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NUCLEOTIDE POLYMORPHISM OF ENHANCED DISEASE RESISTANCE 1 (EDR1) FROM KELAMPAYAN (*Neolamarckia cadamba*)

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DECLARATION

I hereby declare that this thesis presented here is to the best of my knowledge and beliefs of my original work except for quotations and citations, all of which have been duly acknowledged. I would also like to declare that it has not previously been published a degree at UNIMAS or any other institutions.

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LIST OF ABBREVIATIONS

CTAB	Cetyltrimethylammonium Bromide
CTR1	Constitutive Triple Response 1
DNA	Deoxyribonucleic acid
EDR1	Enhanced Disease Resistance 1
EDTA	Ethylenediamine tetracetic acid
Flg22	Flagellin22
HERC2	HECT and RLD domain containing E3 ubiquitin protein ligase 2
HR	Hypersensitive response
InDel	Insertion-Deletion
KEG	Keep On Going
MAPK	Mitogen-activated protein kinase
NaCl	Sodium chloride
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
SAR	Systemic acquired resistance

$\mathbf{T_a}$	Annealing temperature
$\mathbf{T_m}$	Melting temperature
UV	Ultraviolet

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ABSTRACT

Neolamarckia cadamba (Roxb.) Bosser or Kelampayan is well-known for its contribution in economic sector whereby the tree is used as raw materials in plywood, paper and pulp industries and light construction. The enhanced disease resistance 1 (EDR1) gene controls the invasion of pathogens into the living plant, and it also serves as the negative regulator of disease resistance, ethylene-induced senescence and programmed cell death. As conventional plant breeding is costly and time-consuming, new technique such as single nucleotide polymorphism (SNP) is applied as a marker for plant breeding selection. The main objective in this study was to detect the sequence variation caused by SNP in *edr1*. DNA extracted from five selected Kelampayan trees were firstly subjected to polymerase chain reaction. The purified amplicons with ~550 bp were sent for DNA sequencing. However, the attempt to blast the sequenced data was failed and no significant results were found due to the weak and noise sequence data. The main reason that led to the failure was due to the contamination of purified PCR product. Gel extraction should be done in a careful manner to avoid contamination.

Keywords: *Neolamarckia cadamba*, enhanced disease resistance 1 gene (*edr1*), single nucleotide polymorphism (SNP).

ABSTRAK

Neolamarckia cadamba (Roxb.) Bosser atau Kelampayan terkenal dengan sumbangannya dalam sektor ekonomi. Kelampayan mempunyai komersil yang tinggi atas kegunaan kayunya sebagai bahan mentah untuk industri pulpa dan kertas srta industri kayu lapis. Gen enhanced disease resistance (EDR1) mengawal serangan patogen ke dalam tumbuhan dan turut berfungsi sebagai pengatur negatif dalam runtangan terhadap penyakit, cetusan penuaan oleh etilen serta proses kematian sel terprogram. Memandangkan cara konvensional pemilihan tumbuhan bagi tujuan pembiakan genetik adalah mahal dan lama, polimorfisme nukleotida tunggal (SNP) telah digunakan sebagai penanda molekular dalam pemilihan tumbuhan untuk pembiakan. Objektif kajian ini adalah untuk mengenal pasti SNP di gen EDR1 dalam Kelampayan. Kajian ini dimulakan dengan aplikasi tindak balas berantai polimerase (PCR) terhadap lima DNA ekstrak daripada pokok Kelampayan yang terpilih. Selepas itu, ~550 bp amplikon telah dihantar untuk kaedah penjujukan DNA. Akan tetapi, penganalisis jujukan data melalui pemadanan BLAST adalah gagal disebabkan data jujukan mempunyai nisbah isyarat yang banyak dan signalnya lemah. Punca utama kegagalan adalah disebabkan purifikasi produk PCR yang tercemar. Pengekstrakan gel harus dikendali dengan hati-hati untuk mengelakkan kontaminasi.

Kata kunci: *Neolamarckia cadamba,* gen enhanced disease resistance 1(EDR1), polimorfisme nukleotida tunggal (SNP).

1.0 INTRODUCTION

Neolamarckia cadamba (Roxb.) Bosser belongs to the Rubiaceae family. It is known as Kelampayan locally. Kelampayan is a medium to large sized deciduous tree and it is widely distributed throughout India, Southeast Asia and New Guinea (Nair, 2007). Besides that, it is a rapid-growing tree reaching 45 m in height (Joker, 2000). The leaves are simple and the tree produces small, dense, and yellow to orange coloured flowers with globose heads. Kelampayan possess great economic value whereby the parts of the tree are beneficial. For instance the wood is used for pulpwood and light construction while the leaves and barks have medicinal values too.

In natural forest as well as in plantations, the tree species are often invaded by various pathogens such as fungi, bacteria and viruses. The invasion of pathogens generally influences the metabolism of the plant and hence results in the mortality of the plant. Therefore, enhanced disease resistance 1 (EDR1) gene plays a critical role in controlling the invasion of pathogens into the plant. According to Tang *et al.* (2005), *edr1* encodes a CTR-1 like kinase which acts as the negative regulator of disease resistance along with ethylene-induced senescence. In addition, *edr1* also involves in stress response signaling and cell death regulation (Tang *et al.*, 2005). It was reported that even in the absence of pathogens, the mutant form of *edr1* is able to exhibit enhanced stress response and spontaneous necrotic lesions under drought conditions (Tang *et al.*, 2005).

In correlation with the advancement of technology, single nucleotide polymorphism or SNP is a significant biological marker for plant breeding. SNP has gradually become the marker of choice in genetic analysis as well as in agricultural breeding programme. Unlike

conventional selection plant breeding which is time-consuming and expensive, SNP is able to mark the significant allelic differences among species precisely in short period of time. Moreover, SNP is an excellent marker in studying complex genetic traits due to the low mutation rate (Batley *et al.*, 2003). In addition, the potential of SNP in generating high-density genetic maps is an advantageous in developing halotyping systems for genes or regions of interest (Batley *et al.*, 2003).

Hence or otherwise, the SNP is applied in order to detect the sequence variation in the *edr1* gene in Kelampayan. This molecular breeding programme is to reduce the cost expenses and the time taken as compared to the conventional plant selection breeding method. The main objective of this study is to identify the sequence variation caused by single nucleotide polymorphism (SNP) in *edr1*of Kelampayan.

2.0 LITERATURE REVIEW

2.1 *Neolamarckia cadamba* (Roxb.) Bosser

Neolamarckia cadamba (Roxb.) Bosser of the family Rubiaceae is widely distributed throughout India, China, Australia, Indonesia, Malaysia, Papua New Guinea, Phillipines, Singapore and Vietnam (Gautam *et al.*, 2012). Despite growing at its native range, it has been successfully cultivated in foreign countries such as Costa Rica, Puerto Rico, South Africa, Surinam, Taiwan and other tropical countries (Gautam *et al.*, 2012). *Neolamarckia cadamba* is also familiar with the term Kadam in India, Kelampayan in Malaysia and Jabon in Indonesia. Generally, the deciduous Kelampayan can be found near to river and in moist evergreen forests. Kelampayan varies in size, which can be ranged from medium to large sized. Besides that, it is a fast-growing tree as it can grow up to 45 m high (Joker, 2000). According to Mondal *et al.* (2011), the tree trunk measures 2.4 m in width and branches horizontally. Concerning the tree bark, the bark appears to be in gray and smooth in young trees while it is rough and longitudinally cracked in old trees (Gautam *et al.*, 2012). The leaves are glossy green, simple and petiole. As for the flowers, they are bloomed with small, dense, and yellow to orange-coloured with globose head.

The woods of Kelampayan possess great economic values. The centre part of the wood is white and it is characterized with a yellow tinge. On exposure, the yellow tinge is darkened to creamy yellow. According to Choo *et al.* (1999), the wood density ranges from 370 to 465 kg m^{-3} air dry. Moreover, the Kelampayan timber is a lightweight hardwood whereby it is used for the production of plywood and in the pulp industry, producing

intermediate quality papers. In addition, the wood is used in light construction work and wood furniture.

Despite the contribution towards the economic sector, the other parts of the Kelampayan do have precious medicinal values. According to Umachigi *et al.* (2007), it was revealed that the Kelampayan is able to possess "antimicrobial, antioxidant, and wound healing properties, antimalarial and antihepatotoxic activities" (as cited in Alam *et al.*, 2007). For instance the bark of Kelampayan was reported comprising anthelmintic activity whereby the bark contains tonic, astringents, febrifugal, and antidiuretic properties (Mondal *et al.*, 2011). In Orissa state, the Sabara tribes extract the bark juice from Kelampayan in order to treat helminthiasis disease in children (Mondal *et al.*, 2011). As for the flower, the hydroethanolic extract from the flower top exhibits antidiarrhoeal property (Alam *et al.*, 2007). Furthermore, Gautam *et al.* (2012) mentioned that the decoction from the leaves is applied for ulcers, wounds and metorrhea.

2.2 Enhanced Disease Resistance 1 (EDR1) Gene

Enhanced disease resistance 1 (EDR1) gene plays a critical role in controlling the invasion of pathogen into the plant. In the genetic screening that have been conducted on *Arabidopsis* plant, Frye and Innes (1998) discovered that there is a single mutant that exhibited enhanced disease resistance towards the bacterium, *Pseudomonas syringae,* as well as the ascomycete fungus, *Erysiphie cichoracearum* (Tang *et al.*, 2005). Research also revealed that the *edr1* mutant present in *Arabidopsis* confers resistance to powdery mildew disease which caused by the fungus pathogen, *Erysiphie cichoracearum* (Frye *et al.*, 2001). The obligate biotrophic pathogen: powdery mildew fungus grows on the living plant tissue and suppresses host defenses until its life cycle is completed.

In addition, *edr1* portrays an enhanced ethylene-induced senescence phenotype (Frye *et al.*, 2001). It was reported that the *edr1* encodes a putative mitogen-activated protein kinase kinase kinases (MAPKKK) which is found to be similar to the Constitutive Triple Response 1, CTR-1(Frye *et al.*, 2001). The CTR-1 acts as a negative regulator in mediating ethylene responses in *Arabidopsis*. Hence, EDR1 gene is responsible in functioning at the upstream of the MAP kinase cascade and thereby negatively regulates the disease resistance through salicylic acid inducible defenses (Frye *et al.*, 2001).

Apart from that, when the plant is exposed under drought condition, *edr1* may function to regulate the cell death and the growth of the plant. Based on the studies conducted previously, it was discovered that when two plants were grown under standard conditions, there was no much differences between the weight of the *edr1* mutant plant and the wild-type plant (Tang *et al.*, 2005). However, there is a significant weight decrease in

the *edr1* mutant plant compared to the wild-type plant when grown under drought conditions (Tang *et al.*, 2005).

As mentioned previously, *edr1* is responsible in preventing inappropriate initiation of cell death and senescence. Based on the studies conducted by Tang *et al.* (2005), it was observed that pathogen and stress environment eventually caused accumulation of salicyclic acids in the plant (Figure 2.1). The elevated salicyclic acid in plant brings about the hypersensitive response (HR) lesions which results in cell apoptosis. In addition, *edr1* also aids in negative regulation of ethylene-induced senescence (Figure 2.1).

On the other hand, recent study has shown that a specific missense mutation (*keg-4*) in the KEEP ON GOING (KEG) gene can suppress all the *edr1*-mediated phenotypes in *Arabidopsis* (Gu & Innes, 2011). KEG gene encodes several domain proteins including a RING E3 ligase domain, ankryin repeats, and HERC2-like (for HECT and RCC1-like) repeats (Gu & Innes, 2011). Gu and Innes (2011) stated that the interaction between the EDR1 and KEG is mediated by HERC2-like repeats whereby these repeats localized the KEG to the trans-Golgi network or the early endosome vesicles. In other words, the localization of KEG and the recruitment of EDR1 by KEG are highly dependent on the HERC2-like repeats of the KEG. However, the deletion of HERC2-like repeats puts an end to the interaction between the KEG and EDR1 and reduces the localization of the KEG to the trans-Golgi network or early endosome vesicles (Gu & Innes, 2011). The research done eventually portrays that both EDR1 and KEG function together in the regulation of endocytic trafficking when responses to stress environment.

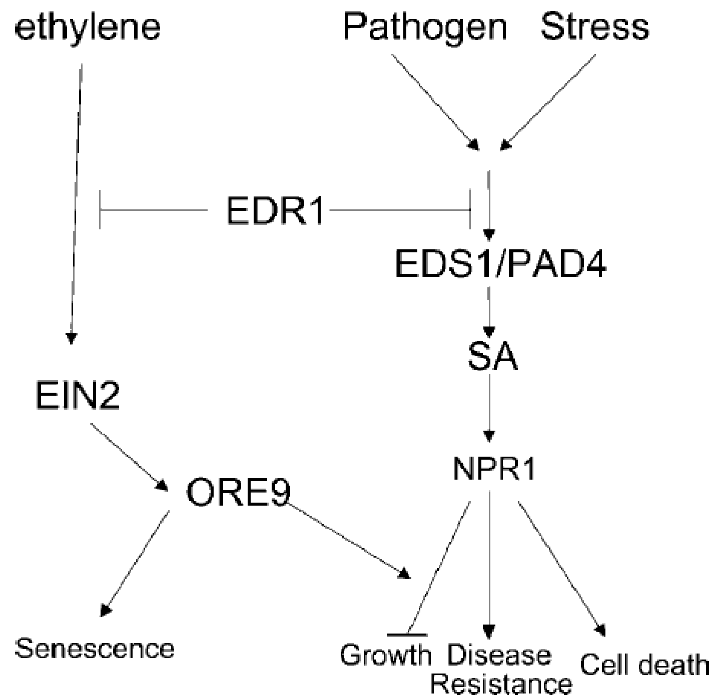

Figure 2.1 Role of EDR1 in programmed cell death, drought tolerance and senescence. The *E. cichoracearum* and drought induce the accumulation of salicyclic acid in plants which in turn promote HR-like lesions. This process is negatively regulated by *edr1* gene to prevent cell apoptosis. *Edr1* gene also negatively regulates the ethylene-induced senescence (Tang *et al.*, 2005).

2.3 Mitogen-activated Protein Kinase (MAPK) Cascades in Plants

MAPK cascades play an extremely significant role in plant. It is crucial for the adaptation as well as for the survival of the plant in responding for the stress condition encounters by the plant. Moreover, MAPK cascades function in the growth, development, programme cell death and response in environmental cues such as "cold, heat, reactive oxygen species, UV, drought and pathogen attack" (Pitzschke *et al.*, 2009).

Plant recognizes the pathogens via pathogen-associated molecular pattern (PAMPs) through the cell surface-located pathogen-recognition receptors (Pitzschke *et al.*, 2009). The intact between the plant receptor and the pathogen receptor eventually provokes the intracellular signaling pathways in plants. This leads to the establishment of PAMP-triggered immunity (Pritzschke *et al.*, 2009). The MAPK cascade is then activated by the PAMP-triggered immunity.

The MAPK cascade is activated by the upstream of the MAPKKs. Generally, the pathogen PAMP comes into contact with the plant PAMP. This in turn activates the MAPKKK to the downstream of the MAPK cascade. The *edr1* and CTR-1 gene belongs to the Raf subfamily of MAPKKKs (Frye *et al.*, 2001). Due to this fact, it is believed that the *edr1* gene will also interact with the upstream of the receptor proteins and this in turn activates the MAPK cascade (Frye *et al.*, 2001).

In *Arabidopsis*, the MAPK mutant *mpk4* and MAPKKK mutant *edr1* exhibit increased resistance to pathogens (Jonak *et al.*, 2002). According to Romeis (2001), the *mpk4* mutant plants have high salicyclic acids level, exhibit constitutive systemic acquired resistance (SAR) and defense-related gene expression. This indicates that the *mpk4* is important in negative regulation in plant defense towards pathogen. On the other hand, the

edr1 mutant in *Arabidopsis* is isolated and it displays enhanced resistance to virulent pathogens *Pseudomonas syringae* and the fungal powdery mildew pathogen *Erysiphie cichoracearum* (Romeis, 2001). The *edr1*gene induced more rapidly in mutant plants than in wild type plants and it functions at the upstream of MAPK signaling cascade, signifying that it also serves as negative regulators of plant defense response similarly to *mpk4* (Jonak *et al.*, 2002).

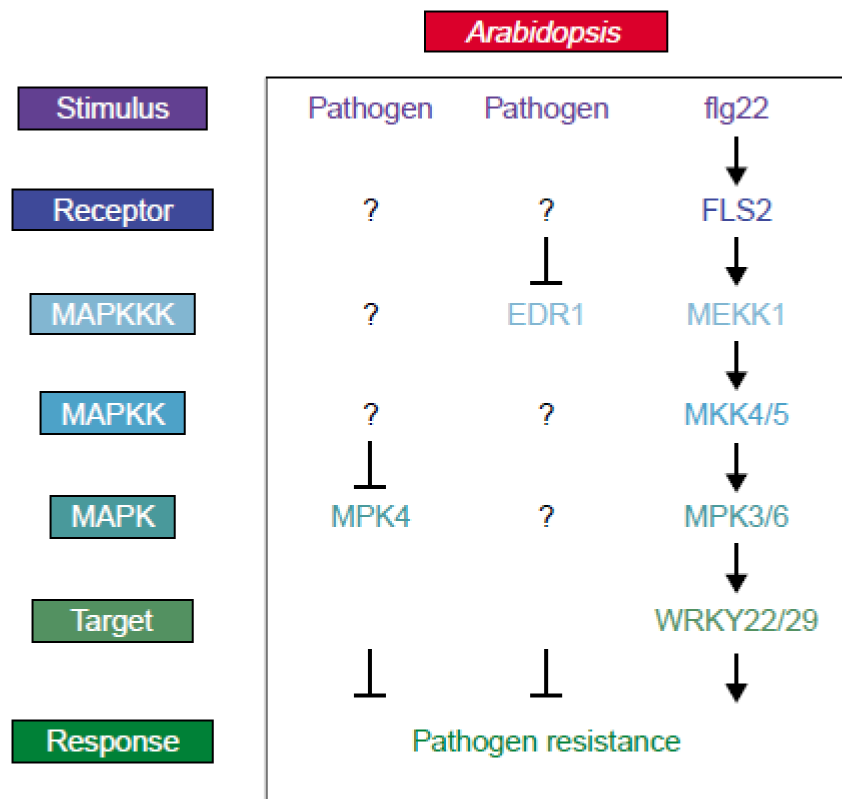

Figure 2.2 MAPK pathways for pathogen responses in plants. In *Arabidopsis*, the MAPK mutant *mpk4* and the MAPKKK mutant *edr1* shows enhanced pathogen resistance when pathogen comes into contact with plants. On the other hand, the flagellin derived peptide flg22 from the bacteria triggers the rapid activation of the downstream MAPK signaling cascade, which comprises of MEKK1, MKK4/5 and MPK3/6 and follows by the transcription induction of WRKY22/29 (Jonak *et al.*, 2002).

2.4 Single Nucleotide Polymorphism (SNP)

Single nucleotide polymorphism (SNP) is the variation in DNA sequencing when a single nucleotide base in the genome sequence is altered from the members of a species (Angaji, 2009). SNP is the simplest form of DNA variation among individuals. According to Perkel (2008), SNPs can occur once every 1000 base pairs and so forth. Nowadays, SNP is broadly used as molecular markers as it is able to mark the significant allelic differences among species precisely.

SNPs occur with a very high frequency. A variation is considered as a SNP when SNP occurs in at least 1% of the population species. Generally, SNPs are the most common form of genetic variation in terms of nucleotide substitution. In other words, nucleotide substitution is a form of point mutation. Nevertheless, single base insertion and deletion or in short, InDel are considered as SNP too. Batley *et al.* (2003) reported that changes of two nucleotides and a range of few small InDel are regarded as SNPs. Studies revealed that both SNPs and InDels are highly abundant in various species including plants (Nasu *et al.*, 2002). The abundance of these SNPs in plants serves as advantages in mapping, marker-assisted breeding and map-based cloning (Gupta *et al.*, 2001; Rafalski, 2002a; Batley *et al.*, 2003b as cited in Semagn *et al.*, 2006).

Nasu *et al.* (2002) had established 213 SNP markers distributed throughout the rice genome which is the *indica* and *Oryza rufipogon*. Among the six strains of rice along each chromosome, it was revealed that chromosome 2, 3 and 10 have a relatively low SNPs rate while chromosome 1, 4, 5, 7 and 11 have high SNPs rate. The SNPs rich region found in the chromosome 1, 4, 5 and 7 are putative intergenic regions except for the SNPs detected on chromosome 11 are transcribed region (Nasu *et al.*, 2002).

Nucleotide substitution is a form of point mutation. The two categories of point mutation include transition substitution and transversion substitution. Transition substitution occurs when a purine base interchanges with another purine (A $\leftrightarrow$G) or a pyrimidine base interchanges with another pyrimidine (C $\leftrightarrow$T). In contrast, transversion substitution involves the interchange between a purine for pyrimidine base and vice versa. According to Wakeley (1996), the occurrence of transition substitution is higher as compared to transversion substitution. This phenomenon is known as transition bias. The SNP scenario varies among species. For instance in human, it was reported that two out of every three SNPs results from a C/T transition change whereas the C/T transitions range between 45% to 55% of the observed SNPs in wheat and maize plants (Henry, 2008).

SNPs can be found in both coding and non-coding regions of the genome. SNPs that occur in the coding region of the genome may or may not alter the protein structure produced by the particular coding region. In other words, SNPs that occur in the coding region can be synonymous or non-synonymous. A synonymous change will not result in a change of amino acid of the encoded protein due to the degeneracy of the genetic code. On the other hand, a non-synonymous change will lead to the formation of different polypeptide sequence. Non-synonymous mutation can either be missense change which leads to formation of different polypeptide or nonsense change which results in termination of the codon. As for the SNPs that occur in the non-coding region of the genome, it affects the level of gene expression which in turn distorting the amount of proteins produced.

3.0 MATERIALS AND METHODS

3.1 Plant Materials

Inner bark tissue samples which were previously extracted by Tchin were used in this study.

3.2 DNA Extraction

3.2.1 Chemicals and Reagents

Reagents that were used for DNA isolation include liquid nitrogen, CTAB extraction buffer [100 mM Tris-Cl pH 8.0; 1.4 M NaCl; 20 mM EDTA pH 8.0; 2% CTAB; 1% polyvinylpyrrolidone (PVP); 2% (v/v) β-mercaptoethanol], chloroform/isomyl alcohol (24:1 v/v), isopropanol, 70% ethanol, TE buffer, and sterile distilled deionized water (ddH_2O).

3.2.2 DNA Isolation Protocol and DNA Purification

The modified CTAB method from Doyle and Doyle (1990) was applied to extract total genomic DNA from Kelampayan while Wizard Genomic DNA Purification Kit (Promega, USA) was used to purify the isolated DNA.

3.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was conducted to detect the presence of genomic DNA which was isolated from Kelampayan. To produce 0.8% of agarose gel, an amount of 0.4g of agarose powder was added to 50 ml of 1×TAE buffer. A total of 2 µl of genomic DNA was mixed with 2 µl of loading dye. Two microlitres of λ *Hind*III (Promega, USA) was used as the DNA marker. The gel was run at 50 V for 120 minutes. Next, ethidium bromide was used to stain the gel for 10 seconds and followed by de-staining with distilled water for 30 minutes. After that, the gel was visualized by using UV transilluminator to observe for the presence of DNA bands.

3.4 Spectrophotometric Quantification of DNA

NanoDrop 2000 Spectrophotometer was used to quantify the concentration of DNA. Absorbance readings were taken at 230 nm (A_{230}), 260 nm (A_{260}) and 280 nm (A_{280}). By calculating the absorbance ratio of A_{260}/A_{280} and A_{260}/A_{230}, the purity of DNA samples was estimated.

3.5 Primer Design

Sequence of EDR1 was obtained from the Kelampayan EST database. Primer Premier 6.0 software (PREMIER Biosoft International, USA) was used to design and analyze primer.

There are several criteria needed to be accomplished when designing an efficient primer. Firstly, the optimal length of the primer should consist of 18-30 nucleotides long. A primer with at least 18 nucleotides in length is considered the best as to lower the chances of problems associated with a secondary hybridization site on the vector or insert (Elsalam, 2003). Secondly, the GC content of the primer is another important criterion as it portrays the strength of annealing. According to Dieffenbach *et al.*, 1995 (as cited in Elsalam, 2003), the GC content should between 45% and 60%. Moreover, an ideal primer will consist of randomly mixed of nucleotides with a 50% GC content and approximately 20 bases long (Vinod, 2004). Thirdly, the concentration of the primer in the amplification reaction should be ranged in 0.1 to 0.5 µM. Studies had shown that primer with 0.2 µM had produced satisfactory result in the PCR assays (Löffert *et al.*, 1997). High concentration primer will result in mispriming and the misprimed molecules will lead to nonspecific PCR products (Löffert *et al.*, 1997). Apart from that, the melting temperature (T_m) for primers works best in the range of 52 °C to 58 °C (Elsalam, 2003). Primers above optimal T_m will lead to secondary annealing.

3.6 Polymerase Chain Reaction (PCR)

Master Cycler Gradient Thermal Cycler (eppendorf, Germany) was used to perform the PCR. The PCR was firstly carried out by using leaf DNA sample as the template in order to determine the optimum annealing temperature for the designed primer. PCR reaction mixture was prepared by adding 20 ng of DNA template; 10 pmol of each primer; 0.2 mM dNTPs (Invitrogen, USA); 1 × PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) (Invitrogen, USA); 1.5 mM $MgCl_2$ (Invitrogen, USA); 4 U *Taq* DNA polymerase (Invitrogen, USA) and ultrapure water to make up 25 μl. The amplification was carried out for 2 minutes at 95 ℃ (pre-denaturation); 30 seconds at 94 ℃ (denaturation); 30 seconds at gradient temperature 64.3 ℃ (annealing); 72 ℃ for 30 seconds (extension) and subsequently 10 minutes at 72 ℃ for final extension.

3.7 PCR Product Purification

The PCR products were examined on 1.5% (w/v) agarose gel. Electrophoresis was performed at 60V and 65A for 2 hours. The desired single band was extracted under UV transilluminator and it was transferred to a pre-weight microcentrifuge tube. The gel slice with the desired fragment was purified according to the protocol in Wizard® SV Gel and PCR Clean-Up System (Promega, USA).