbate peroxidase (APX1) were isolated and expression levels were determined using protein blot. Because these APX proteins are ROS scavenging enzymes, treatment of oxidative stress elevated expression of these APX proteins. Double mutation deficient in cytosolic APX1 and tylakoid APX did not show expression in these two APX proteins, but expression of stromal/mitochondrial APX remained elevated. This indicated that these ROS-scavenging enzymes are potential sensor to indirectly detect presence of intracellular ROS (Miller et al., 2007).

ROS treatment and abiotic stress treatment caused changes in the expression of ROS-responsive genes that encode zinc finger proteins Zat12 and Zat7, cytosolic ascorbate peroxide APX1, and WRKY transcription factor (WRKY25). Using RNA blots, the expression of these ROS-responsive genes was determined which in turn detect the presence of intracellular ROS. Application of H_2O_2 induced the expression of these genes while untreated sample did not showed expression. Application of abiotic stresses such as heat stress and light stress elevated the expression indicated these abiotic stresses cause intracellular production of H_2O_2 (Rizhsky et al., 2004).

Levels of expression ROS-responsive genes can be determined using RNA blots and proteins blots which in turn allows quantification of intracellular ROS produced during oxidative stress. However, these methods require collection of sample from the

plants which destroys the collected tissue. It does not allow *in vivo* monitoring of ROS signalling in regulating physiological processes such as stomatal closure. Easily degraded RNA molecules can also possibly lead to false negative result.

2.4.3 Detection of ROS using Reporter Gene System.

Treatment of H_2O_2 enhanced level of β-glucuronidase (GUS) expressed from the *PR-1a* promoter. A construct of *GUS* gene fused with *PR-1a* gene was made and treatment of H_2O_2 activates *PR-1a* promoter and expressed PR-1 protein and GUS simultaneously. After the treatment of H_2O_2, intact plant materials were treated with histochemical staining to detect the presence of GUS (Blee, Yang, & Anderson, 2004). Hence, ROS can be detected using this kind of construct.

Various researches used the construct in which luciferase (Luc) reporter gene fused with *Zat12* promoter to detect the accumulation of ROS in the plants and study the signalling role of ROS in regulating plant physiological processes, as well as defence mechanism. *Zat12* promoter is activated by H_2O_2 and luciferase is expressed. Luciferase, after induction of oxidative stress and abiotic stress, was expressed and monitored through bioluminescence illuminated after introduction of luciferin. Rapid systemic signal induced by mechanical wounding was monitored using transgenic *Zat12::Luc* plants. The result revealed the systemic signal induced by H_2O_2 travelled at the rate of up to 8.4 cm min^{-1} (Miller et al., 2009). In the study of role of Zat12 protein in response to oxidative stress and abiotic signalling, transgenic *Zat12::Luc* plants were used and treatment of abiotic stress caused bioluminescence of luciferase. Through the aid of this

Zat12::Luc construct, these result indicated the importance of ROS-responsive *Zat12* gene during oxidative burst (Davletova et al., 2005b).

2.5 Functions of Zinc Finger Protein Zat12 in Plants.

Zinc finger protein Zat12 was first isolated in the study of light acclimatization response in *Arabidopsis*, which known as zinc finger protein RHL41 due to its responsiveness toward high light (Iida et al., 2000). Various researches have been conducted and showed its role toward different abiotic stress such as heat stress, high light stress, wounding, paraquat application, osmotic stress, and cold acclimatization (Davletova et al. 2005b; Vogel et al., 2005).

In the study of Zat12 transcription factor toward expression of cytosolic ascorbate peroxidase (APX1), the expression of *Zat12* gene was elevated upon treatment of plants with H_2O_2 and abotic stress such as paraquat, heat shock, high light, wounding, and osmotic stress. These result suggested *Zat12* is a ROS-responsive gene which is expressed when the plants suffered from environmental stress (Rizhsky et al., 2004). The study also showed its role in expression of ROS-scavenging enzyme APX1 to combat the elevation of intracellular ROS from causing cellular damage.

In the study of relationship of Zat12 transcription factors toward cold acclimatization of *Arabidopsis*, constitutive expression of Zat12 in the *Arabidopsis* plant caused increasing in freezing tolerance. Experimental data showed expression of Zat12 transcription factor has negative regulatory effect toward expression of *CBF* gene, which is responsible in cold response pathways (Vogel et al., 2005).

Using the construct of promoter::reporter where *Zat12* promoter is fused with luciferase reporter gene, role of Zat12 protein in response to cold, heat, salinity, drought, wounding, and superoxide-generating agent methl violegen was studied. Expression of luciferase was enhanced in response to different abiotic stress indicated *Zat12* promoter is activated by ROS and Zat12 protein is involved in the response of plants toward these stresses. Furthermore, both wild type and Zat12-overexpressing plants showed elevation of 465 transcripts upon treatment with H_2O_2. This result indicated its role in regulating the expression of these signal transduction, ROS-related, and defence-related transcripts (Davletova et al. 2005b). Davletova et al. (2005b) proposed that Zat12 protein may not directly involved in stress-tolerance but serves as transcriptional regulator during these stresses. In another words, it regulates collection of transcripts involved in these environmental stresses.

2.6 Green Fluorescent Protein (GFP) for *in vivo* Cell Imaging

Green fluorescent protein was first discovered from *Aequorea* jellyfish by Shimomura, Johnson, and Saiga (1962) which they described as "a protein giving solutions that look slightly greenish in sunlight through only yellowish under tungsten lights, and exhibitiong a very bright, greenish fluorescence in the ultraviolet of a Mineralite, has been isolated from squeezates." (as cited in Tsien, 1998). Various variants of GFP were also described after the discovery. The properties of wild type GFP and variants such as αGFP, S65T, EGFP, and EBFP were described by Patterson et al. (1997). As described by Ehrhardt (2003), fluorescent proteins such as GFP can be genetically

fused with any protein sequences and expressed by one of the available regulatory sequences. Discovery of GFP and its variants permits *in vivo* imaging of living tissue.

In the study of rearrangement of cortical microtubule in living epidermal cells, construct of microtubule binding domain of the mammalian microtubule-associated protein 4 (*MAP4*) gene fused with the green fluorescent protein (*GFP*) gene was used. Using laser scanning confocal microscope with excitation at 488 nm, the movement of microtubules, localized microtubule reorientations, and global microtubule reorganizations in transgenic *Vicia faba* were revealed (Marc et al., 1998).

Fusion protein between GFP and second actin-binding domain (fABD2) of *Arabidopsis thaliana* fimbrin, AtFIM1 revealed structural detail not seen with other labelling methods. Expression of GFP-fABD4 in transgenic plants revealed a dense network of free actin filaments in cortex and perinuclear region of hypocotyls, pavement epidermal, and trichome cells. Masses of bundled filaments were observed in transvasuolar strands of pavement epidermal cells and cortex of mature root epidermal cells. This fusion protein also revealed the continual reorganization of actin filaments network (Sheahan, Staiger, Rose, & McCurdy, 2004).

Unlike other reporter gene system such as luciferase reporter gene system, expression of GFP within the living cells can be detected *in vivo* without usage of substrates to form bioluminescence. Dynamic of intracellular components, localization of proteins, interaction between proteins can be studied using GFP reporter gene system where the imaging can be done through excitation at a particular wavelength such as 488 nm for variants S65T GFP (Ehrhardt, 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Equipments and Materials

All chemicals and equipments were obtained from UTAR Laboratory. Table 3.1 and Table 3.2 showed list of equipments and chemicals used, respectively.

Table 3.1: List of equipments used, together with their model name.

No	Equipment	Models/Manufacturers
1	ABJ Analytical Balance	KERN
2	Autoclave Sterilizer	HIRAYAMA HVE-50 (Made in Japan)
3	Computer	AcerPowerPE (Made in China)
4	Electronic balance	KERN
5	Electrophoresis System	Electrophoresis system Mupid®-2plus (TAKARA BIO INC.)
6	Electroporation Cuvette	Eppendorf
7	Electroporator	Eppendorf (Electroporator 2510)
8	Evaporator	EYELA Centrifugal Evaporator cve-2000
9	Hotplate Stirrers	Stuart
10	Imaging System	UVP (MultiDoc-It Digital Imaging System)
11	Incubator	Memmert
12	Laminar Flow (Horizontal)	ESCO Laminar Flow Cabinet (Model: AHC-4DI)
13	Microcentrifuge	Thermo Electron Corporation (Sorvall Legend Micro 17 Centrifuge)
14	Micropipette	i. ViPR Ω Mechanical Pipette ii. Eppendorf iii. ThermoScientific iv. Gilson (Pipetman)
15	Microwave	SHARP (Japan) Model: R-218H(S)
16	Nanophotometer	Implen
17	PCR machine	Bio-RAD (MyCycler™ Thermo Cycler)
18	Refrigerator	Toshiba
19	Shaking Incubator	N-Biotek
20	Spectrophotometer	BIO-RAD SmartSpec™

No	Equipment	Models/Manufacturers
21	Table Top Refrigerated Centrifuge	Dynamica (Velocity 14R centrifuge)
22	UV Transilluminator	UVP
23	Water bath	Memmert

Table 3.2: List of chemicals used, together with their manufacturers**.**

No.	Materials/Chemicals	Manufacturers
1	1 kb DNA marker	Fermentas GeneRuler 1kb DNA ladder 0.5µg/ml, 50µg
2	95% Ethanol	Copens Scientific (M) Sdn. Bhd.
3	Absolute Ethanol	HmbG® Chemical
4	Acetic Acid	SYSTERM®
5	Agar Powder	R & M
6	Agarose Powder	Vivantis
7	Ampicilin Sodium	Wako
8	Boric Acid	QRëC™
9	Calcium Chloride Dehydrate	QRëC™
10	DNA Polymerase	i. Takara PrimeSTAR HS DNA polymerase (Code R010A Lot N170/DA) 250U, 2.5u/µl ii. Fermentas Taq DNA Polymerase (recombinant) (Lot 00046416 #EP0402) 500U, 5u/µl iii. Takara Ex Taq
11	DNA polymerase buffer	i. Fermentas 10X Taq Buffer with $(NH_4)_2SO_4$ ((Lot 00044197 #EP0402) 1.25ml ii. Takara 5X PrimeSTAR Buffer-$Mg2^+$ plus (Lot A2701A) 1ml
12	dNTPs	TOYOBO (2 mM each)
13	Ethidium Bromide	BIO BASIC INC.
14	Ethylenediaminetetraacetic Acid	QRëC™
15	Gentamycin Sulfate	BIO BASIC INC.
16	Glucose (Dextrose) (D-Glucose)	Rdeh
17	Glycerol	QRëC™
18	Hydrochloric Acid	MERCK
19	Hygromycin B	Wako
20	Isopropanol	MERCK

No.	Materials/Chemicals	Manufacturers
21	Kanamycin Sulfate	Wako
22	Magnesium Chloride	QRëC™ Fermentas 25mM $MgCl_2$ (Lot 00037863) 1.25ml
23	Phenol:Chloroform:Isoamylalcohol-25:24:1	CALBIOCHEM
24	Potassium Acetate	MERCK
25	RNaseA	
26	Sodium Acetate	SYSTERM®
27	Sodium Chloride	SYSTERM®
28	Sodium Hydroxide	R & M
29	Tris	Vivantis
30	Tris-HCl	BIO BASIC INC.
31	Tryptone	CONDA pronadisa
32	Yeast Extract	Scharlau

3.2 Culture Medium and Solutions Preparation

3.2.1 Preparation of Culture Media

3.2.1.1 Preparation of Luria Bertani Medium

To prepare 1 L of Luria Bertani medium, 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl were dissolved in 900 mL of distilled water. Distilled water was added to 1 L and pH value was adjusted to pH 7.0. The media was sent for autoclave at 15 min, 121°C, and 15 psi (Sambrook, Fritsch, & Maniatis, 1989).

3.2.1.2 Preparation of 2× YT Medium

To prepare 1 L of 2× YT medium, 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl were dissolved in 900 mL of distilled water. Distilled water was added to 1 L

and pH value was adjusted to pH 7.0. The media was sent for autoclave at 15 min, 121°C, and 15 psi (Sambrook et al., 1989).

3.2.1.3 Preparation of S.O.C. Medium

To prepare 1 L S.O.C. medium, 20 g of tryptone, 5 g of yeast extract, and 0.5 g of sodium chloride were dissolved in 950 mL distilled water. After all the components were dissolved, 10 mL of sterile 250mM potassium chloride was added and the pH was adjusted to 7.0 using 5 N sodium hydroxide. The volume were adjusted to 1 L and sterilized by autoclaving at 15 min, 121°C, and 15 psi. Just before use, 5 mL of sterile 2 M magnesium chloride and sterile 1 M of glucose were added (Sambrook et al., 1989).

3.2.2 Preparation of Other Solutions

3.2.2.1 Preparation of Solution 1

To prepare 100 mL of solution 1, 0.9 g of glucose, 2.5 mL of 1 M Tris-HCl, and 2 mL of 0.5 M EDTA were dissolved in 90 mL distilled water. The volume was adjusted to 100 mL and autoclaved at 15 min, 121°C, and 15 psi (Micklos & Frever, 2003).

3.2.2.2 Preparation of Solution 2

To prepare 15 mL of solution 1, 3 mL of 1 N sodium hydroxide, and 1 mL of 15% sodium dedocyl sulphate was dissolved in 10 mL distilled water. The volume was adjusted to 15 mL and filter sterilization was used to sterilize it (Micklos & Frever, 2003).

3.2.2.3 Preparation of Solution 3

To prepare 100 mL solution 3, 60 mL of 5 M potassium acetate and 11.5 mL acetic acid were dissolved in 90 mL distilled water. The volume was adjusted to 100 mL and autoclaved at 15 min, 121°C, and 15 psi (Micklos & Frever, 2003).

3.2.2.4 Preparation of TE Buffer

To prepare 100 mL TE Buffer, 1 mL of 1 M Tris-HCl (pH 8.0) and 200 µL of 0.5 M EDTA (pH 8.0) were added into distilled water to give final volume of 100 mL. It was autoclaved at 15 min, 121°C, and 15 psi (Micklos & Frever, 2003).

3.2.2.5 Preparation of 5× TBE Buffer

To prepare 1 L of 5× TBE buffer, 54 g of Tris, 27.5 g of boric acid, and 20 mL of 0.5 M EDTA were dissolved in 900 mL distilled water. The volume was adjusted to 1 L and autoclaved at 15 min, 121°C, and 15psi (Micklos & Frever, 2003).

3.3 Extraction and Purification of DNA Sample

3.3.1 Extraction and Purification of Plant Genomic DNA

An amount of 0.08 g of young leaf of *Arabidopsis thaliana* (cv. Columbia) was obtained and was ground using mortar after addition of 250 µL of pre-chilled DNA extraction buffer [200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, and 0.5% SDS]. All the liquid, including the crushed leaf tissue, was transferred to 1.5 mL microcentrifuge tube and was added with 550 µL of DNA extraction buffer. The mixture was homogenized by vigorously vortex for 3 min at room temperature. After vigorous vortexing, the sample was centrifuged at 14000 rpm for 10 min using Velocity 10 Benchtop Centrifuge by Dynamica. The supernatant was recovered into another 1.5 mL microcentrifuge without disturbing the cell debris at the bottom of the microcentrifuge tube. Equal volume of phenol: chloroform: isoamyl alcohol solution (25: 24: 1) was added and homogenized by briefly vortexing the mixture. The mixture was centrifuged at 14000 rpm for 5 min at room temperature. Upper aqueous layer was recovered into another microcentrifuge tube and 3/4 volume of chilled isopropanol was added and mixed by vortexing. The mixture was centrifuged at 14000 rpm for 10 min and the supernatant was discarded without disturbing the pellet. The pellet was rinsed with 1 mL of 70% ethanol and centrifuged at 14000 rpm for 5 min. The supernatant was removed and the pellet was dried using EYELA Centrifugal Evaporator cve-2000 to remove remaining ethanol. The pellet was dissolved using 100 µL TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)].

3.3.2 Mini-Preparation of Plasmid DNA

A volume of 3 mL overnight bacterial culture was harvested into a 1.5 mL microcentrifuge tube by centrifuging at 14000 rpm at 4°C for 15 min. Supernatant was discarded without disturbing the cell pellet. Cell pellet was re-suspended with 100 µL of chilled Solution 1 [50 mL Glucose, 25 mM Tris-HCl (pH 8.0), and 10 mM EDTA] and homogenized by vortexing. After the cell pellet has fully suspended, 200 µL of Solution 2 (0.2 N NaOH and 1% SDS) was added. The tube was inverted five times to ensure complete lysis. The tube was left on ice for 5 min and followed by addition of chilled Solution 3 (3 M potassium acetate and 11.5% acetate acid). The mixture was homogenized by vortexing briefly and left on ice for 5 min. The mixture was centrifuged at 14000 rpm at 4°C for 15 min. The supernatant was recovered into new 1.5 mL microcentrifuge tube without disturbing the while precipitate. Equal amount of PCI solution was added and homogenized by briefly vortexing the mixture. The mixture was centrifuged at 14000 rpm for 5 min at room temperature and the upper aqueous layer was recovered into new 1.5 mL microcentrifuge tube. Two volumes of absolute ethanol was added and left on ice for 2 min. The mixture was centrifuged at 14000 rpm at 4°C for 15 min. The supernatant was removed and 1 mL of 70% ethanol was used to rinse the pellet. The sample was centrifuged at 14000 rpm at 4°C for 5 min. The supernatant was removed and the pellet was dried using vacuum evaporator to remove remaining ethanol. The pellet was dissolved using 100 µL TE buffer.

3.3.3 Midi-Preparation of Plasmid DNA

Midi-preparation of plasmid DNA was carried out using HiSpeed® Plasmid Midi Kit by QIAGEN, according to HiSpeed® Plasmid Purification Handbook.

A volume of 120 mL of overnight bacterial culture was harvested by centrifugation at 14000 rpm at 4°C for 15 min. The cell pellet was re-suspended in 6 mL of Buffer P1. The suspended bacterial cells was added with 6 mL of Buffer P2 and mixed by inverting the tube 4 – 6 times. The mixture was incubated at room temperature for 5 min. After 5 min of incubation, 6 mL of Buffer P3 was added into the mixture and mixed by inverting the tube 4 – 6 times.

Outlet nozzle of the QIAfilter Cartridge was screwed with the cap and the cell lysate was poured into the barrel. The mixture was incubated in room temperature for 10 min without inseting the plunger. A HiSpeed® Midi Tip was equilibrate by applying 4 mL of Buffer QBT and allowed the column to empty by gravity flow. The cap was removed from QIAfilter Cartridge outlet nozzle and the plunger was inserted into the QIAfilter Cartridge and filtered the cell lysate into the equilibrated HiSpeed® Midi Tip. The cleared lysate was allowed to pass through the resin by gravity flow. The HiSpeed® Midi Tip was washed with 20 mL of Buffer QC. The DNA was eluted with 5 mL Buffer QF, into a 15 mL centrifuge tube.

The eluted DNA was mixed with 3.5 mL isopropanol and incubated at room temperature for 5 min. QIAprecipitator was attached onto outlet nozzle of a 20 mL syringe after removing the plunger. The eluate DNA/isopropanol was transferred into the 20 mL syringe and filtered into waste bottle, by inserting plunger to the syringe. The

QIAprecipitator and then the plunger were removed from the syringe and the QIAprecipitator was reattached back to the syringe. Exactly 2 mL of 70% ethanol was added into the syringe and plunger was inserted to press the ethanol through the QIAprecipitator. The QIAprecipitator and then the plunger were removed from the syringe and the QIAprecipitator was reattached back to the syringe. The plunger was inserted to press air through the QIAprecipitator quickly and forcefully. The plunger was removed from a 5 mL syringe and the QIAprecipitator was attached to the outlet nozzle of the syringe. Exactly 1 mL of TE buffer was added into the 5 mL syringe and the plunger was inserted to elute the DNA sample into a 1.5 mL collection tube. The QIAprecipitator and then the plunger were removed from the syringe and the QIAprecipitator was reattached back to the syringe. Eluted DNA sample was transferred back to the same 5 mL syringe and eluted into the same 1.5 mL collection tube.

3.3.4 Purification of DNA Sample from RNA Contamination.

Approximately 1/100 volume of 10 mg/mL of RNase A was added into the DNA sample and incubated in 37°C water bath for 30 min. After 30 min incubation, the DNA sample was added with equal volume of PCI solution and mixed by vortexing briefly. The mixture was centrifuged at 14000 rpm at room temperature for 5 min. Upper aqueous layer was transferred into new microcentrifuge tube. Exactly 1/10 volume of 3 M sodium acetate and 2.5 volume of absolute ethanol were added into the sample. The mixture was homogenized by vortex briefly and then left on bench for 30 min. After 30 min, the mixture was centrifuged at 14000 rpm at 4°C for 30 min. The supernatant was discarded

and the pellet was rinsed with 1 mL of 70% ethanol. The sample was centrifuged at 14000 rpm at 4°C for 2 min. The supernatant was discarded and the pellet was dried using vacuum evaporator. The pellet was dissolved in appropriate amount of TE buffer. Concentration and ratio A_{260}/A_{280} of the DNA sample was determined using Nanophotometer by Implen. The DNA sample was stored at -20°C until future use.

3.4 Polymerase Chain Reaction (PCR)

3.4.1 Amplification of *proZat12* using PrimeSTAR™ HS DNA Polymerase by Takara

Zat12 promoter region (*proZat12*) comprises of 1,000 base pairs and was amplified by PCR with the following primers: *proZat12F*, 5'-CACCCTCATTGCTGACGGTAGTTTTTAGAG - 3' and *proZat12R*, 5' – TTTTCTTCTGATGATGATGATTAAAACG - 3'. Table 3.3 showed components of the PCR reaction mixture.

Table 3.3: PCR reaction mixture using PrimeSTAR™ for amplification of *proZat12*.

Components	Volume
5× PCR Buffer	4.0 µL
2.0 mM dNTP Mixture	2.0 µL
10 µM *proZat12F*	0.8 µL
10 µM *proZat12R*	0.8 µL
***A. thaliana* Genomic DNA**	1.5 µL
PrimeSTAR™ (2.5 units/µL)	0.2 µL
Sterilized ddH_2O	Up to 20.0 µL

3.4.1.1 Gradient PCR of *proZat12*

Gradient PCR of *proZat12* was done using GenePro Thermal Cycler by BIOER, by setting the annealing temperature ranging from 55°C to 60°C. Table 3.4 showed thermocycling condition for gradient PCR of *proZat12*.

3.4.1.2 Amplification of *proZat12* at a Specific Annealing Temperature

After suitable annealing temperature for amplification of *proZat12*, was determined using gradient PCR, this promoter was amplified using that temperature to obtain higher amount of product for cloning purposes (i. e. 58.6°C). Table 3.5 showed thermocycling condition for amplification of *proZat12* after determination of suitable annealing temperature.

Table 3.4: Thermocycling condition for gradient PCR of *proZat12*.

Temperature	Time	Number of cycles
96°C	15 sec	1 cycle
96°C	15 sec	30 cycles
55°C - 60°C	15 sec	
70°C	60 sec	
70°C	60 sec	1 cycle
10°C	∞	1 cycle

Table 3.5: Thermocycling condition for amplification of *proZat12*.

Temperature	Time	Number of cycles
96.0°C	15 sec	1 cycle
96.0°C	15 sec	30 cycles
58.6°C	15 sec	
70.0°C	60 sec	
70.0°C	60 sec	1 cycle
10.0°C	∞	1 cycle

3.4.2 Colony PCR

After transformation, colonies toothpicked onto master plate before putting into the different PCR tubes. The toothpicks were removed and 10 µL of "PCR Pre-mix" into the tubes. Colony PCR was done using (recombinant) *Taq* DNA Polymerase by Fermentas. Table 3.6 showed the general PCR reaction mix for colony PCR. Tubes containing bacterial cells were boiled at 100°C for 10 min for PCR. Table 3.7 showed themocycling condition for colony PCR.

The primer set used for colony PCR varies depending on the vector used for cloning. To screen the recombinant pENTR™/D-TOPO® with correct orientation of *proZat12*, two option of primer sets can be used: (1) M13F and *proZat12R*; (2) M13R and *proZat12F* (refer to Appendix D). To screen the recombinant pGWB4 (GATEWAY® cloning) carrying *proZat12*, forward and reverse primers for *proZat12* were used (Invitrogen, 2006).

Table 3.6: General "PCR-Premix" for colony PCR.

Components	**Volume**
10× PCR Buffer	1.00 µL
25 mM Magnesium Chloride	0.60 µL
2.0 mM dNTP Mixture	1.00 µL
10 µM Forward Primer	1.00 µL
10 µM Reverse Primer	1.00 µL
(recombinant) *Taq* DNA Polymerase (5 units/µL)	0.05µL
Sterilized ddH_2O	Up to 10.0 µL

Table 3.7: General thermocycling condition for colony PCR.

Temperature	**Time**	**Number of cycles**
95.0°C	600 sec	1 cycle
95.0°C	30 sec	35 cycles
55.0°C	60 sec	
72.0°C	120 sec	
72.0°C	600 sec	1 cycle
10.0°C	∞	1 cycle

After the screening by the first colony PCR, the colonies carrying recombinant plasmid with correct orientation of insert was extracted and screen for second times to confirm the result by substituting the colonies with small amount of purified plasmid (0.3 µL).

3.4.3 Purification of PCR Product

Zat12 promoter (*proZat12)*, which was amplified through PCR method, was purified for cloning purposes. By using Montage® PCR Centrifugal Filter Device by Millipore Corporation, the amplified *proZat12* was purified from primer dimer before cloning into pENTR™/D-TOPO®. Montage PCR sample reservoir was inserted into one of the two vials provided and 320 µL of TE buffer was added. Exactly 80 µL of PCR product was added into the reservoir and the reservoir was sealed with attached cap. The Montage PCR unit was centrifuged at 1000 x *g* for 15 min. After the centrifugation, 20 µL of TE buffer was added into the sample reservoir and placed into a clean vial in inverted manner. The Montage® PCR unit was centrifuged at 1000 x *g* for 2 min. The purified PCR product was kept in -20°C until future use (Millipore Corporation, 2005).

3.5 DNA Sequencing

Purified plasmid pENTR™/D-TOPO® carrying *proZat12* was sequenced with M13F and M13R in both direction, using BigDye Terminator Cycle Sequencing (Applied Biosystems), by First Base Laboratories Sdn Bhd.

Sequences of plasmid DNA samples were aligned with published sequence using web-based software BLASTN 2.2.25+ (Zhang, Schwartz, Wagner, & Miller, 2000).

3.6 Competent Cells Preparation

3.6.1 Competent Cells Preparation with Calcium Chloride

Single colony (DH5α and DB3.1) was picked from overnight culture plate and inoculated into 2 mL of 2× YT broth medium for 16 hrs incubation with incubator shaker at 37°C with 220 rpm. After 16 hrs incubation, the OD_{600} of the culture were measured and dilution to $OD_{600} \approx 0.1$ was made within centrifuge tube. The diluted culture was placed in incubator shaker and grown to $OD_{600} \approx 0.6$ with agitation at 220 rpm with 37°C.

The centrifuge tube with bacterial culture was centrifuged at 3000 x *g*, 4°C, for 15 min and supernatant was discarded. The cell pellet was re-suspended with 0.1 M calcium chloride twice. The cells were centrifuged at 3000 x *g*, 4°C, for 15 min after each washing and supernatant was discarded without disturbing the pellet. The pellet was suspended with salt mixture (20 mM $CaCl_2$ and 80 mM $MgCl_2$) and 10% glycerol. Exactly 50 µL of suspended cells was transferred into each microcentrifuge tubes. The cells were frozen in liquid nitrogen and stored at -80°C until future use.

3.6.2 Electrocompetent Cells Preparation

Single colony (DH5α and DB3.1) was picked from overnight culture plate and inoculated into 2 mL of 2× YT broth medium for 16 hrs incubation with incubator shaker

at 37°C with 220 rpm. After 16 hrs incubation, the OD_{600} of the culture were measured and dilution to $OD_{600} \approx 0.1$ was made within centrifuge tube. The diluted culture was placed in incubator shaker and grown to $OD_{600} \approx 0.6$ with agitation at 220 rpm with 37°C.

The centrifuge tube with bacterial culture was centrifuged at 3000 x *g*, 4°C, for 15 min and supernatant was discarded. The cell pellet was re-suspended with equal volume of 10% glycerol. The suspended cells were centrifuged at 3000 x *g*, 4°C, for 15 min and supernatant was discarded. The cell pellet was re-suspended with half volume of 10% glycerol. The suspended cells were centrifuged at 3000 x *g*, 4°C, for 15 min and supernatant was discarded. The cell pellet was re-suspended with 1/25 volume of 10% glycerol. The suspended cells were centrifuged at 3000 x *g*, 4°C, for 15 min and supernatant was discarded. The cell pellet was re-suspended with appropriate volume of 10% glycerol. Exactly 50 µL of suspended cells was transferred into each microcentrifuge tubes. The cells were frozen in liquid nitrogen and stored at -80°C until future use.

3.7 Transformation

3.7.1 Calcium Chloride-Mediated Transformation

A volume of 1 µL plasmid or ligation reaction mix was added into 50 µL of competent cells prepared with calcium chloride. The mixture was left on ice for 10 min. The cells were then incubated at 42°C for 90 sec. The cells were immediately left on ice for 10 min. A volume of 250 µL S.O.C. medium was added and incubated at 37°C for 30 min with agitation at 220 rpm using incubator shaker. The cells were centrifuged at

8000 x *g* for 15 min and 200 µL of supernatant was discarded. The cell pellet was re-suspended with the remaining medium in the tube and transformation mixture was transferred onto agar plate with appropriate antibiotics for selection. The cells were incubated at 37°C for overnight.

3.7.2 Electroporation

A volume of 1 µL plasmid or ligation reaction mix was added into 50 µL of electrocompetent cells. The mixture was left on ice for 10 min. The mixture was transferred to chilled electroporation cuvette by Eppendorf and pulsed at 1.55 volts using Electroporator 2510 by Eppendorf. A volume of 1 mL S.O.C. medium was added into the cuvette and transferred to a microcentrifuge tube. The cells were incubated at 37°C with agitation for 45 min. A volume of 100 µL transformation mixture was plated on agar plate with appropriate antibiotics. The cells were incubated at 37°C for overnight.

3.8 Cloning of *proZat12*

GATEWAY® cloning was used to allow the insertion of *proZat12 at* upstream and in frame with green fluorescence protein (GFP) gene, in correct orientation. The cloning procedure involves two parts: (1) insertion of *proZat12* into entry vector; and (2) insertion of *proZat12* from entry vector into destination vector using LR recombination reaction.

3.8.1 Insertion of *proZat12* into Entry Vector (pENTR™/D-TOPO®)

pENTR™ cloning was performed by following pENTR™ Directional TOPO® Cloning Kits use manual with slight modifications which were the extended incubation time from 5 min to 30 min, and TE Buffer was used for ligation reaction instead of sterile water. Table 3.8 showed the reaction mix for pENTR™ cloning for both chemically-prepared competent cells and electrocompetent cells. Reaction mix was mixed gently and incubated 30 min at 23°C before transforming into competent cells (Invitrogen, 2006).

3.8.2 Insertion of *proZat12* into Destination Vector (pGWB4)

Zat12 promoter (*proZat12*) was inserted from pENTR™/D-TOPO® into pGWB4 through GATEWAY™ Cloning System by Invitrogen. Entry vector containing correct orientation of insert, destination vector, and LR clonase reaction buffer were mixed. Table 3.9 showed volume of each component for LR recombination reaction mixture. LR Clonase enzyme mix was mixed by vortexing briefly after transferred on ice for 2 min for thawing. Exactly 1 µL of LR Clonase enzyme mix was added into LR recombination reaction mixture and mixed well by vortexing briefly. The reaction mix was incubated at 23°C for overnight. After overnight incubation, 1 µL of the Proteinase K (2 µg/µL) was added into the reaction tube and incubated at 37°C for 10 min before transforming into competent cells (Invitrogen, 2004).

Table 3.8: Reaction mix for pENTR™ cloning**.**

Reagents	Chemically-Prepared Competent Cells	Electrocompetent Cells
proZat12	2 µL	2 µL
Salt Solution	1µL	--
Dilute Salt Solution (1:4)	--	1 µL
pENTR™/D-TOPO®	1 µL	1 µL
TE Buffer	Add up to final volume 6µL	Add up to final volume 6µL

Table 3.9: Volume of each component for LR recombination reaction mixture.

Components	Volume
Entry vector, pENTR™/D-TOPO®	2µL
Destination vector, pGWB4	1µL
LR Clonase Reaction Buffer	1µL
TE Buffer	Add up to 5µL

3.9 Agarose Gel Electrophoresis

Gel electrophoresis was used to analyze PCR product, extracted plasmid, and extracted genomic DNA. Concentration of agarose gel was determined based on the expected size of the DNA sample to be analysed. 5× TBE was prepared and diluted to working 0.5× TBE. Electrophoresis System Multi®-2Plus by Takara was used for electrophoresis. All electrophoresis was conducted with voltage at 100 V. After electrophoresis, the gel was stained with ethidium bromide (EtBr) for 15 min and UV-illuminated using UVP UV Transilluminator and image was captured using computer with camera built inside the UVP UV Transilluminator.

3.10 Experimental Design for Molecular Cloning of *proZat12*.

Zat12 promoter was isolated from purified *Arabidopsis thaliana* genomic DNA using PCR. The blunt end PCR product was cloned into entry vector pENTR™/D-TOPO®. Plasmid pENTR™/D-TOPO® carrying *proZat12* in correct orientation was sequenced. Using LR recombination reaction, *proZat12* was cloned into destination vector/binary vector pGWB4. Figure 3.1 showed the schematic diagram for the experimental design in cloning of *proZat12* into binary vector pGWB4.

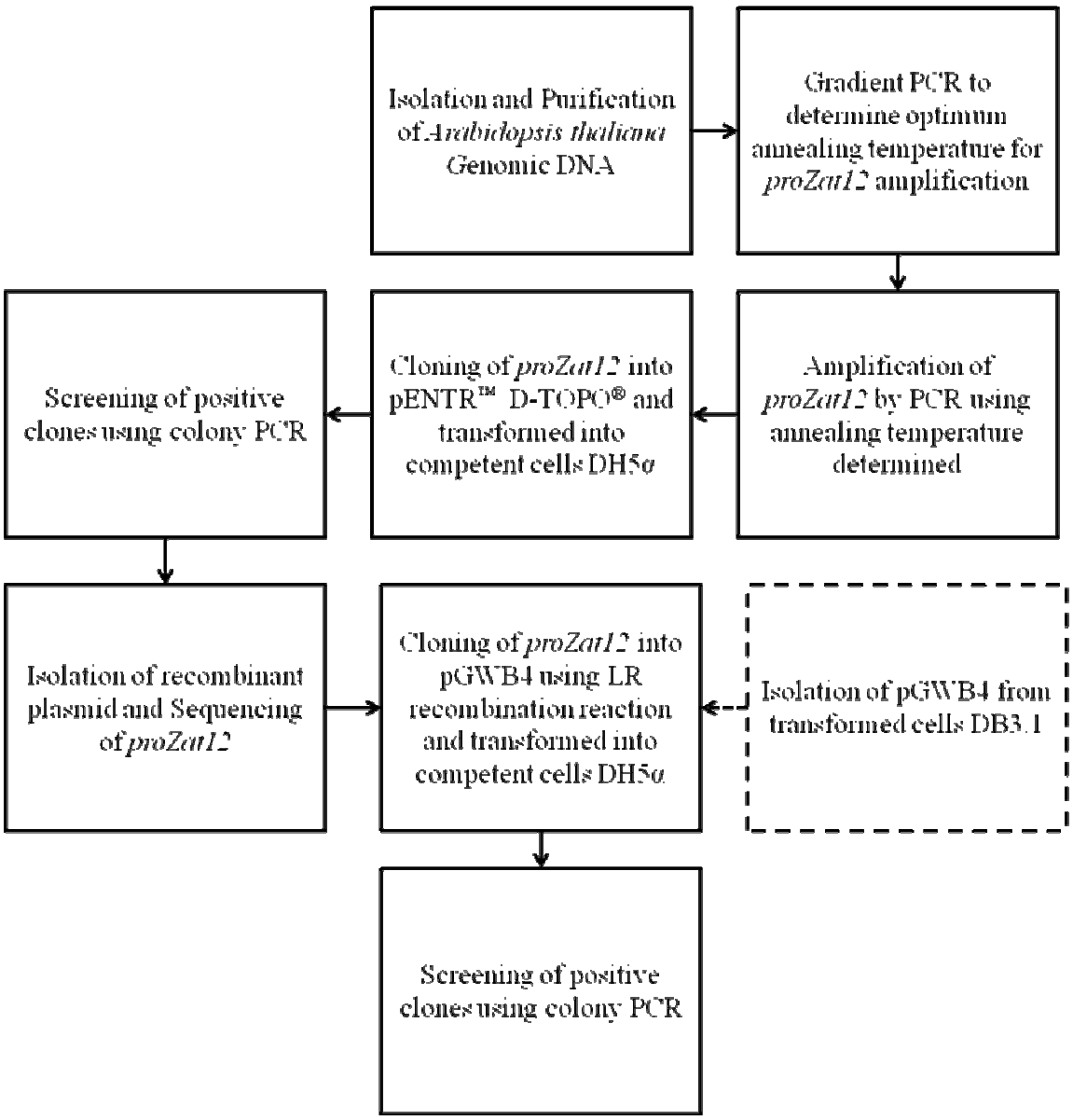

Figure 3.1: Work flow for molecular cloning of *proZat12* into binary vector pGWB4.

CHAPTER 4

RESULTS

4.1 Amplification of *proZat12* using PCR Method

RNase-treated *A. thaliana* genomic DNA was used as template for amplification of *proZat12* using PCR method. Appropriate annealing temperature (T_a) was determined using gradient PCR with a range of annealing temperature. Using the algorithm that was preset in the GenePro Thermal Cycler by BIOER, series of annealing temperatures was generated, ranging from 55°C to 60°C. Thermocycling condition for gradient PCR was shown in Table 3.4. One microlitre of gradient PCR products were loaded into 0.7% agarose gel for electrophoresis and showed that a single band with size of 1 kb at EtBr-stained agarose gel, for all the annealing temperatures tested. Figure 4.1 showed the image of 0.7% agarose gel loaded with gradient PCR products, after EtBr staining.

Annealing temperature at 58.6°C was used for the amplification of *proZat12*. Four replicates of the same reaction mixture were made. Thermocycling condition for PCR was shown in Table 3.5. One microlitre of PCR products were loaded into 0.7% agarose gel for electrophoresis and a distinct band with size of 1 kb was observed in all lanes. Faint band with size smaller than 250 bp was also observed. Figure 4.2 showed the image of 0.7% agarose gel loaded with PCR products, after EtBr staining.

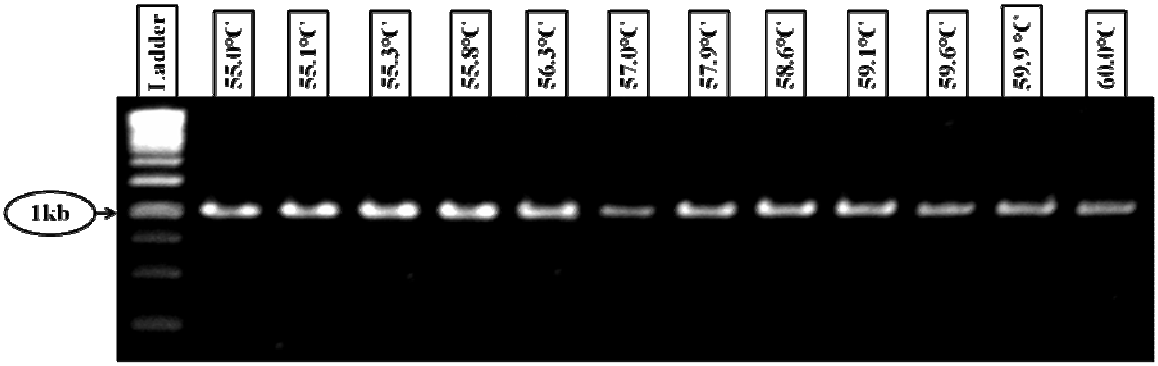

Figure 4.1: Gradient PCR of *proZat12* using *proZat12*F and *proZat12*R with different annealing temperature (T_a), ranging from 55°C to 60°C, generated by GenePro Thermal Cycler by BIOER. Lane 1: Fermentas GeneRuler 1 kb DNA Ladder; Lane 2: T_a = 55.0°C; Lane 3: T_a = 55.1°C; Lane 4: T_a = 55.3°C; Lane 5: T_a = 55.8°C; Lane 6: T_a = 56.3°C; Lane 7: T_a = 57.0°C; Lane 8: T_a = 57.9°C; Lane 9: T_a = 58.6°C; Lane 10: T_a = 59.1°C; Lane 11: T_a = 59.6°C; Lane 12: T_a = 59.9°C; Lane 13: T_a = 60.0°C.

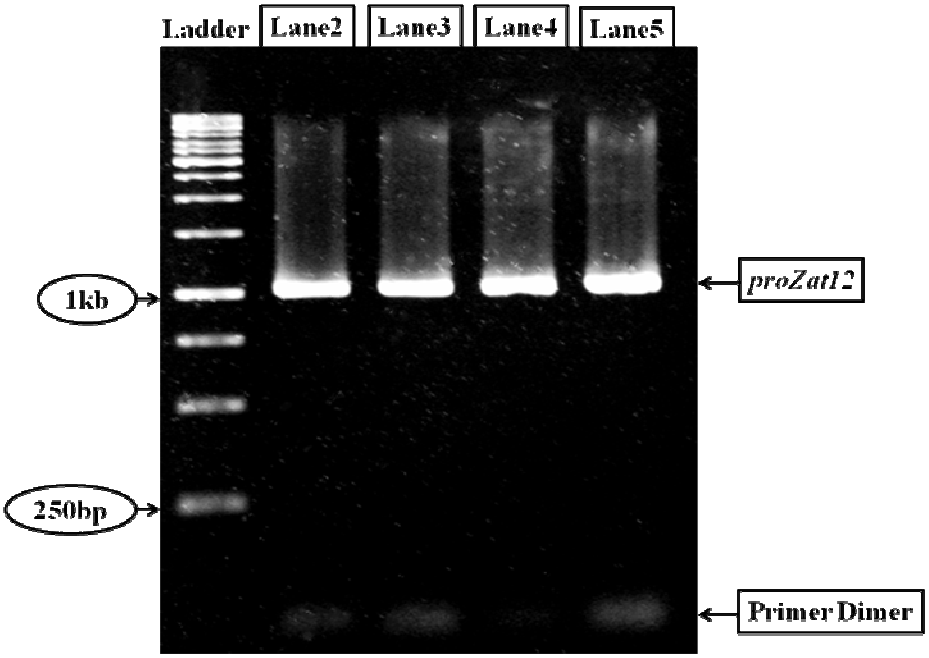

Figure 4.2: Amplification of *proZat12* using *proZat12*F and *proZat12*R at annealing temperature of 58.6°C. Lane 1: Fermentas GeneRuler 1 kb DNA Ladder; Lane 2-5: PCR product from amplification of *proZat12*.

PCR products, *proZat12*, were pooled and purified using Montage® PCR Centrifugal Filter Device by Millipore Corporation. One microlitre of purified *Arabidopsis thaliana* genomic DNA and purified PCR product were loaded into 0.7% agarose gel for electrophoresis. A band with size greater than 10 kb was observed in lane loaded with purified *A. thaliana* genomic DNA. A band with size of 1 kb was observed in lane loaded with purified PCR product. Figure 4.3 showed the image of 0.7% agarose gel loaded with purified *Arabidopsis thaliana* genomic DNA and purified PCR product, after EtBr staining.

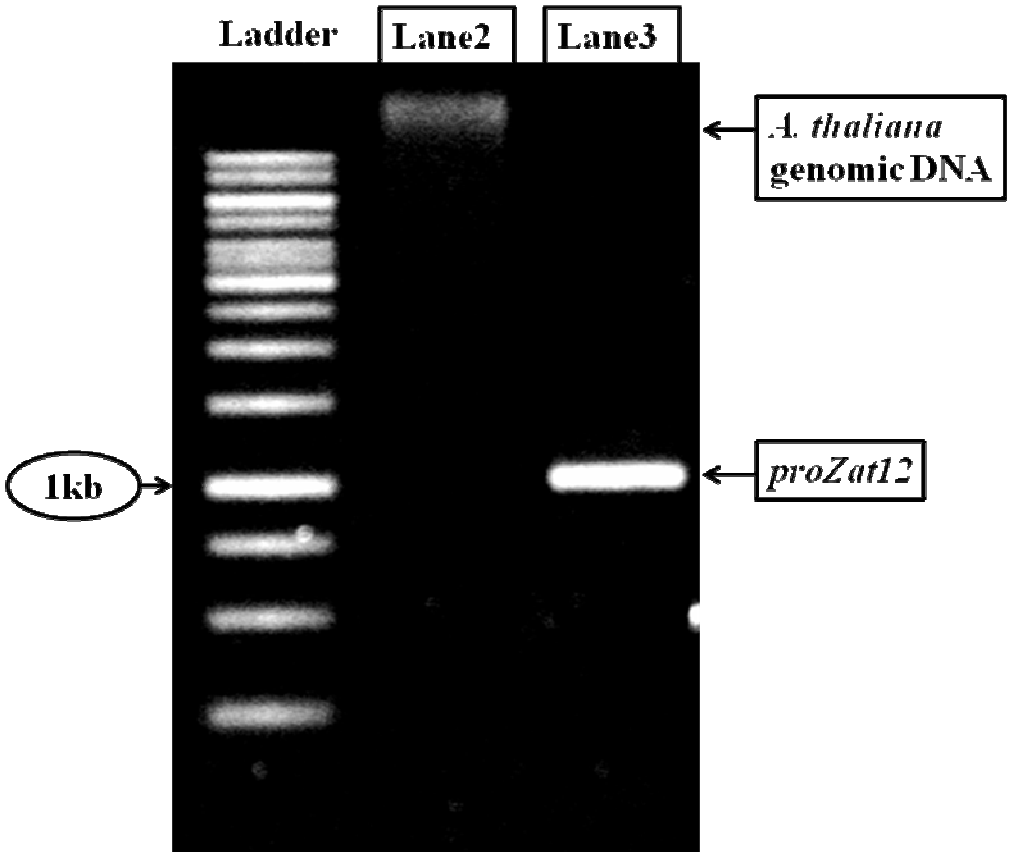

Figure 4.3: Purified *Arabidopsis thaliana* genomic DNA and purified *proZat12*. Lane 1: Fermentas GeneRuler 1kb DNA Ladder; Lane 2: Purified *Arabidopsis thaliana* genomic DNA; Lane 3: Purified *proZat12*

4.2 Colony PCR of pENTR™/D-TOPO® Carrying *proZat12*

Colony PCR was used to screen transformed cells *E. coli* DH5α carrying recombinant pENTR™/D-TOPO® with *proZat12* at correct orientation. Primers M13F and *proZat12*R were used and 23 bacterial colonies were picked randomly (coded S1-S23) for the colony PCR. Thermocycling condition for colony PCR was shown in Table 3.7. One microlitre of colony PCR products was loaded into 0.7% agarose gel for electrophoresis. All lanes showed smearing and/or a distinct band with size greater than 10 kb, except lane 4, 5, 9, and 12. Lane 5 and 12 showed a band with size slightly greater than 1 kb. Figure 4.4 showed the image of 0.7% agarose gel loaded with colony PCR products, after EtBr staining.

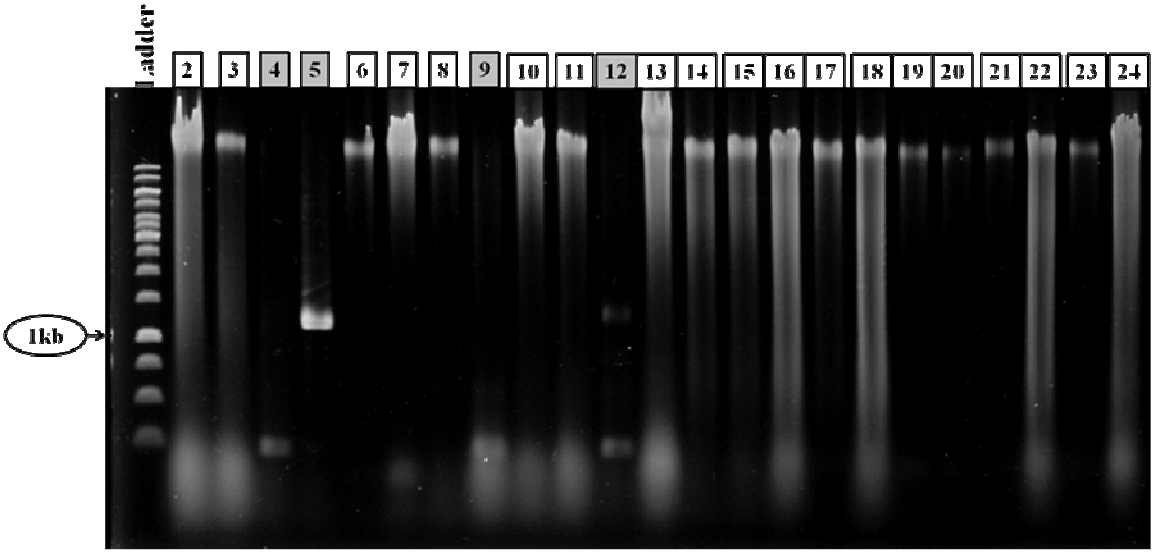

Figure 4.4: Colony PCR using M13F and *proZat12*R as primers to screen recombinant pENTR™/D-TOPO® carrying *proZat12*. Lane 1: Fermentas GeneRuler 1kb DNA Ladder; Lane 2-24: Colony PCR products with different colonies (coded from S1-S23).

4.3 Extraction of Recombinant pENTR™/D-TOPO® Carrying *proZat12*

Plasmid pENTR™/D-TOPO® carrying *proZat12* was isolated from colonies coded S3, S4, S8, and S11. One microlitre of isolated DNA samples were loaded into 0.7% agarose gel for electrophoresis, without treated with RNase. Three bands and smears below the bands were observed in all the lanes loaded with extracted DNA. Figure 4.5 showed the image of 0.7% agarose gel loaded with plasmid DNA samples extracted from colonies coded S3, S4, S8, and S11 (without RNase treatment), after EtBr staining.

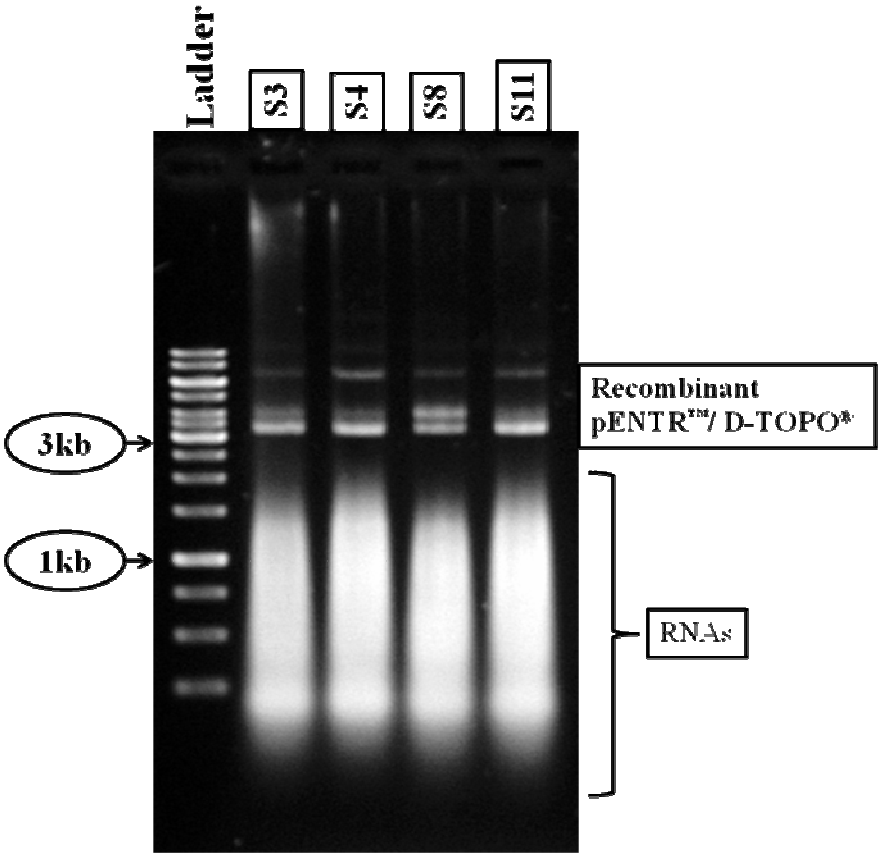

Figure 4.5: Extracted recombinant pENTR™/D-TOPO® carrying *proZat12*. Lane 1: Fermentas GeneRuler 1kb DNA Ladder; Lane 2: plasmid extracted from colony coded S3; Lane 3: plasmid extracted from colony coded S4; Lane 4: plasmid extracted from colony coded S8; Lane 5: plasmid extracted from colony coded S11.

Plasmid DNA samples extracted from colonies S3, S4, S8, and S11 were treated with RNase. One microlitre of RNase-treated plasmid DNA samples was loaded into 0.7% agarose gel for electrophoresis. Smears have been removed with some leftover at position smaller than 250 bp. A faint band between 2 distinct bands was observed in all lanes loaded with RNase-treated plasmid DNA samples. Figure 4.6 showed the image of 0.7% agarose gel loaded with RNase-treated plasmid DNA samples extracted from colonies coded S3, S4, S8, and S11, after EtBr staining.

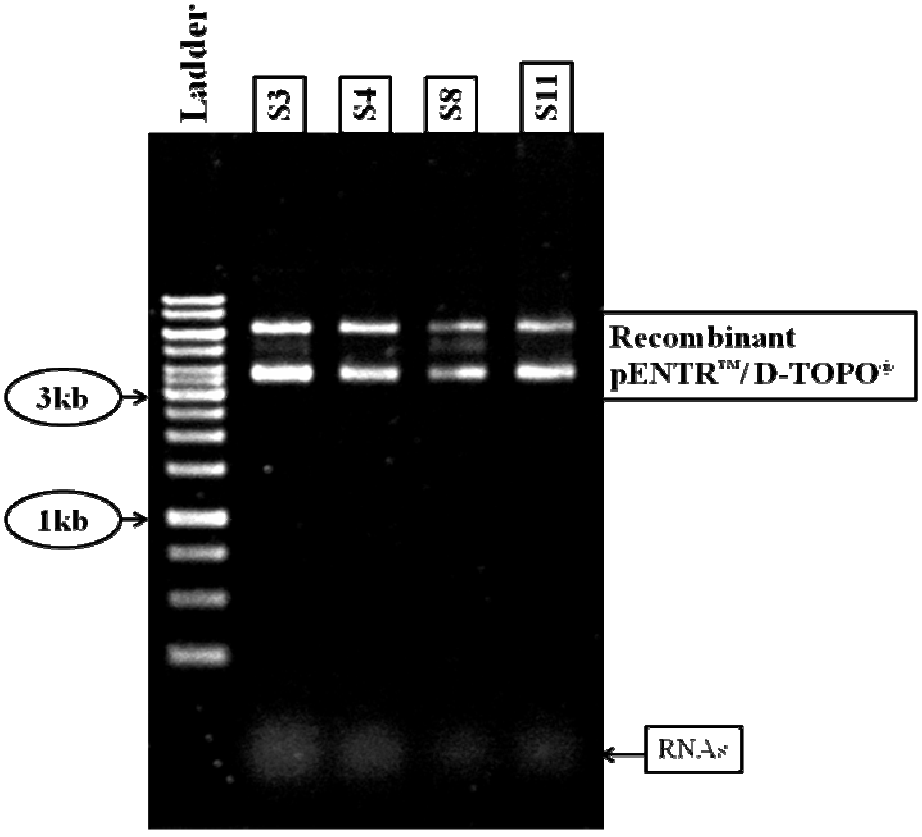

Figure 4.6: Extracted recombinant pENTR™/D-TOPO® carrying *proZat12* (after RNase treatment). Lane 1: Fermentas GeneRuler 1kb DNA Ladder; Lane 2: plasmid extracted from colony coded S3; Lane 3: plasmid extracted from colony coded S4; Lane 4: plasmid extracted from colony coded S8; Lane 5: plasmid extracted from colony coded S11.

Purified plasmid DNA samples were used as template for PCR with three different sets of primers: (1) M13F and *proZat12R*; (2) M13R and *proZat12F*; and (3) *proZat12*F and *proZat12*R. Thermocycling condition for the PCR, same as colony PCR, was shown in Table 3.7. One microlitre of PCR products was loaded into 0.7% agarose gel for electrophoresis. Lanes loaded with PCR products that were amplified with M13F and *proZat12R*, and M13R and *proZat12*, showed a band with size slightly greater than 1 kb. Lanes loaded with PCR products that were amplified with *proZat12*F and *proZat12*R showed a band with size of 1 kb. Figure 4.7 showed the image of 0.7% agarose gel loaded with PCR products amplified using three different primer sets.

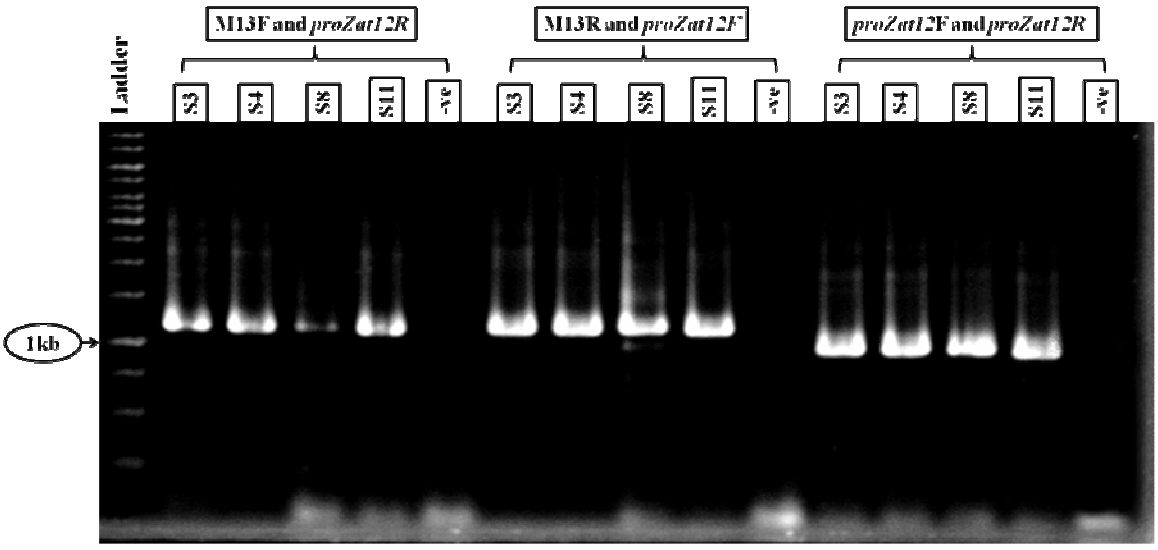

Figure 4.7: PCR of recombinant pENTR™/D-TOPO® carrying *proZat12*. Lane 1: Fermentas GeneRuler 1kb DNA Ladder; Lane 2, 7, 12: plasmid extracted from colony coded S3 was used as template; Lane 3, 8, 13: plasmid extracted from colony coded S4 was used as template; Lane 4, 9, 14: plasmid extracted from colony coded S8 was used as template; Lane 5, 10, 15: plasmid extracted from colony coded S11 was used as template; Lane 6, 11, 16: negative control where sterilize de-ionized water was used as template; Lane 2-6: M13F and *proZat12*R were used; Lane 7-11: M13R and *proZat12*F were used; Lane 12-16: *proZat12*F and *proZat12*R were used.

CHAPTER 5

DISCUSSION

5.1 Amplification of *proZat12* using PCR method

Annealing temperature for the PCR amplification of *proZat12* was determined by using gradient PCR. Determination of optimum annealing temperature for PCR is essentially important, especially when total genomic DNA was used as substrate or template for PCR (Rychlik, Spencer, & Rhoads, 1990). According to the gradient PCR result (Figure 4.1), annealing temperatures ranging from 55°C to 60°C were suitable for amplification of *proZat12*, which is 1 kb in size. All lanes displayed a distinct band with the size of 1 kb suggested that the amplified fragment was *proZat12*. Hence, any annealing temperature in this range was suitable for amplification of *proZat12*. The result suggested that both *proZat12*F and *proZat12*R were able to bind at the expected binding sites in the *A. thaliana* genomic DNA.

Annealing temperature at 58.6°C, therefore, was chosen for amplification of *proZat12* using primer *proZat12*F and *proZat12*R. As shown in Figure 4.2, four replicates of same reactions were made and all reaction showed a distinct band with size of 1 kb, similar to expected *proZat12* size. The result suggested successful amplification of *proZat12* from Arabidopsis thaliana genomic DNA using *proZat12*F and *proZat12*R at annealing temperature of 58.6°C. The PCR reactions were carried out in four tubes to obtain high yield of PCR product, without increasing the reaction volume in each tube. Low PCR reaction volume promotes heat transfer and thermal distribution within the

reaction mixture which in turn improves heating/cooling rate of reaction that reduces PCR reaction time (Neuzil, Zhang, Pipper, Oh, & Zhuo, 2006).

Faint band with size smaller than 250 bp was primer-dimers that were formed due to primer-primer complementation during PCR reaction. Edge of these primer-dimers may have the same sequence as the amplified PCR products (*proZat12*) (Brownie et al., 1997). This similarity may affect subsequent cloning efficiency as these primer-dimers may compete with PCR product in inserting into cloning site. Hence, PCR products were purified using Montage® PCR Centrifugal Filter Device by Millipore Corporation. Gel image in Figure 4.3 showed the successful in elimination of primer-dimers from PCR product.

5.2 Colony PCR of pENTR™/D-TOPO® Carrying *proZat12*

Transformed bacterial cells DH5α were selected using 40 µg/mL kanamycin due to the presence of kanamycin resistance gene in pENTR™/D-TOPO®. Non-transformed bacterial cells DH5α are susceptibile to effect of kanamycin, hence, could not survive on kanamycin selective agar plate. Colony PCR on the randomly selected bacterial colonies was conducted using primers M13F and *proZat12*R to identify transformed bacteria that carry recombinant pENTR™/D-TOPO® with insert *proZat12* at desired orientation. Based on the gene map shown in pENTR™ Directional TOPO® Cloning Kits User Manual, M13F binds on the vector upstream of the cloning site and *proZat12*R should theoretically binds to *proZat12* only. Hence, orientation of the insert could be determined. Also, the amplified fragment is approximately 1.15 kb, included *proZat12* (refer to

Appendix D) (Invitrogen, 2006). Based on the Figure 4.4, lane 5 and 12 displayed a band with size slightly above 1 kb, approximately 1.15 kb. Furthermore, lane 4, 5, 9, and 12 showed similarity in the absence of band with size greater than 10 kb. The result suggested that colonies coded S3 (lane 4), S4 (lane 5), S8 (lane 9), S11 (lane 12) may carry recombinant pENTR™/D-TOPO® with insert *proZat12* at desired orientation.

The assumption was tested through isolation of plasmid from colonies coded S3, S4, S8, and S11, which followed by another round of PCR. Figure 4.5 and Figure 4.6 showed differences in untreated extracted plasmids and RNase-treated extracted plasmids, respectively. The observed bands are recombinant pENTR™/D-TOPO® with different confirmations. Plasmids present in different confirmation inside the cells (Dale & von Schantz, 2007). Besides, RNase-treated plasmids showed removal of RNAs from the DNA samples. However, there was leftover of RNAs in the samples which can be observed as faint band at the bottom of the gel. RNAs must be removed from DNA samples as it may cause interference during DNA quantification as RNA and DNA able to absorb same wavelength. This similarity will cause higher A_{260} value and affects DNA concentration calculation (Dale & von Schantz, 2007). Furthermore, RNA contamination may interfere with DNA sequencing process. Further purification step was not conducted to prevent further loss of plasmid DNA.

Purified plasmids S3, S4, S8, and S11 were used as substrate for another round of PCR. Primers M13F and *proZat12*R, as well as primers M13R and *proZat12*F, were used to determine the orientation of the insert *proZat12* at the cloning site of pENTR™/D-TOPO®. PCR reactions using both primers sets showed a band with size slightly above 1 kb, at approximately 1.15 kb (Figure 4.7). The result agreed with gene map published

by Invitrogen (2006) in pENTR™ Directional TOPO® Cloning Kits User Manual. Hence, the result suggested that the *proZat12* was inserted into cloning site of pENTR™/D-TOPO® at desired orientation. PCR reactions using *proZat12*F and *proZat12*R showed a band with size of 1 kb (refer to Appendix D). The result further confirmed that these plasmid DNA samples were pENTR™/D-TOPO® with insert *proZat12* in the cloning site at desired orientation.

5.6 Quantification of DNA Samples

Purified plasmid S3, S4, S8, and S11 have relatively higher ratio A_{260}/A_{280} compared to other DNA samples. This may be due to the leftover RNA molecules in these samples that absorbed the same wavelengths as DNA molecules, causing higher A_{260} and higher ratio A_{260}/A_{280}. PCR products (*proZat12*) that was purified using Montage® PCR Centrifugal Filter Device by Millipore Corporation may not be sufficient to remove DNA polymerase in PCR reaction. The remaining DNA polymerase absorbed wavelength at 280 nm, causing increase in A_{280} and lower ratio A_{260}/A_{280}.

CHAPTER 6

CONCLUSIONS

Zat12 promoter (*proZat12*) was amplified with primers *proZat12*F and *proZat12*R from *Arabidopsis thaliana* by polymerase chain reaction (PCR), with annealing temperature of 58.6°C. Amplified fragment was cloned into pENTR™/D-TOPO®. Recombinant pENTR™/D-TOPO® with *proZat12* in desired orientation was extracted from transformants after screening with colony PCR. DNA sequencing using M13F direction showed the amplified *proZat12* was 99% identical to the sequence published by Nakamura et al. (1998) with 1000 nucleotides aligned. DNA sequencing using M13R direction showed 98% identical to published sequence with 134 nucleotides aligned. However, based on the chromatograms, the DNA sequencing result can be ambiguous, probably due to RNA contamination. *Zat12* promoter was transferred into pGWB4 using LR reaction of transformants were screened using colony PCR. Screening of transformants using colony PCR using *proZat12*F and *proZat12*R showed 12 out of 21 colonies carried recombinant binary vector pGWB4 with *proZat12*.

Further purification of extracted recombinant pENTR™/D-TOPO® with *proZat12* should be conducted prior to resubmission for DNA sequencing. Orientation of *proZat12* in pGWB4 should be further confirmed by colony PCR using different primers such as combinantion of *proZat12R* primer and primer that bind at *attB1* site in pGWB4 vector. The recombinant pGWB4 with *proZat12* in desired orientation should then be extracted and send for DNA sequencing to further verify the orientation. Functional test of

proZat12::*sGFP* construct should be conducted through transient expression in the plant. The *proZat12*::*sGFP* construct can be infiltrated and transformed into plants such as tobacco *Nicotiana benthamiana* through *Agrobacterium*-mediated transformation, together with co-infiltration of ROS-inducing plasmid carrying *OsrbohB* gene, for example. Alternatively, ROS can be induced through application of H_2O_2 and other abiotic stresses. Expressed GFP, due to accumulation of ROS, can be detected through fluorescence microscopy. Development of stable *proZat12*::*sGFP* transgenic plants is suggested as well. This *proZat12*::*sGFP* construct is essentially important in study of ROS signaling pathways, such as cross tolerance and stress acclimatization, to improve plants' survival in harsh environmental conditions.

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