CHAPTER 1

INTRODUCTION

Rice is a staple food for almost half of the world's population and especially in Asia, providing 40–70% of the total food calories consumed (Swapan, 2004; Miura *et al.*, 2010). The population of the world is expected to reach eight billion by 2020 while less land and water will be available in the future. Therefore, strategies to increase the yield of rice are really vital and necessary (Swapan, 2004).

Oryza rufipogon, wild red rice, is designated as a putative ancestor of Asian cultivated rice (Song *et al.*, 2003). The phenotypes of *O. rufipogon* rice are not agronomically desirable although it contains a large portion of genetic variation that can be improved to solve the problem of gene pool narrowing in modern rice cultivars (Xiao *et al.*, 1998a).

Genetically modified (GM) foods are obtained from GM organisms. Genetically modified organisms are produced through some changes in their DNA by genetic engineering. These foods are usually transgenic plant products including those from soybean, corn, canola, cotton and rice (Kang and Leaf, 2007). The genetic modifications are performed in these plants to obtain the changes in their quality and quantity. So GM rice is genetically modified rice that is produced for developing more valuable rice varieties from the aspects of nutrition, yield or for the study of gene function (Kang and Leaf, 2007).

A jump in rice yield occurred over the past three decades because of the Green Revolution, which refers to the transformation of agriculture that began in 1945. The yield increase did help to avoid famine and prevent a greater disruption of the food supply in Asia (Swapan, 2004).

Functional genomics can be explained as the development and application of global experimental approaches to discover gene function. This target is obtained by applying the information and reagents provided by structural genomics (Hieter and Boguski, 1997). One of the available approaches for functional genomics for rice is to reduce or knock out expression of a gene with the goal of obtaining a phenotype that is suggestive of its function. Two major techniques applied are insertional mutagenesis and RNA interference (RNAi). RNAi has several advantages in comparison with the other methods. One of the important advantages is the specific selection of a gene or genes of interest, as RNAi is a homology (i.e. nucleotide specific sequence) dependent process (Matthew, 2004).

The leucine rich repeat (*LRR*) Receptor-Like-Kinase (*RLK*) family was identified as potential candidate genes relating to yield increase in rice (Zha *et al.*, 2009). Based on this, a research project at University of Malaya included a putative *CLAVATA1* Receptor Kinase (putative *CLV1*) as a potential yield related gene to study gene function in rice. A part of a putative *CLAVATA1* (*CLV1*) receptor kinase-like protein gene from the Malaysian wild rice, *O. rufipogon* (IRGC105491) was cloned in an RNAi vector (pANDA vector) for *Agrobacterium tumefaciens* mediated transformation of the same plant variety. In this thesis project, the aims are to analyze the putative transgenic plants over two generations towards the elucidation of the role of this gene in rice.

RNAi (also described as Post Transcriptional gene silencing or PTGS in plant) refers to the process of sequence-specific negative regulation of gene expression by RNA degradation (or inhibition of translation) that is started by the formation of dsRNA. This can be introduced into a plant by virus or transgene (Thakur, 2003; Dillon *et al.*, 2005). PTGS in plants can be defined as post transcriptional down-regulation of gene expression by targeting specific RNAs. Transgenes are subject to suppression by PTGS, as are other genes that share significant sequence homology with the silenced genes (Thakur, 2003). Currently RNAi is one of the most powerful techniques in molecular biology and involves various methods such as: microprojectile bombardment with dsRNA or intron-containing hairpin RNA (ihpRNA)-expressing vectors; infiltration of plant tissue with an *Agrobacterium* strain carrying a T-DNA expressing an ihpRNA transgene; virus induced gene silencing (VIGS) (Matthew, 2004).

Presently, rapid progress of transgenic biotechnology has meaningfully promoted the development and production of GM crops in the world (Lu, 2008). One of the principal concerns about GM plants into the environment is the possibility of transgene escape by means of gene flow into the non-transgenic plant populations (Snow *et al.*, 2005).

In plants, pollen has a crucial role in the flow of genes, specifically in out-crossing plants. It is very important to investigate the pollen when a transgenic plant is formed and to compare it with non-transgenic plants (Bengtsson, 2006). These considerations are vital to reveal risk assessment regarding pollen-mediated gene flow while it has extreme importance for any future release of transgenic cultivars (Wang *et al.*, 2004).

Based on the importance of GM technology for its potential in rice improvement and as part of a larger ongoing research program which has developed a rice GM line, the objectives of this project are:

i. To confirm the presence of the transgene within the genome of putative transgenic plants over two generations
ii. To determine transgene copy number of T1 transgenic plants
iii. To evaluate pollen viability towards establishment of biosafety data for these plants

CHAPTER 2

LITERATURE REVIEW

2.1 Rice

2.1.1 Wild Rice

Rice is the largest food crop in the world and provides the daily caloric needs of millions of people (Sweeney and McCouch, 2007). The genus *Oryza* is categorized under the tribe Oryzeae, subfamily Oryzoideae in the family Poaceae (*Gramineae*) (Lu, 1999). This genus is divided into four distinct species complexes including *O. officialis*, *O. granulata* species, *O. ridelyi* and *O. sativa* (Vaughan *et al*., 2003).

The genus *Oryza* is composed of 23 species that have two types of genome: diploid genomes (AA, BB, CC, EE, FF, GG; 2n = 24) or tetraploid genomes (BBCC, CCDD, HHJJ; 2n = 48) (Vaughan *et al.*, 2003). Seven species of *Oryza* have the AA genome, including two domesticated species (Khush, 1997). Despite the fact that interspecific crossing is feasible within each complex of *Oryza* genus members, it is hard to obtain fertile descendants from crosses across complexes (Vaughan *et al.*, 2003; Sweeney and McCouch, 2007).

The *O. sativa* complex is classified into two domesticated species and five or six wild species. *O. sativa* and *O. glaberrima* are domesticated species, while *O. longistaminata*, *O. barthii*, *O. meridionalis*, *O. glumaepatula*, *O. rufipogon* and *O. nivara* (also take into account as an ecotype of *O. rufipogon*) are wild species (Lu, 1999; Vaughan *et al.*, 2003; Sweeney and McCouch, 2007). These domesticated species and their relative wild species are classified as AA genome (2n=24) (Ge *et al.*, 1999; Vaughan *et al.*, 2003).

O. rufipogon had been the name greatly used to accommodate the wild Asian A-genome taxa up to the time which *O. nivara* was identified (Sang and Ge, 2007). *O. nivara* was accepted for populations with specifications such as, insensitivity to photoperiod, self-fertilization in a predominant manner and adaptation to seasonally dry habitats. On the other hand, *O. rufipogon* was accepted for populations that were perennial, photoperiod sensitive, largely cross-fertilized, and adapted to persistently wet habitats (Sharma *et al.*, 2000).

Although wild species are inferior from the phenotypic point of view , They play an important role as a reservoir of strategic genes for resistance to main abiotic and biotic stresses (Xiao *et al.*, 1998b; Brar and Khush, 2006). Wild species present a greatly unutilized resource of important genes in agriculture that have potential to eradicate many problems in cultivated rice production such as yield, drought and disease and insect resistance (Wing *et al.*, 2007). For example, resistance gene to grassy stunt virus was identified from *O. nivara* and rice bacterial blight resistance gene (Xa-23) identified from *O. rufipogon* (Fu *et al.*, 2010). Wild species also contain a large considerable genetic variation that can be manipulated to overcome the problem of gene pool narrowing in modern rice cultivars by breeding programs (Xiao *et al.*, 1998a).

2.1.2 Genetically modified rice

Genetically modified (GM) rice is produced through some changes in genomic DNA content of rice plant. The main target of these modifications is to produce more valuable rice that lead to modify the nutrition and the yield of the rice plant. Also, GM rice has been used as an important source for functional genomic studies in rice plant (Kang and Leaf, 2007).

Golden Rice (GR) is genetically modified rice that contains beta-carotene (the vitamin A precursor) in the endosperm of the grain. This name is given due to the yellow colour of grain that is observable after milling and polishing of raw grain (Al-Babili and Beyer, 2005). The grain of Golden Rice contains provitamin A because of genetic transformation. Therefore, Golden Rice can be consumed in developing countries where there is a wide vitamin A deficiency among poor people (Dawe, 2002).

In transgenic plants, *Bacillus thuringiensis* (Bt) uses as the major source to create pest resistance. These pest resistant transgenic plants are comprised of truncated forms of Bt genes that code Bt toxin protein against pests (Wang *et al*., 2006). Bt rice is a genetically modified rice that contains cry1Ab gene from *Bacillus thuringiensis* for production of insecticidal toxin to eliminate lepidopteran pests (Wu *et al.*, 2004).

2.2 Functional Genomics

Functional genomics can be defined as the development and application of global technologies to discover gene function that take advantage of the information and reagents given by structural genomics (Hieter and Boguski, 1997; Bouchez and Hofte, 1998). Genetic information have been produced in a large scale quantity by the means of plant genome and EST sequencing efforts, such as the complete genomic sequencing projects of *Arabidopsis thaliana* and rice. On the other hand, this valuable information has provided a necessity for effective methods to use such a data for functional genomic (Matthew, 2004).

As shown in Figure 2.1, after obtaining the genome sequence and discovery of gene, the next step to connect the sequence and function of a specific gene is gene functional annotation that is feasible through four main methods. Mutant analysis is one

of the basic methods in functional genomics that comprised of two major approaches: forward and reverse genetics (Peters *et al.*, 2003; Alonso and Ecker, 2006). In forward genetics, researchers start the study based on organism phenotype leading eventually to the study of DNA sequence including the gene for this phenotype (Gupta, 2007). In other words, gene function in classical forward genetics is determined on the basis of mutant phenotype analysis that is happened by the mutation disturbing the activity of a gene in comparison with that of the wild type (Robles *et al.*, 2010). In contrast, researchers start the study with DNA segment in reverse genetics that its phenotypic effect is unknown following introduction of DNA into the plant or animal and then finally study its phenotypic effects (Gupta, 2007).

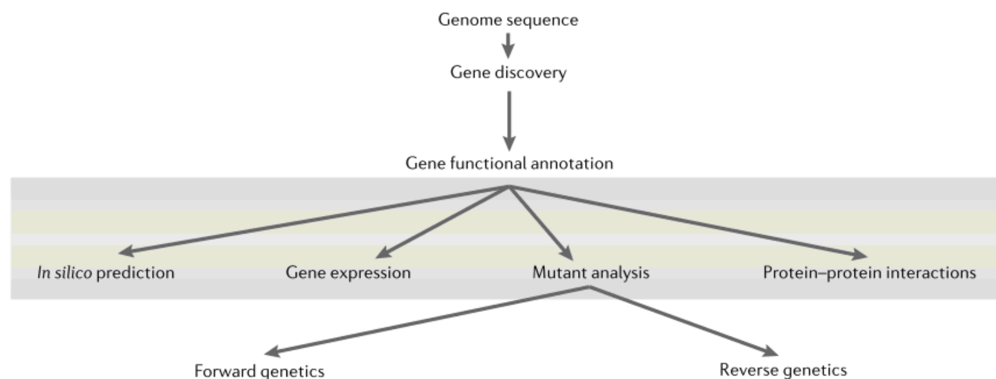

Figure 2.2: From genome sequence to gene function (Alonso and Ecker, 2006).

2.2.1 Functional genomics based on reverse genetics in rice

Rice has been known as an important model system in the field of plant biology because (a) the rice genome is the smallest one among crop plants; (b) substantial colinearity of rice is common with grass family members (c) effective methods are available for transformation; (d) there are lots of genetic stocks and germplasm recourses for rice; (e) the genome of rice is completely sequence at least in two species (Moore *et al.*, 1995; Xing and Zhang, 2010).

Functional characterization of all the genes of the rice genome has an extreme importance in agriculture, and, especially, the identification of genes and genomic regions regulating agronomically significant traits like high yield or disease resistance is a huge challenge for rice researchers. Strategies taking advantage from sequence information of other genomes, like Arabidopsis, has been successfully proved to shortcut both forward and reverse genetics in rice plant (Leister, 2005).

A number of reverse genetic approaches have been developed to determine the function of genes in rice, including insertional mutagenesis, homologous recombination and anti-sense or RNAi suppression (An *et al.*, 2005). Insertional mutagenesis is one of the most widely used techniques among reverse genetic approaches that provide more quick way for cloning the mutated gene (An *et al.*, 2005). In plants, DNA elements that are capable of random insertion into the chromosomes, such as T-DNA of *Agrobacterium tumefaciens* or transposons, have been used as a mutagen to produce loss-of-function mutations (Bouchez and Hofte, 1998). Although the insertional mutagenesis has been used as a throughput tool in plant functional genomics, especially in *Arabidopsis thaliana*, this approach has several limitations. For example, the investigation of functions is not possible for duplicated genes, and a great number of mutant phenotypes in these lines are induced by division to genes other than those into which the DNA tag is inserted (Waterhouse and Helliwell, 2003).

In plants, the control of homologous recombination has reported highly difficult because the illegitimate recombination events are prevalent. Although some successful experiments have been reported, gene replacement through recombination is not possible on a large scale, and functional analysis for large number of genes has to be carried out along with other strategies (Puchta and Hohn, 1996).

In the post-genome era, finding the function of all the genes has been a serious challenge in plant genomes. One approach to solve this problem is to reduce or knock out a gene expression that hopefully leads to any change in plant phenotype suggestive for its gene function (Matthew, 2004). Currently, there are two major techniques to change the gene expression and function: insertional mutagenesis and RNAi (Bevan, 2002). The principle of RNAi application in plant functional genomics is not complicated. The dsRNA is introduced into the plant cell that activates DICER gene and RISC complex finally leading to gene silencing (Thakur, 2003). RNAi has several benefits over insertional mutagenesis, and the first and foremost benefit is the ability to specifically make silent the target gene. This is because of the homology-dependent nature of the RNAi process (Matthew, 2004).

2.3 Quantitative Trait Loci (QTLs)

Quantitative traits are phenotypic traits such as quality, yield and some forms of disease resistance in plants that are controlled by multiple genes (Collard *et al.*, 2005), only occasionally some of these individual genes have major effects (Falconer, 1965). The region inside the genome that includes genes responsible for manifestation of a particular quantitative trait is known as quantitative trait locus (QTL)(Collard *et al.*, 2005) and can be identified by the means of molecular markers, which should also segregate according to the Mendelian law of segregation (Gupta, 2002).

Identification of QTLs are usually difficult due to the lack of distinct phenotypic segregation and also because each gene participating in a complex trait has comparatively small phenotypic effects (Yano and Sasaki, 1997). More than 75 years ago, the concepts of QTLs detection were developed (Sax, 1923), and these years the accessibility of linkage maps and DNA markers has led to substantial progress of QTL

mapping in plants (Lander and Botstein, 1989; Paterson *et al.*, 1989). In 1980, development of DNA markers was a major achievement in the characterization of quantitative traits that led to creation of opportunities to select for QTLs (Collard *et al*., 2005). Hybridization of selective parental lines, different in one or more quantitative traits, and analyzing the segregating progeny in order to link the quantitative trait locus to identified DNA markers is known as QTL analysis (Asins, 2002). A variety of methods are used for QTL analysis in crop plants that the earliest and easiest methods for QTL analysis involved single-marker regression or tests of the independency in segregation of DNA marker and the trait of interest (Gupta, 2002).

QTL mapping is defined as the process of constructing linkage maps and conducting QTL analysis to recognize genomic regions associated with traits (McCough and Doerge, 1995). On the other hand, QTL mapping is a set of procedures with these two main targets: first detecting and the second locating a QTL (Sofi and Rather, 2007). A plenty of factors can influence the detection of QTLs segregation in a target population that the main ones are environmental effects, genetic properties of QTLs and experimental error (Tanksley, 1993; Asins, 2002; Collard *et al.*, 2005).

Advanced backcross (AB) QTL is an efficient approach to improve wild species through detection and transfer of worthwhile QTLs from unadapted germplasm into elite breeding lines (Tanksley and Nelson, 1996). This strategy has been successfully effective for some crops such as rice (Xiao *et al.*, 1998b; Moncada *et al.*, 2001) and tomato (Tanksley and Nelson, 1996; Bernacchi *et al.*, 1998). AB-QTL approaches recently have been applied to identify and introduce the QTL of yield enhancing alleles from wild species into high-yielding elite cultivars species of rice (Fu *et al.*, 2010).

2.3.1 Yield-Related QTL in Rice

A considerable part of crop improvement programmes is to increase the yield of them. While Wild progenitors consider an important gene pool in breeding programs, there is deep concern due to the limited diversity of current high yielding varieties and hybrids in plants (Swamy and Sarla, 2008). Plant breeding conventionally uses crosses that have phenotypically promising genotypes in yield improvement programmes. However, molecular mapping studies suggest that wild species with poor phenotype can donate genes to improve yield (Xiao *et al.*, 1996; Ori *et al.*, 1997; Tanksley and McCouch, 1997; Gur and Zamir, 2004).

Yield is considered a complex trait that is governed by many loci. These QTLs have been mapped to a few sections on almost all chromosomes (Price, 2006; Swamy and Sarla, 2008). The most known components of grain yield include number of grains per panicle, grain weight and number of panicles per plant (Song *et al*., 2007). Rice yield has taken advantages from two major genetic improvements: hybrid production that exploits hetrosis, and using semidwarf genes to improve harvest index and plant architecture. Therefore, rice yield has increased more than double in most regions of the world and even tripled in special countries in the last five decade (Xing and Zhang, 2010). QTLs of nine yield related traits have been mapped from three different species of rice including *O. glumaepatula* (2n=24, AA), *O. Grandiglumis* (2n=48, CCDD) and *O. Rufipogon* (2n=24, AA) (Swamy and Sarla, 2008). Gn1a is a yield-related QTL in rice that has been cloned and functionally characterized, and determined as a QTL for number of grains per panicle (Ashikari *et al.*, 2005). Song *et al*. (2007) reported a new QTL called GW2 that controls rice grain weight and width. This QTL encodes an unknown RING-type protein that has E3 ubiquitin ligase activity.

Yield-related QTLs are not distributed in a random manner in rice chromosomes. More yield QTLs interestingly identified on chromosomes 1, 2, 3 and 4 (Swamy and Sarla, 2008). Table 2.2.1 displays the chromosome-wise distribution of yield-related QTLs and related traits from wild species of rice. On the one hand, there are favourable alleles of yield-related traits in wild species but the expression of these alleles is frequently masked because of the presence of other harmful loci. On the other hand, nearly one half of the QTLs taken from wild species were trait enhancing (Swamy and Sarla, 2008). Molecular maps are helpful tools to identify suitable loci that probably exist in the most undesirable gene pool among wild species (Paterson and Press, 1998).

Tomato and rice are the most extensively studied crops for yield QTLs in wild species (Tanksley and McCouch, 1997). However yield-enhancing QTLs have been recognized in the O. nivara and *O. rufipogon*, progenitor wild species of rice, no yield QTL was discovered in *O. grandiglumis* and *O. glumaepatula*. In rice, it seems that the closely related wild species are more probably to have applicable novel alleles (Blair *et al.*, 2006; McCouch *et al.*, 2007). Several yield-related QTLs have been identified and mapped by researchers in *O. rufipogon* that some of them were enhancing QTLs. Xiao *et al*. (1998b) reported 68 QTLs out of which 35 identified as trait enhancing. Septiningsih *et al.* (2003) reported 42 QTLs, of which 33% determined beneficial. Thomson *et al*. (2003) Mapping quantitative trait loci for yield, yield components reported 76 QTLs, 53% of which were desirable at one or the other location tested.

Table 2.3.1: Chromosome-wise distribution of yield-related QTL and related traits from wild species of rice (Xiao *et al.*, 1998b; Moncada *et al.*, 2001; Brondani *et al.*, 2002; Septiningsih *et al.*, 2003; Thomson *et al.*, 2003; Marri *et al.*, 2005; Yoon *et al.*, 2006).

Trait	**QTLs on chromosome**												**Total QTLs**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	
Yield per plot	1	1	–	–	–	–	–	5	–	–	–	–	7
Yield per plant	6	7	4	4	1	3	2	–	2	–	3	–	32
Yield per panicle	3	3	1	6	4	1	2	–	–	–	3	–	23
Tillers per plant	1	1	–	1	1	–	–	–	–	–	–	–	4
Panicles per plant	4	4	1	–	–	–	1	–	–	–	2	–	12
Spikelets per plant	1	–	–	–	–	–	–	–	–	–	–	–	1
Spikelets per panicle	7	4	2	2	–	1	1	1	2	–	–	1	21
Grains per plant	5	4	–	1	3	1	–	4	–	–	1	1	20
Grains per panicle	3	3	2	2	1	1	–	–	1	–	1	3	17

2.3.2 Yield-Related Genes in Rice

Meaningful advance has been performed to elucidate the plant genes that are capable of improving yield. Although there is a doubt if identified genes will lead to increased performance in the fields, but this doesn’t decrease the importance of the finding that single discovered genes can have deep effects on complicated, multifactorial traits like yield (Sakamoto and Matsuoka, 2008).

Analysis of significant agronomic traits, like grain yield, have extremely facilitated by the useful information and tools derived from The International Rice Genome Sequencing Project (Ashikari and Matsuoka, 2002). Using these information and tools, rice researches have revealed several genes that regulate grain size, grain number, tiller number and plant height (Sakamoto and Matsuoka, 2008). Some important traits for increasing yield have recently been distinguished in rice, such as plant height, grain number, and leaf erectness. These properties are regulated primarily by three phytohormones: gibberellins, cytokinins, and brassinosteroids. So the

producing genes of these hormones can be used as strategies for increasing the yield (Sakamoto, 2006).

In rice, the grain yield potential can be divided into four major components including grain number per panicle, panicle number per plant, grain weight and ratio of filled grains (Matsushima *et al.*, 1966). Recently, an increasing grain number QTL named Gn1 has been identified on the chromosome 1 in rice. This QTL has two loci including Gn1a and Gn1b that the first one is a gene for cytokinin (CK) oxidase/dehydrogenase (CKX), OsCKX2 (Ashikari *et al.*, 2005; Sakamoto and Matsuoka, 2008). The activity of OsCKX2 enzyme is degradation of phytohormone cytokinin. Reduction of endogenous OsCKX2 expression, with antisense OsCKX2 cDNA, led to cytokinin accumulation in inflorescence meristems that increases the number of sex organs and finally enhancing grain yield (Ashikari *et al.*, 2005).

GS3, encoding a transmembrane protein, is a major QTL for grain weight and length that is located on the pericentromeric part of chromosome 3 (Fan *et al.*, 2006). Song *et al*. (2007) also reported the cloning and characterization of a new QTL called GW2 on the short arm of chromosome 2 that controls grain weight and width. Their data showed that GW2 encodes a RING-type ubiquitin E3 ligase. They stated that the lack of GW2 function increases cell numbers that leads to enhance grain width, weight and yield.

TEOSINTE BRANCHED1 (TB1) considers as a major gene in the domestication programme of maize that is involved in lateral branching. The morphology of tb1 mutants and the expression pattern of TB1 imply the negative regulation role of TB1 in the outgrowth of maize axillary buds (Doebley *et al.*, 1997).

On the other hand, The rice ortholog OsTB1/FINE CULM1 (FC1) exhibits the same characteristics and hence also regulates rice tillering negatively (Takeda *et al.*, 2003).

2.4 Receptor Like Kinase (RLK)/ *Pelle* Family

Signal perception via different classes of cell-surface receptors is a common feature among all living systems (Shiu and Bleecker, 2001). In plants, various types of cell-surface receptors receive and process diverse stimuli and signals from the environment (Shiu and Bleecker, 2003). Some of these receptors like the receptor histidine kinases have not been detected in metazoan genomes (Shiu and Bleecker, 2001). On the other hand, there are a large number of transmembrane kinases that are similar to growth factor receptor tyrosine kinases in animals indicating one of the similarities of signal transduction between animals and plants (Fig. 2.3.1) (Initiative, 2000). These transmembrane proteins are called receptor-like kinases (RLKs) that are defined by the presence of putative versatile N-terminal extracellular domains and C-terminal intracellular kinase domains (Shiu and Bleecker, 2003).

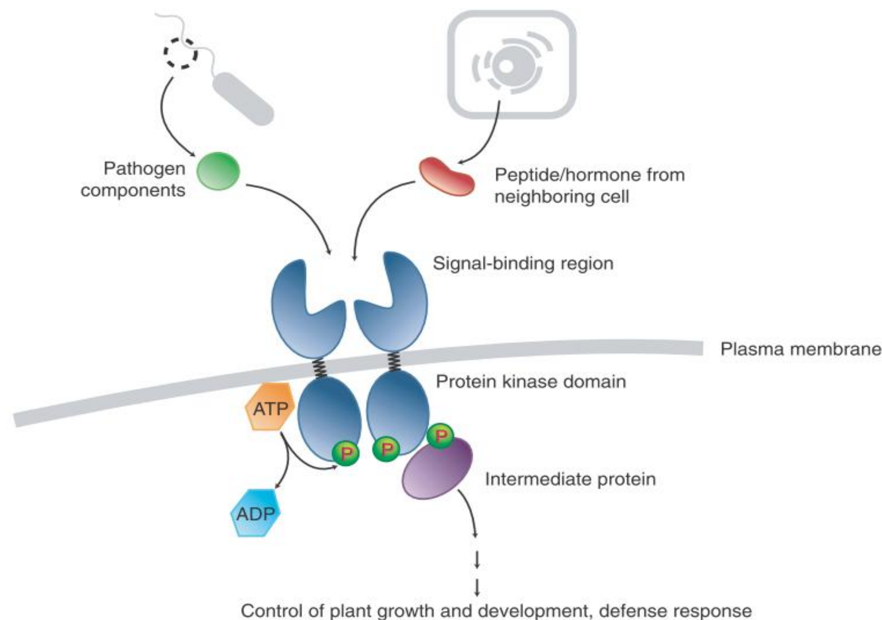

Figure 2.4: Common themes in plant and animal receptor protein kinases (Shiu and Bleecker, 2001).

Almost 20 years ago, Walker and Zhang (1990) reported the first plant RLK in maize utilizing degenerate PCR primers to the protein kinase domains. Afterwards, RLKs have been detected in many plant species and have been proposed to play different roles in plant biology history (Shiu *et al.*, 2004).

In plants, intracellular kinase domains of RLKs belong to RLK/Pelle gene family of *Drosophila melanogaster*. Therefore plant RLK grouped into the RLK/Pelle gene family (Shiu and Bleecker, 2001). The plant RLK*/Pelle* family can be classified into three fundamental groups including transmembrane receptor kinase, receptor-like cytoplasmic kinase (RLCK) and receptor like protein (RLP) (Shiu and Bleecker, 2001). Transmembrane receptor includes an extracellular domain that a single pass transmembrane helix connects it to a cytoplasmic serine/theronine (ser/ther) protein kinase (Shiu and Bleecker, 2001). On the other hand, RLCK proteins lack an extracellular domain while RLP proteins lack an intracelluar cytoplasmic kinase domain (Tor *et al.*, 2009).

The functions of plant RLK/Pelle family members can be divided into two major categories (Shiu and Bleecker, 2001). The first category includes those RLKs that control the growth and development of plant (Becraft, 2002). The second category consists of RLKs involved in defence responses and plant-microbe interactions (Shiu and Bleecker, 2001). RLK/Pelle members participate in a broad range of plant developmental processes, such as the organ specification, regulation of meristem proliferation, hormone signal transduction and reproduction (Lehti-Shiu *et al.*, 2009). RLK/Pelle members participate in a broad range of plant developmental processes, such as the organ specification, regulation of meristem proliferation, hormone signal transduction and reproduction (Lehti-Shiu *et al.*, 2009). Several RLKs have been

recognized in plants that are involved in growth and developmental processes including maize CRINKLY4, Petunia pollen-expressed receptor-like kinase 1(PRK1), Arabidopsis BRI1, *CLAVATA1* and ERECTA (Shiu and Bleecker, 2001).

PRK1 was identified as a receptor-like kinase protein expressing by a pollen-expressed gene in *Petunia inflata* (Mu *et al.*, 1994). Down regulation of the PRK1 plant gene in antisense PRK1-expressing lines led to pollen abortion and the loss of the nuclei. These events suggested the key role of PRK1 in a signal transduction pathway that regulates the postmeiotic development of microspores in *P. inflata* (Lee *et al.*, 1996).

BRs (Brassinosteroids) are a group of plant steroidal hormones that regulate plant growth and development (Xu *et al.*, 2008). BR-insensitive-1 (BRI1), a leucine-rich repeat receptor (LRR) protein kinase, functions as BRs receptor (Friedrichsen *et al.*, 2000). Mutations in the greatly expressed Arabidopsis BRI1 gene cause reduction of fertility, dwarfism, delayed senescence and light independent development due to lack of sensitivity to the brassinosteroid (Li and Chory, 1997). This event represents the first instance of RLK conservational function between dicots (Arabidopsis) and monocots (rice) (Yamamuro *et al.*, 2000).

A number of RLK family members have been involved in plant-microbe interactions or in controlling disease resistance. These RLKs are expressed by several isolated and characterized genes like Xa21 gene of rice, LRK10 gene of wheat, Pti gene of tomato, LRK10 gene of wheat and FLS2 gene of Arabidopsis (Shiu and Bleecker, 2001). Rice Xa21 belongs to a member of a multigene family including seven XA21 genes called A1, A2, B, C, D, E, and F that are located on chromosome 11 (Song *et al.*,

1995). The XA21 gene gives resistance against *Xanthomonas oryzae pv. oryzae* in a race specific manner (Song *et al*., 1995). The predicted protein of this gene contains leucine-rich repeats in the supposed extracellular domain and a serine/threonine kinase in the supposed cytoplasmic domain suggesting that LRR-containing RKs have a key role in plant disease resistance (Wang *et al*., 1998).

2.4.1 CLAVATA genes involving in plant growth and development

In plants, the origin of all above-ground mature organs is the shoot meristem that must provide a population of undifferentiated cells to function as a source of uninterrupted organ formation (Yu *et al.*, 2000). The family of CLAVATA-like genes may include 20 members that three of them, *CLV1*, *CLV2* and *CLV3*, are the best-characterized genes. The lack of activity in any of these three genes results in an increase in the size of the shoot meristem as stem cell proliferation surpasses differentiation (Clark *et al.*, 1997).

CLAVATA1(*CLV1*) is a previously characterized gene in *Arabidopsis thaliana* that encodes a putative receptor kinase, suggesting a role in signal transduction, containing 21 tandem LRRs in the extracellular domain of its structure (Clark *et al.*, 1997). *CLV1* is expressed particularly in the L2 and L3 layers of plant shoot apical meristem, and exists as a 185-kDa disulfide-linked multimer in plant extraction (Clark *et al.*, 1993; Clark *et al.*, 1997; Trotochaud *et al.*, 1999).

CLV3 encodes a small protein that cooperates with *CLV1* in regulating meristem proliferation (Fletcher *et al.*, 1999). As a matter of fact, *CLV3* considers as a ligand for *CLV1* (Trotochaud *et al.*, 2000). *CLV2* encodes an LRR protein, with a signal peptide and a transmembrane domain, which gives disease resistance against pathogenic fungi

in plants (Jeong *et al.*, 1999). The expressed protein of this gene probably participates in *CLAVATA1*-CLAVATA3-mediated developmental activities in an independent pathway controlling the development of organ (Kayes and Clark, 1998). Both of the *CLV1* and *CLV2* are necessary for CLAVATA3 function. On the other hand, *CLV1* interaction with *CLV3* takes place with the presence of *CLV2* (Trotochaud *et al.*, 1999; Brand *et al.*, 2000).

CLV1 and *CLV3* are recognized in identical ~450-kD complex to kinase-associated protein phosphatase (KAPP), and a putative Rho GTPase (Trotochaud *et al.*, 1999). KAPP acts as a negative regulator for *CLV1* function, and also has interactions with other RLKs (Braun *et al.*, 1997). *CLV1* negatively regulates two recently discovered genes called WUSCHEL (WUS) and POLTERGEIST (POL) that are involved in signal transduction pathway of CLAVATA genes (Shiu and Bleecker, 2001). WUSCHEL acts as a homeodomain transcripion factor that its expression is repressed by *CLV1* activity. On the other hand, its expression influences the expression of *CLV3* that forms a feedback loop in regulating the proliferation of the meristem. (Shiu and Bleecker, 2001). The POLTERGEIST gene, which encodes a protein phosphatase 2C (PP2C), is a key regulator in CLAVATA pathways that controls stem cell identity of shoot and flower meristems in Arabidopsis plant (Yu *et al.*, 2003).

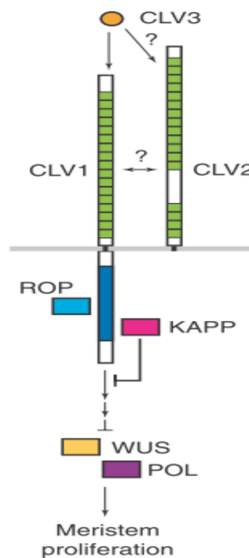

Figure 2.4.1: *CLV1*-mediated signalling pathway involves the activation of *CLV1* by the ligand *CLV3* and also the interference of *CLV2* with unknown exact role. The intracellular domain (blue) may interact with KAPP and Rho GTPase (ROP). Generally, when *CLV1* gets active, this event prevents the function of POLTERGEIST and WUSCHEL and negatively controls meristem proliferation in plant (Shiu and Bleecker, 2001).

2.5 RNA interference

RNA interference is known as a mechanism for gene regulation that affects the transcript level by either transcriptional suppression (TGS) or by the activation of a sequence-Specific RNA degradation process [PTGS/RNA interference (RNAi)] (Agrawal *et al.*, 2003). At the post-transcriptional level, a number of gene silencing events were reported in different species such as animals, plants, fungi and ciliates during the 1990 (Baulcombe, 2000; Matzke *et al*., 2001).

The first manifestation of RNA silencing was observed in 1990 when Jorgensen and Mol were attempting to up-regulate the activity of a pigment-producing gene: chalcone synthase by introduction of exogenous transgenes into petunias to deepen the purple color of flowers (Napoli *et al.*, 1990; Agrawal *et al.*, 2003). They expected to obtain darker color in comparison with pink or violet flowers by the experiment.

Unexpectedly, the experiment led to variegation with complete loss of color (fully or partially white flowers) in many plants that indicated not only transgenes were inactive, but also the added DNA sequences effected endogenous loci expression (Hannon, 2002).

In plants, RNA silencing is identified as a cellular mechanism to control the post-transcriptional regulation of plant gene expression with producing microRNA (miRNA) (Bartel and Bartel, 2003; Tenea, 2009) and also serves as a natural defense reaction against invading viruses (Voinnet, 2001).

In recent years, research has applied RNA interference as a powerful tool to decipher gene function by specific targeting of gene expression and complementary study of the silenced phenotype (Matthew, 2004).

2.5.1 The Biology and Mechanism of RNA interference

Currently, three natural pathways have been identified for RNA silencing in plants that have been revealed by genetic and molecular analysis. The miRNA and viral RNA pathways are two of them in which the targets are RNA that is silenced as a result of turnover and/or an effect at the level of translation. The third pathway, targeting DNA, is led to DNA methylation and heterochromatin formation. These pathways all involve the cleavage of a double-stranded RNA (dsRNA) into short 21–26-nucleotide RNAs by an enzyme called Dicer. These RNAs are known as short interfering RNAs (siRNAs) and microRNAs (miRNAs) according to the origin of dsRNAs (Baulcombe, 2004).

The existence of multigene families is affected on diversity of silencing pathways for silencing genes in plants. For instance, DCL3 is required for nuclear pathway While DCL1 is implicated in the miRNA silencing (Xie *et al.*, 2004). The core of RNA interference system is composed of several protein families, including ARGONAUTE (AGO), DICER (DCR) or DICER-LIKE (DCL) and RNA-dependent RNA Polymerase (RDR) (Baulcombe, 2004; Chapman and Carrington, 2007). Dicer includes dual RNase III motifs, helicase domain and a region homologous to the protein of QDE2 or RDE1 or ARGONAUTE family (Bernstein *et al.*, 2001).

The RNA interference system acts in two main steps, the initiation and the execution steps. Double-stranded RNAs (dsRNAs) are identified as initiating factors for RNAi with either endogenous or exogenous sources. They are processed into 21 to 28 nucleotide RNA duplexes which then direct the cellular silencing machinery to complementary single-stranded RNAs like messenger or RNAs viral genomic RNAs (Meister and Tuschl, 2004; Zambon *et al.*, 2006).

When the dsRNA is exogenous like a virus with an RNA genome, the RNA is either transported into the cytoplasm, or introduced there and cleaved to short double-stranded fragments named small interfering RNAs (siRNAs) by Dicer enzyme. siRNAs are then divided into single-strand molecules and joined to an active RNA-induced silencing complex (RISC) (Bagasra and Prilliman, 2004; BURLIBAŞA *et al.*, 2008). After integration of siRNAs into the RISC, They base-pair to the complementary target mRNAs, and this event suppress mRNA to be used as a template in translation process (Parker *et al.*, 2006). The active component of RISC is called argonaute protein that plays a crucial role in gene silencing with cleavage of the complementary target mRNA strand (Gregory*et al.*, 2005; BURLIBAŞA *et al.*, 2008).

Gene regulation is one of the recognized roles of RNAi through the usage of different type of endogenously encoded RNAs that are produced from the genome of organism. One type of these RNA precursors is processed into pre-miRNA in the nucleus. This structure, in the shape of stem-loop, is exported from nucleolus to the cytoplasm to be transformed to miRNA by dicer cutting activity, then miRNA associate with the RISC complex for further manipulation (Pasquinelli *et al.*, 2005). Therefore both RNAi pathways, with the exogenous and endogenous dsRNA origins, come together in the step of RISC complex activity (Voorhoeve *et al.*, 2006).

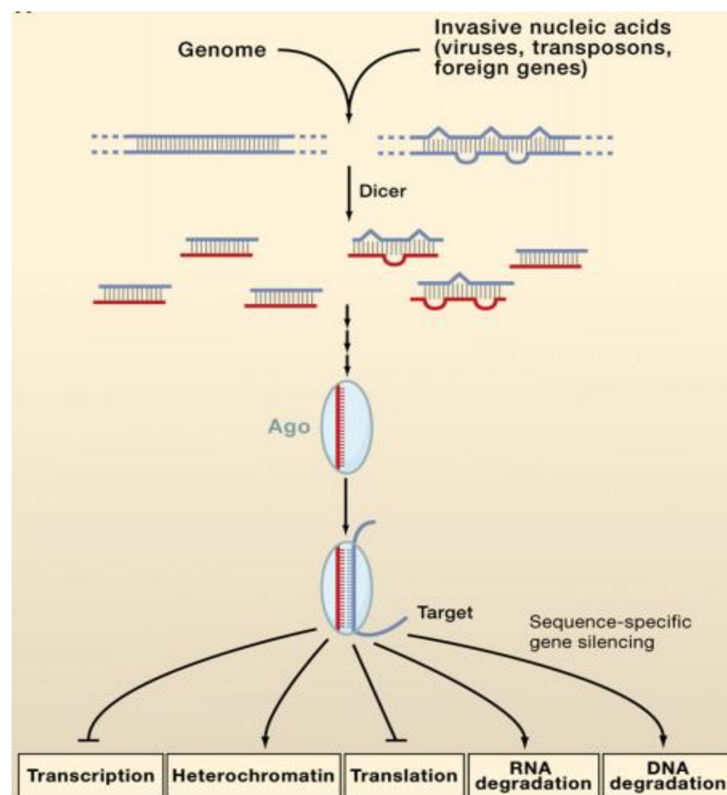

Figure 2.5.1: Core features of siRNA and miRNA silencing (Carthew and Sontheimer, 2009).

2.5.2 Hairpin RNA (hpRNA)–mediated gene silencing in plants

RNA silencing can be achieved easily by introducing a gene fragment into the cell that can produce complementary dsRNA with target gene mRNA (Thakur, 2003). There are several methods that dsRNA can be transformed or produced in the plants such as tissue infiltration with an Agrobacterium strain having a T-DNA expressing an hairpin RNA (hpRNA) transgene, microprojectile bombardment with hpRNA vectors or dsRNA and viruse-induced gene silencing (VIGS) (Waterhouse and Helliwell, 2003).

Hairpin RNA (hpRNA)–mediated gene silencing takes advantage from the cellular mechanisms in which double-stranded RNA (dsRNA) identify and expose homologous mRNA to degrade in a sequence-specific manner. (Cazzonelli and Velten, 2004). Some researches has described that hpRNA constructs, producing dsRNA, has a considerable potential to silence interested gene expression (Akashi *et al.*, 2001; Wesley *et al.*, 2003; Scattat *et al.*, 2004).

hpRNA vector requires a fragment of target gene that is cloned as an inverted repeat sequence behind an appropriate promoter such as the maize ubiquitin promoter for monocots and the 35S CaMV promoter for dicots (Kusaba, 2004). In *Escherichia coli*, the presence of spacer between the arms of inverted repeat is helpful to increase the stability of vector (Smith *et al.*, 2000). ihpRNA vectors, containing intron, can induce silencing more effective that lead to 90-100% silencing in produced independent transgenic lines (Wesley *et al.*, 2001). Currently, several different vectors use to induce RNAi in the functional genomics field such as binary vectors to express GFP and GUS proteins, pHELLSGATE high-throughput vector and tobacco rattle virus (TRV) based Virus-induced gene silencing (VIGS) vector (Matthew, 2004).

Miki and Shimamoto (2004), developed a Gateway vector, pANDA, for RNA interference of rice genes. In this developed construct, hairpin RNA is produced from a given gene fragment and transcribed from a maize ubiquitin promoter. They observed more than 90% suppression in the transgenic plants mRNA expression and short interfering RNA was recognized as RNA silencing evidence in silent plants (Miki and Shimamoto, 2004).

2.6 Biosafety of Transgenic Plants

Currently, rapid advances in transgenic biotechnology have meaningfully promoted the development and production of GM crops. The vast global production of GM crops has created great benefits like solving the food security of the world although it has introduced considerable biosafety concerns (Lu, 2008). One of the principal concerns about GM plants production is the possibility of transgene escape via gene flow into the non-transgenic plants populations (Snow *et al.*, 2005).

Gene flow is the movement and incorporation of genes from the gene pool of one population into the gene pool of another (Futuyma, 1998). Transgene escape is defined as a process in which a transgene moves from a GM plant to its non-GM plant counterpart or to its wild relatives via gene flow (Lu, 2008). Gene flow may take place through two main processes including transgene introgression into non-transgenic plants because of hybridization and the invasion of GM plants to accidental regions. The scattering of pollen and seeds, from GM plant populations, are the key basic mechanisms for gene flow (Eastham *et al.*, 2002; Messeguer, 2003; Snow *et al.*, 2005; Chapman and Burke, 2006). As a result, the key basic mechanisms for gene flow are the scattering of pollen and seeds from GM plant populations (Haygood *et al.*, 2004; Hails and Morley, 2005; Snow *et al.*, 2005).

From the human or animal health point of view, the possible risks of a particular transgene and its product can be tested and measured. On the other hand, the effects of transgene escape is more sophisticated (Kuvshinov *et al.*, 2001). The risks of gene flow depend on the effects of transgenes on recipient and the effects of GM plants on the ecosystem in the habitat that transgenes may invade (Snow *et al.*, 2005; Chapman and Burke, 2006).

Transgene escape usually happens when following requirements occur in agreement: (i) spatially, transgenic plant and its non-transgenic varieties or wild relatives should have sympatric distribution for example they grow in the same area; (ii) temporally, the flowering time of transgenic plant and its non-transgenic varieties or wild relatives should take place at the same time; and (iii) biologically, transgenic and its wild relative species should have an adequately close relationship that the resulting interspecific hybrids can be capable of reproduction in a normal way (Baorong *et al.*, 2003).

There are three principal ways that a transgene may escape. First, transgenic plant may persist after harvesting as seed or vegetative form. These forms can create populations that are capable of spreading to near cultivated land or neighbour native habitats. Second, the transgene could be transferred by the means of pollination to a non-specific crop or naturalized population of the identical species and scatter beyond the borders of agriculture. Last, the transgene could be transferred through pollination and interspecific hybridization to another crop species or to a nearly related wild species, resulting in transgene escape from containment (Armstrong *et al.*, 2005).

Various factors can determine the frequency of transgene escape, such as the presence of related species, appropriate geographical conditions, the efficiency of pollen transfer and suitability of the created seed and hybrid (Groot *et al.*, 2003; Bots and Mariani, 2005). In plants, pollen has a crucial role in the flow of genes, particularly in out-crossing plants. It is specifically important to investigate the pollen when a transgenic plant is formed and to compare it with non-transgenic plants. As a risk assessment is necessary before releasing of transgenic cultivars, different methods can be used to get a good risk assessment of the plant, e.g. pollen viability (Bengtsson, 2006).

Pollen-mediated sexual reproduction can be one of the ways of transgene escape, and hence pollen viability may consider a critical factor to determine the frequency of transgene escape. Analysis of pollen viability can be used as a method to estimate the probable role of pollen viability in transgene escape (Bots and Mariani, 2005). Pollen viability is defined as the ability of the pollen grain to carry out its delivery function of sperm cells to the embryo sac that happens during compatible pollination (Shivanna *et al.*, 1991).

The identification of a proper method is the key to an exact estimation of pollen viability (Rodriguez-Riano and Dafni, 2000). The examination of mature stained pollen with iodine-potassium iodide solution is a common approach to estimate pollen viability in rice. *In vitro* germination also has been used as another method even though difficulties were reported in obtaining an optimal culture medium (Khatun and Flowers, 1995).

Generally, it is accepted that a more in-depth understanding of gene flow, pollen flow and species biosystematic relationships will make easier effective prediction of transgene escape and the environmentally potential risks, in addition to proper management of environmental risks (Baorong *et al.*, 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Materials

The seeds of *Oryza rufipogon* IRGC105491 were obtained from the GenBank center of the Malaysian Agricultural Research and Development Institute (MARDI) in Seberang Perai, Penang, Malaysia. These seeds were used to produce putative *CLV1* transgenic plants via *Agrobacterium tumefaciens* mediated transformation during a previous project at University of Malaya (Law, 2011) that led to the production of eight individual transgenic plants (A1, A2, A3, A5, A6, A7, A8, and A9) as confirmed by PCR. Afterwards, the seeds of these T0 transgenic plants were planted to produce a T1 generation of transgenic plants in eight lines based on the initial T0 plants. Six to eight seeds from each T1 line (derived respectively from T0 lines A1, A2, A3, A5, A6, A7, A8, and A9), and also confirmed to contain transgenes by PCR, were selected to produce T2 transgenic plants. Control seeds used in the study were *Oryza rufipogon* IRGC105491 obtained from GenBank center of the Malaysian Agricultural Research and Development Institute (MARDI). All the plants involved in the project are shown in Table 3.1.

Table 3.1: All the transgenic and non transgenic control plants involved in the project

T0 events	T1 Lines	T2 Lines
A1	A1.1, A1.2, A1.3, A1.4, A1.5, A1.6, A1.7, A1.8 **(Line A1)**	A1.1.1, A1.1.2, ... A1.1.19, A1.1.20 **(Line A1.1)**
A2	A2.1, A2.2, A2.3, A2.4, A2.5, A2.6, A2.7, A2.8 **(Line A2)**	A2.2.1, A2.2.2, ... A2.2.21, A2.2.22 **(Line A2.2)**
A3	A3.1, A3.2, A3.3, A3.4, A3.5, A3.6, A3.7, A3.8 **(Line A3)**	A3.2.1, A3.2.2, ... A3.2.20, A3.2.21 **(Line A3.2)**
A5	A5.1, A5.2, A5.3, A5.4, A5.5, A5.6, A5.7, A5.8 **(Line A5)**	A5.2.1, A5.2.2, ... A5.2.19, A5.2.20 **(Line A5.2)**
A6	A6.1, A6.2, A6.3, A6.4, A6.5, A6.6 **(Line A6)**	No plant was available because no seed was planted from line A6 (T1)
A7	A7.1, A7.2, A7.3, A7.4, A7.5, A7.6 **(Line A7)**	A7.4.1, A7.4.2, ... A7.4.23, A7.4.24 **(Line A7.4)**
A8	A8.1, A8.2, A8.3, A8.4, A8.5, A8.6 **(Line A8)**	A8.5.1, A8.5.2, ... A8.5.19, A8.5.20 **(Line A8.5)**
A9	A9.1, A9.2, A9.3, A9.4, A9.5, A9.6, A9.7, A9.8 **(Line A9)**	A9.2.1, A9.2.2, ... A9.2.23, A9.2.24 **(Line A9.2)**
Controls		Control Plant Lines
Ci	Ci1, Ci2, Ci3, Ci4, Ci5, Ci6, Ci7, Ci8 **(Ci Control Line)**	Ci6.1, Ci6.2, Ci6.3, ... Ci6.15, Ci6.16 **(Ci6 Control Line)**
Cii	No T1 Cii plant available because Cii was a control line that its seeds was planted in the same time with T2 seeds	Cii1, Cii2, Cii3, Cii4, Cii5, Cii6, Cii7, Cii8, Cii9 **(Cii Control Line)**

3.2 Plant Growth Conditions

The rice seeds were soaked in water on wet tissue paper in Petri dishes for 4 days to obtain primary seedlings. After that, they were transferred to pot trays (Dimensions: Diameter: 7cm, Height: 6.5cm, Volume: 0.19 liter) containing brown soil and compost for 25 days for further growth. The pot trays were flooded to three centimeters depth with water and placed in a greenhouse with natural lightning (13 h

day/ 11 h night), in a controlled environment of 30°C/25°C and relative humidity of 70% (Ohnishi *et al*., 2011). After 25 days, the rice seedlings were transferred to individual pots (Dimensions: Diameter: 11cm, Height: 12cm, Volume: 1.00 liter) containing brown soil and compost. After transfer, one gram of Sodium: Potassium: Phosphorus (15:15:15) fertilizer pellets were added each week until flowering stage. Automatic drip watering system of greenhouse was used to keep the standard humidity of the soil in the pots during the whole time.

3.3 DNA extraction

Plant DNA isolation to obtain DNA templates for PCR screening was carried out using DNeasy Plant Mini Kit (QIAGEN, Germany) according to the user's manual.

3.4 DNA quantity and quality measurements

DNA was analyzed using standard methods for agarose gel electrophoresis (Sambrook *et al*., 1982). The DNA quantity and quality was further estimated spectrophotometrically by Bio photometer (Eppendorf, Germany) at different UV absorbances to obtain values for A260/280 and A260/230.

3.5 Polymerase Chain Reaction (PCR)

A master mix including all of the necessary PCR components (See Table 3.5.2), with the exception of DNA template, was prepared in a 2 ml centrifuge tube and mixed well by a Vortex Mixer (Gilson GVLab), then the master mix was aliquotted into 0.5 ml microcentrifuge tubes (PCR tubes). After that, the DNA template was placed in each PCR tube and the whole solution was centrifuged (WEALTEC, USA). One tube was prepared with all the necessary PCR components without adding DNA template as a negative control. Finally, the tubes were transferred immediately after preparation to a

DNA Thermocycler (Eppendorf, Germany). The DNA was initially denatured at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 sec, 30 sec at 56 °C, and 35 sec at 72 °C. After 35 cycles, the reaction was followed by a final extension at 72 °C for 10 min.

Two pairs of primers, forward and reverse, were used to amplify 636 bp fragment of transgene. The primers were designed by Miki and Shimamoto (2004) for the amplification of hairpin loop region of pANDA vector and their sequences are shown in Table 3.5.1.

Table 3.5.1: The primers sequences for Polymerase Chain Reaction

Gus linker (Miki and Shimamoto, 2004)	Forward: 5' CATGAAGATGCGGACTTACG 3'
	Reverse: 5' ATCCACGCCGTATTCGG 3'

Table 3.5.2: The reaction mixture for Polymerase Chain Reaction

PCR stock solution (Promega, USA)	**Volume (µl)**	**Final concentration**
25 mM MgCl2	1.5	1.0-4.0mM
5X Green GoTaq® Flexi Buffer	5	1X
10 mM dNTPs	0.50	0.2Mm each dNTP
Gus linker forward primer	1	0.1-1.0µM
Gus linker reverse primer	1	0.1-1.0µM
GoTaq® Flexi DNA Polymerase (5u/µl)	0.25	1.25u
dd H2O	13.75	-
DNA template	2	<0.25µg/25µl
Total	25	-

3.6 Analysis by Agarose Gel Electophoresis

3.6.1 TBE buffer preparation

For agarose gel preparation and sample loading, 1X TBE buffer was used. One hundred millilitres of 1X TBE is made from 10.8 g Tris Base, 5.5 g boric acid and 4 ml of 0.5 M EDTA dissolved by mixing with 85 ml autoclaved distilled water.

3.6.2 Agarose gel preparation

Two different percentages, 1 and 0.7% of molecular biology Analytical Grade agarose were used for the electrophoretic separation of nucleic acids. For the preparation of 1% agarose gel with diameter of 6 millimeter, 0.24g agarose was mixed with 24 ml 1X TBE which was prepared with nuclease-free water in conical flask and weighed. Then the solution was microwaved until dissolved and weighed again followed by replacement of evaporated water with nuclease free water. After cooling the solution to about 60ºC, ethidium bromide (0.5µg/µl) was added and poured into a casting tray containing a sample comb and allowed to solidify at room temperature. This concentration of agarose gel was used for analysis of isolated DNA samples and PCR products.

The low percentage agarose gel was prepared to run digested DNA. The low percentage agarose gel makes easier the transfer of digested DNA from gel to positively charged nylon membrane (Roche, Germany) in the blotting process. For this purpose, the agarose gel with 0.7% concentration was prepared by mixing 0.35g agarose with 50 ml TBE according to the 1% agarose gel preparation that was explained previously.

3.6.3 Preparation of DNA samples

Based on optical density, 1µg of isolated DNA was mixed with 1µl of 6X loading dye Buffer (Fermentas, Canada) and electrophoresed on 1% LE Analytical Grade Agarose (Promega, USA). The buffer (1X TBE) was used as the reservoir buffer and also to prepare the agarose gel with ethidium bromide (EtBr). GeneRuler™ 1kb DNA Ladder (Fermentas, Canada) was incorporated in each gel as DNA size marker. Gel was run at 120 V for 30 minutes. The DNA bands were visualised by exposure to short wave UV light and photographed using an Alphaimager™ 2200 (Alpha Innotech, USA).

3.6.4 PCR products

As 5X Green GoTaq® Flexi Buffer (Promega, USA) was used in Polymerase Chain Reaction, there was no need to mix PCR product with loading dye. Therefore, PCR products were directly loaded into 1% LE Analytical Grade Agarose (Promega, USA) including ethidium bromide (EtBr). GeneRuler™ 100bp DNA Ladder (Fermentas, Canada) was incorporated in each gel as DNA size marker. Gel was run at 120 V for thirty eight minutes, and DNA bands were visualised by exposure to short wave UV light and photographed using an Alphaimager™ 2200 (Alpha Innotech, USA).

3.7 Purification of PCR products

The PCR product was purified for sequencing and probe production procedures according to the user's manual of High Pure PCR Product Purification Kit (Roche, Germany).

3.8 Gel extraction

The gel extraction procedure was done according to the user manual of QIAquick® Gel Extraction Kit (QIAGEN, Germany) to extract PCR product as an intermediate step in probe production.

3.9 Sequencing

A commercial DNA sequencing service was provided by 1st BASE Inc., Malaysia using gene specific primers (See Table 3.4.1) to sequence randomly selected PCR products of T1 and T2 transgenic plants for confirmation of PCR results.

3.9.1 Analysis of sequences

The analysis of sequencing results was performed by aligning application of nucleotide blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and BioEdit v7.0.9 (http://www.mbio.ncsu.edu/bioedit/page2) softwares.

3.10 Southern blot analysis

3.10.1 Conventional DNA extraction

DNA was isolated using a CTAB (Cetyl Trimethyl Ammonium Bromide) method (Murray and Thompson, 1980) with some modifications. About 1 g of fresh leaf sample harvested from transgenic plants was ground in liquid nitrogen by mortar and pestle. After that, the sample was transferred into a 15 ml polycarbonate centrifuge tube containing 3 ml of pre-warmed (65 °C) CTAB buffer (Appendix A) and 10 µl β-mercaptoethanol (β-ME). The mixture was mixed vigorously by Vortex Mixer (Gilson GVLab). The mixture was incubated in a water bath for 60 min at 65 °C. After 65 °C incubation, the mixture was transferred to a 37 °C water bath and 6 µl RNase (10 mg/ml) was added and incubated for 10 min. Next, an equal volume of chloroform:

isoamyl-alcohol (24:1) was added and mixed gently by inversion. The mixture was centrifuged at 2200 × g for 30 minutes. The upper aqueous phase was transferred into a new 15 ml polycarbonate centrifuge tube. This step was repeated twice while the amount of aqueous phase was estimated in the first time. According to the volume of aqueous phase in the first step, 0.08 volume of cold ammonium acetate and immediately 0.50 volume of cold isopropanol were added and mixed gently for 20 to 30 times to precipitate the DNA pellet at -80 °C overnight. The next day, the mixture was centrifuged at 2200 × g for 30 min. The DNA pellet was washed with 4 ml of 70 % (v/v) ethanol and centrifuged at 2200 × g for 10 min. After that, 4 ml 95 % (v/v) ethanol was added to the tube and supernatant was discarded and centrifuged at 2200 × g for 10 min. the supernatant was discarded and the pellet was air-dried for 10 min. finally, The DNA pellet was resuspended in 500 µl distilled water and stored at -20°C.

The concentration and purity of the DNA were determined by spectrophotometer (Eppendorf) and the DNA quality was analyzed using a 1 % (w/v) agarose gel by gel electrophoresis technique to confirm the intact structure of extracted genome.

3.10.2 Probe synthesis and labelling

A specific probe was synthesized for the southern hybridization. This probe was synthesized by the amplification of 636 base pair fragment of transgene using a DNA Thermocycler (Eppendorf, Germany). One microgram of genomic DNA, from positive transgenic plants, was used as DNA template while a pair of specific primers (See Table 3.4.1) added in PCR master mix (See Table 3.4.2) to produce interest probe with 636 base pair length. After that, the PCR product was analyzed by gel electrophoresis (See section 3.6.2). Finally, the PCR product was extracted (See section 3.8) and analyzed by Biophotometer (Eppendorf) to recognize the quality of produced probe. The labelling

process was performed based on the user manual of DIG DNA Labelling and Dectection Kit (Roche, Germany) to produce DIG-labelled DNA probe.

3.10.3 The sequence analysis of the probe target

The probe target sequence was analyzed by Restriction Summary software that is a JavaScript online programme under Sequence Manipulation Suite 2 online programmes (http://biochimica.unipr.it/home/tools/sms2/index.html). This analysis was done to prove the lack of the specific restriction sites in the target sequence. These specific restriction sites could be cut by the restriction enzymes that were chosen to digest the genomic DNA in the digestion step of southern blotting process. In this case, the probe couldn't attach to its target because it was digested by the restriction enzymes.

3.10.4 Genomic and plasmid DNA digestion

Mixtures of restriction digestion reaction components (Table 3.10.1) were prepared in 1.5 ml microcentrifuge tubes while three different restriction enzymes *SacI(*0.25U/µl), *SpeI*(0.2U/µl) and *Hind*III(0.3U/µl) (Fermentas, Canada) separately added to each tube. Then, the tubes were incubated at 37 °C overnight to complete digestion. Finally, the analysis of 5 µl digested genomic DNA was performed with agarose gel electrophoresis and a gel detection system (Alphaimager™ 2200) to check for the complete digestion of each sample.

A mixture with the same reaction components for *HindIII* digestion reaction was prepared, but thirty microgram of non-transgenic genomic DNA added to the tube as a control negative sample instead of thirty microgram transgenic DNA.

As a control positive sample, 120 nanogram of plasmid DNA (pANDA vector), including transgene, was added to a digestion reaction along with *HindIII* restriction enzyme and other components for restriction digestion. The mixture was incubated at 37 °C for three hours, and digested plasmid analyzed by agarose gel electrophoresis and gel detection system (Alphaimager™ 2200).

Table 3.10.4.1: Restriction digestion of genomic DNA by *SacI* restriction enzyme

Digestion stock solution	**Volume (µl)**	**Final concentration**
dd H2O	57.5	-
10x Buffer *SacI* (Fermentas,Canada)	10	1X
Transgenic DNA template (1µg/µl)	30	0.3µg/µl
SacI restriction enzyme (10u/µl) (Fermentas, Canada)	2.5	0.25U/µl
Total	100	-

Table 3.10.4.2: Restriction digestion of genomic DNA by *SpeI* restriction enzyme

Digestion stock solution	**Volume (µl)**	**Final concentration**
dd H2O	58	-
10x Buffer Tango (Fermentas,Canada)	10	1X
Transgenic DNA template (1µg/µl)	30	0.3µg/µl
SpeI restriction enzyme (10u/µl) (Fermentas, Canada)	2	0.2U/µl
Total	100	-

Table 3.10.4.3: Restriction digestion of genomic DNA by *HindIII* restriction enzyme

Digestion stock solution	Volume (µl)	Final concentration
dd H2O	57	-
10x Buffer *HindIII* (Fermentas,Canada)	10	1X
Transgenic DNA template (1µg/µl)	30	0.3µg/µl
HindIII restriction enzyme (10u/µl) (Fermentas, Canada)	3	0.3U/µl
Total	100	-

Table 3.10.4.4: Restriction digestion of plasmid DNA by *HindIII* restriction enzyme

Digestion stock solution	Volume (µl)	Final concentration
dd H2O	13	-
10x Buffer *HindIII* (Fermentas,Canada)	2	1X
Plasmid DNA (30ng/ µl)	4	6ng/ µl
HindIII restriction enzyme (10u/µl) (Fermentas, Canada)	1	0.5U/µl
Total	20	-

3.10.5 Purification and precipitation of digested genomic DNA

After the confirmation of complete digestion, molecular grade phenol solution was added in an equal volume to each digested sample to begin the purification process. The mixture was mixed gently by Vortex Mixer (Gilson GVLab) and centrifuged at 12,000 for 3 min. subsequently; supernatant was transferred to a 1.5 ml microcentrifuge tube, and the same volume of chloroform: isoamylalcohol (24:1) was added to it, mixed and centrifuged at 12,000 × g for 3 min. After that, 0.1 volume of 3 M sodium acetate and 2.5 volume of cold absolute ethanol were added into the supernatant to precipitate the digested genomic DNA at -80 °C for overnight. The next day, the mixture was centrifuged at 12,000 × g for 15 min at 4 °C to recover the DNA. The pellet was washed with 1 ml of cold 70 % (v/v) ethanol air-dried for 5 minutes. The pellet was resuspended in 25 µl of sterile dH_2O.

3.10.6 Gel treatment and blotting procedure

The digested genomic DNA (three transgenic samples and one nontransgenic sample) and digested plasmid DNA were electrophoresed along with DIG-labeled DNA Molecular Weight Marker III (Roche, Germany) on 0.7 % (w/v) agarose gel without ethidium bromide. The gel electrophoresis was performed in 35 V for 5 hours. Then, the gel was submerged in 250 mM HCl with shaking at room temperature for 10 min and rinsed with sterile distilled water for sevral times. After that, the gel was submerged in Denaturation Solution (0.5 M NaOH, 1.5 M NaCl) for 20 min (twice) at room temperature with gentle shaking and rinsed for 2 min with sterile distilled water. Subsequently, the gel was submerged in Neutralization Solution (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 20 min (twice) at room temperature. Finally, the gel was equilibrated in 20 × SSC for 10 min.

Based on the protocol by Sambrook *et al*. (1982), the blotting pyramid was assembled to capillary transfer of DNA from treated agarose gel to nylon membrane (Roche, Germany) for overnight. The next day, nylon membrane was rinsed with 2 × SSC for 5 min and wrapped in a whatman filter paper (Whatman, USA) to bake at 80 °C for three hours.

3.10.7 Hybridization and Visualisation of Genomic DNA Blot

The membrane was soaked in 10 ml of pre-warmed DIG Easy Hyb (Roche, Germany) at 45 °C for 30 min for pre-hybridization. Subsequently, 500 ng of DIG-labeled DNA probe was denaturated by boiling for 10 min and rapidly cooling in the ice for 5 min. The denaturated DIG-labeled DNA probe was added to 1.5 ml pre-warmed (45 °C) DIG Easy Hyb and centrifuged shortly to mix by centrifuge machine (WEALTEC, USA). Then, probe/hybridization mixture was added to the membrane

while the pre-hybridization solution was poured off. The membrane was hybridized at 45 °C for 20 hours.

After hybridization, the membrane was washed with 20 ml Low Stringency Buffer (2 × SSC containing 0.1 % SDS) for 5 min (twice) at room temperature under constant agitation. Then, the membrane was washed with 20 ml preheated High Stringency Buffer for 15 min (twice) at 65 °C under constant agitation.

The membrane was transferred and washed in 20 ml Washing Buffer for 5 min at room temperature with gentle shaking. Then, the membrane was transferred and incubated in 50 ml Blocking Solution for 30 min with gentle shaking. Later, the membrane was transferred and incubated in 20 ml Antibody Solution for 30 min, with gentle shaking. Next, the membrane was washed with 50 ml Washing buffer for 15 min (twice). The membrane was equilibrated in 20 ml Detection Buffer for 3 min. Then, 200 μl of NBT (Nitro blue tetrazolium chloride) stock solution was added into 10 ml of Detection Buffer to make Colour Substrate Solution. The 10 ml Colour Substrate Solution was added and covered whole membrane completely without shaking in darkness for a few hours. Finally, the reaction was stopped by washing the membrane for 5 min with 50 ml of sterile double distilled water. The results were documented by Olympus digital camera after drying the membrane in the room temperature.

3.11 Pollen viability tests

Pollen viability test was done to determine if there were any differences between control and transgenic plants concerning pollen viability, physiology and pollen growth (Bengtsson, 2006a).

3.11.1 *In vitro* pollen tube germination

The pollen germination test was performed on newly opened flowers. Medium for the pollen tube germination was made by mixing 1.0 mM $CaCl_2$, 1.0 mM KCl, 0.8 mM $MgSO_4$, 1.6 mM H_3BO_3, 0.03% casein, 0.3% 2-(N-morpholino) ethanesulfonic acid, 10.0% sucrose and 12.5% polyethylene glycol (Xu *et al.*, 2008). Pollen grains with three different time intervals after anther dehiscence-i.e. 1, 15 and 30 min were germinated in 2 ml microcentrifuge tubes containing 60 µL medium (Song *et al.*, 2001). One hour after incubation of the anthers and stigma from newly-opened flowers at room temperature, 20 µL medium was placed on a microscope slide and the number of germinated pollen grains was counted under the light microscope (Leica, Germany). The pollen was considered germinated when the pollen tube length was longer than the diameter of the pollen grain (Wang *et al*., 2004). The germination rate was averaged from three observation fields under the microscope while the test was done with three replicates.

3.11.2 Iodine potassium iodide Staining

One drop of iodine potassium iodide solution (I_2-KI) was placed on a microscope slide this solution was prepared by dissolving one gram of iodine and two gram of potassium iodide in 100 ml of water. The anthers and stigma from newly-opened flowers were dipped into the drop by help of forceps, and then they were crushed by a needle to release the pollen grains. After removing the debris, a cover slip was placed and the slide was observed under the light microscope (Leica, Germany). The entire slide was scanned in three random different fields and black-stained pollen was considered viable.

CHAPTER 4

RESULTS

4.1 DNA extraction

Total genomic DNA was successfully extracted from all T1, T2 and control plants. The extracted DNA samples were labelled as shown in Tables 4.1.1 and 4.1.2. Agarose electrophoresis images of total genomic DNA extracted from T1 and T2 leaf samples showed intact and single bands with molecular weight of more than 10,000 bp according to the reference band of 1kb ladder that was loaded with each group of DNA samples (Figure 4.1).

Table 4.1.1: The extracted DNA samples from T1 plants

T1 plant lines	**Number of plants**	**Names of DNA samples**
A1	8	A1.1, A1.2, … A1.8
A2	8	A2.1, A2.2, … A2.8
A3	8	A3.1, A3.2, … A3.8
A5	8	A5.1, A5.2, … A5.8
A6	6	A6.1, A6.2, A6.3, A6.4, A6.5, A6.6
A7	6	A7.1, A7.2, A7.3, A7.4, A7.5, A7.6
A8	6	A8.1, A8.2, A8.3, A8.4, A8.5, A8.6
A9	8	A9.1, A9.2, … A9.8
Ci (control line)	8	Ci1, Ci2, Ci3, Ci4, Ci5, Ci6, Ci7, Ci8

Table 4.1.2: The extracted DNA samples from T2 plants

T2 plant lines	**Number of plants**	**Names of DNA samples**
A1.1	20	A1.1.1, A1.1.2, … A1.1.20
A2.2	22	A2.2.1, A2.2.2, … A2.2.22
A3.2	21	A3.2.1, A3.2.2, … A3.2.21
A5.2	20	A5.2.1, A5.2.2, … A5.2.20
A7.4	24	A7.4.1, A7.4.2, … A7.4.24
A8.5	20	A8.5.1, A8.5.2, … A8.5.20
A9.2	24	A9.2.1, A9.2.2, … A9.2.24
Ci6 (Control)	16	Ci6.1, Ci6.2, Ci6.3, ... Ci6.15, Ci6.16
Cii (new control)	9	Cii1, Cii2, Cii3, … Cii8, Ci9

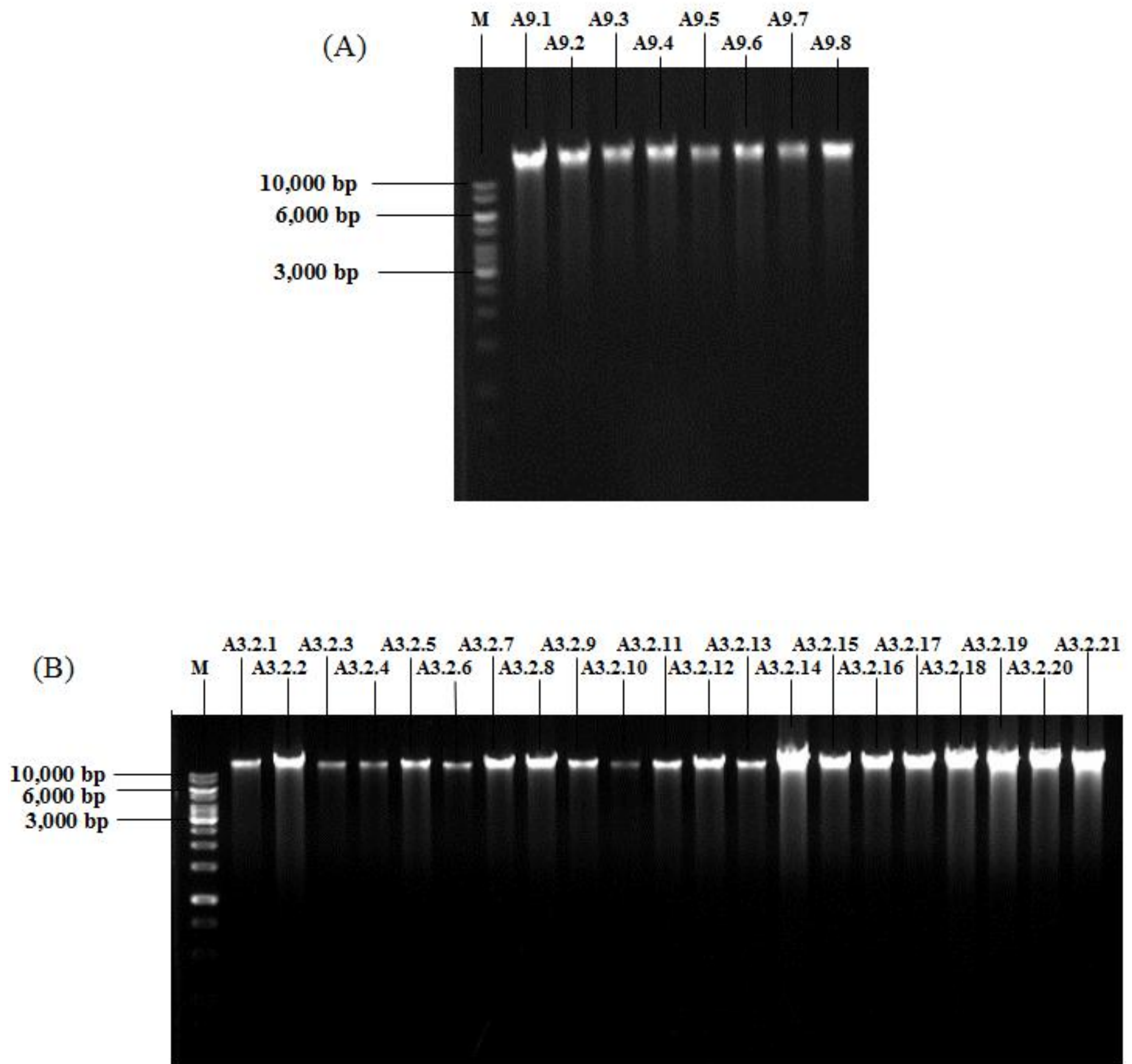

Figure 4.1: Agarose electrophoresis (1%) of total genomic DNA extracted from eight samples of A9 line (T1 plants) (A) and twenty-one samples of A3.2 line (T2 plants) (B). Lane M: GeneRuler™ 1kb DNA Ladder (Fermentas, Canada).

4.2 DNA quantity and quality measurements

The expected standard quality and quantity of DNA, according to the DNeasy Plant Mini Kit (QIAGEN, Germany) standards, was obtained for each sample. The range of the yields of total DNA from different extracted DNA samples was 0.015-0.063 µg/µl. The range of $A_{260/280}$ absorbance was 0.99-2 while the range of $A_{260/230}$ absorbance was 0.91-1.20.

4.3 PCR amplification of DNA samples obtained from T1 plants

After DNA extraction of T1 plants, the DNA samples were subjected to PCR amplification to confirm the presence of the transgene in the genome of T1 plants. The expected band, with 636 bp length, was detected after gel electrophoresis of PCR products for all T1 transgenic lines. Figures 4.3.1 and 4.3.2 show the presence of the 636 bp expected band for A5 line samples and control-untransformed plants.

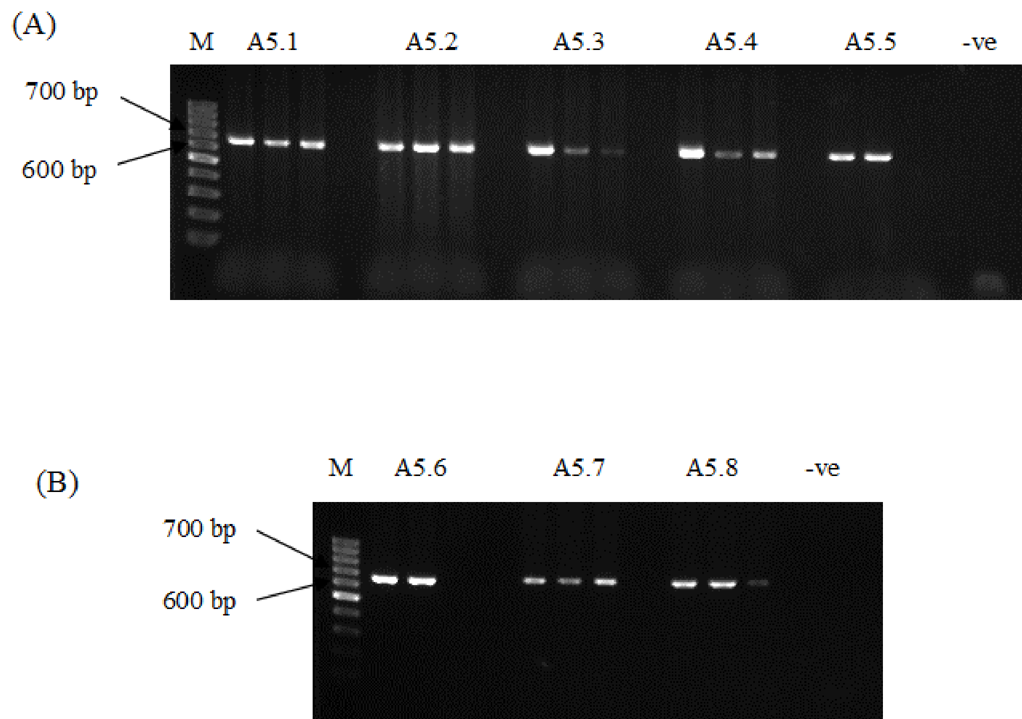

Figure 4.3.1: PCR screening of A5 samples with *Gus* linker primer (A and B). PCR amplification was done for each DNA sample in three replicates. Lane M: GeneRuler™ 100bp DNA Ladder (Fermentas, Canada); Lane -ve: Control negative (no DNA sample).

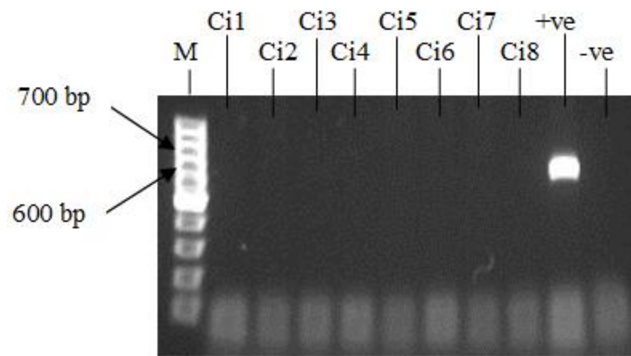

Figure 4.3.2: PCR screening of Ci line as control untransformed plants with *Gus* linker primers. Lane M: GeneRuler™ 100bp DNA Ladder (Fermentas, Canada); Lane +ve: Control positive (PCR amplification of pANDA vector); Lane -ve: Control negative (no DNA sample).

4.4 Germination of T1 plant's seeds

One transgenic plant was selected from each line of T1 plants for seed collection. Around thirty seeds from each plant were grown to produce T2 transgenic plants. These chosen transgenic plants, confirmed by PCR, were A1.1, A2.2, A3.2, A5.2, A7.4, A8.5 and A9.2 as shown in Figure 4.3.1. The germination percentage varied from ~70-100% and was 85.18% for Ci control seeds and 100% for Cii control seeds (Table 4.4).

Table 4.4: Germination percentage of T1 seeds and control non-transformed seeds (Ci6 and Cii)

Plants	Total number of seeds	Germinated seeds	Percentage of germination
A1.1	32	25	78.12
A2.2	34	27	79.41
A3.2	34	24	70.58
A5.2	28	25	89.28
A7.4	30	27	90
A8.5	28	24	85.71
A9.2	29	27	93.10
Ci6	27	23	85.18
Cii	9	9	100

4.5 PCR amplification of DNA samples obtained from T2 plants

The expected 636 bp band, indicating the presence of *Gus* linker sequence in the genome of transformed plant, was observed at a high frequency ranging from 59% to 85% for the T2 lines, with the exception of the A3.3 line (19%). PCR was repeated for the samples that gave a negative result and the final results for the presence of the *Gus* linker are shown in Table 4.5. Figure 4.5.1 shows the PCR screening result of A2.2 and A3.2 lines whilst the Figures for the other lines are in Appendix B. No band was observed for any of the negative control (untransformed) plants. Table 4.5 shows the percentage samples where the *Gus* linker was present among the representatives of each line. The Chi-square values were calculated for each line. Segregation analysis of Transgenic T1 rice seeds showed that typical 3:1 Mendel’s segregation ratio was obtained in the all of the transgenic lines with the exception of A3.3 line that showed a non-Mendelian inheritance pattern.

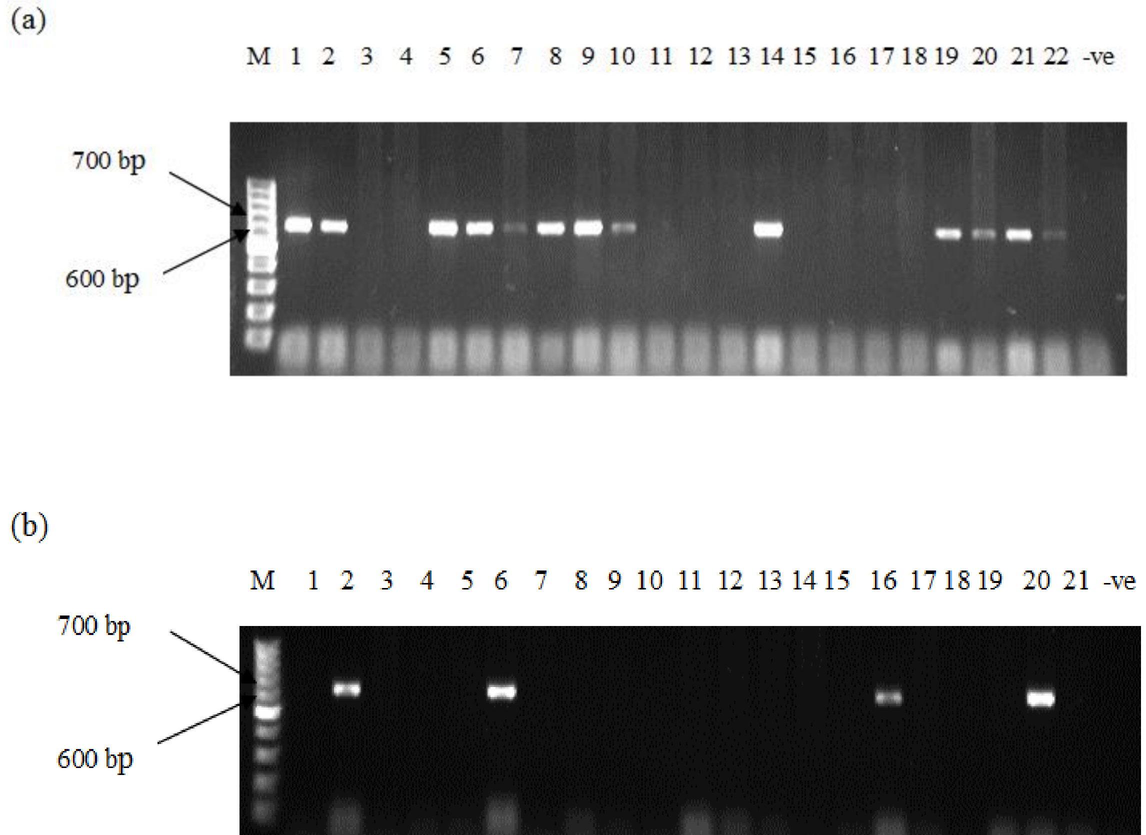

Figure 4.5.1: PCR screening of A2.2 (a) and A.3.2 (b) lines with *Gus* linker primers. Lane M: GeneRuler™ 100bp DNA Ladder (Fermentas, Canada); Lane -ve: Control negative (no DNA sample).

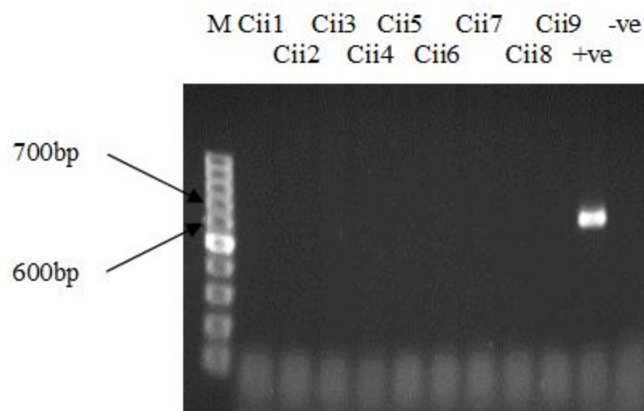

Figure 4.5.2: PCR screening of Cii line control untransformed plants with *Gus* linker primers. Lane M: GeneRuler™ 100bp DNA Ladder (Fermentas, Canada); Lane +ve: Control positive (PCR amplification of pANDA vector); Lane -ve: Control negative (no DNA sample).

Table 4.5: Segregation ratio of transgene presence (*Gus* linker) in transgenic T1 rice seeds

Transgenic line	**Total**	***Gus+/Gus-***	***Gus+%***	**Expected ratio**	**χ^2 value**	**P**
A 1.1	20	14/6	70%	3:1	0.066	<3.84
A 2.2	22	13/9	59%	3:1	0.740	<3.84
A 3.2	21	4/17	19%	3:1	8.760	>3.84
A 5.2	20	14/6	70%	3:1	0.066	<3.84
A 7.4	24	19/5	79%	3:1	0.050	<3.84
A 8.5	20	17/3	85%	3:1	0.260	<3.84
A 9.2	24	18/6	75%	3:1	0.000	<3.84

4.6 Sequencing analysis of *Gus* sequences cloned from transformed rice plants

The alignment results of DNA sequences from PCR amplified samples of the *Gus* linker sequence disclosed a very high identity ranging from 96% to 99%. This degree of similarity confirmed the presence of transgenes into the transformed plants. The alignment released by the nucleotide blast software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) is shown in the Appendix C.

4.7 Southern Blotting

As a large amount of DNA was needed for southern blotting, the DNA of eight T1 transgenic plants including A1.1, A2.2, A3.4, A5.2, A6.3, A7.3, A8.4 and A9.1 were extracted from their leaves by CTAB conventional method that has proved to produce a highly concentrated DNA in comparison with DNA extraction kit. The obtained DNA bands, for the extracted samples, were intact and single after gel electrophoresis as shown in Figure 4.7.1. The expected standard quality and quantity of DNA, according to the CTAB DNA isolation standards, was obtained for each sample with a yield of total isolated DNA, for ranging between 1.45-1.84 µg/µl and $A_{260/280}$ absorbance from 1.70-1.92.

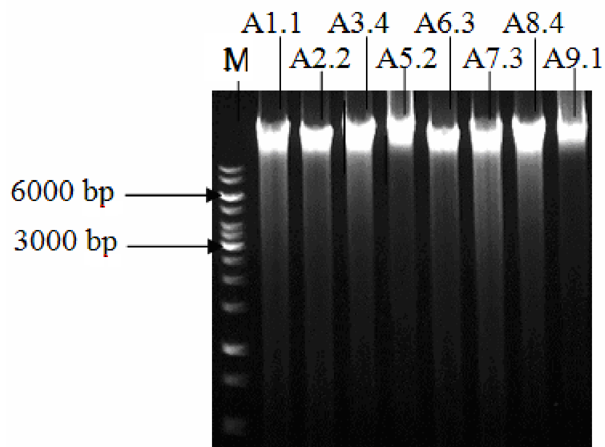

Figure 4.7.1: Agarose electrophoresis (1%) of total genomic DNA extracted from eight T1 transgenic plants. Lane M: GeneRuler™ 1kb DNA Ladder (Fermentas, Canada).

Before probe synthesis, the sequence of the probe complementary fragment within the transgenic plants, i.e. 636 bp of *Gus* linker sequence forming part of the transgene construct, was analyzed to confirm the absence of restriction sites for *Hind*III, *Sac*I and *Spe*I enzymes. This was because these restriction enzymes were used to digest the total DNA samples during the digestion step of the southern blotting procedure and the presence of their restriction site within the transgene sequence could lead to failure in transgene detection. Figure 4.7.2 shows the synthesized probe in three replicates for production of extra source of probe while the expected band size for the probe was confirmed by comparison of the probe bands and 100 bp molecular ladder (Fermentas, Canada).

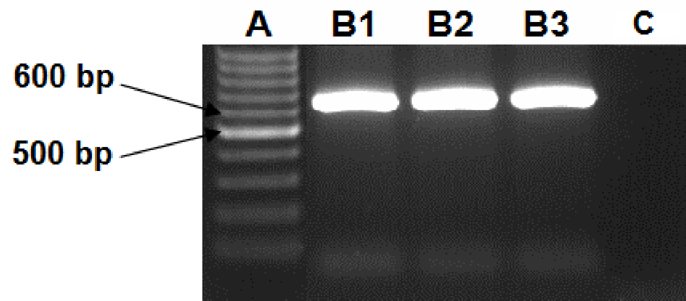

Figure 4.7.2: *Gus* linker probe synthesised by PCR amplification of 636 bp fragment of transgene. Lane A: GeneRuler™ 100bp DNA Ladder (Fermentas, Canada); Lanes B1, B2 and B3: three replicates of synthesized probe; Lane C: -ve control-untransformed sample.

Restriction digestion was also optimized by using three different enzymes including *Hind*III, *Sac*I and *Spe*I that each led to complete digestion of total genomic DNA of transgenic samples shown in Figure 4.7.3.

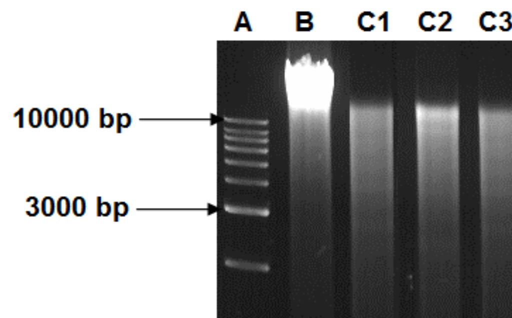

Figure 4.7.3: The optimized restriction digestion of transgenic DNA sample by three different restriction enzymes. Lane A: GeneRuler™ 1kb DNA Ladder (Fermentas, Canada); Lane B: undigested transgenic DNA sample; Lane C1: digested transgenic DNA sample by *Hind*III; Lane C2: digested transgenic DNA sample by *Spe*I; Lane C3: digested transgenic DNA sample by *Sac*I.

After this, the southern blotting process was successfully optimized for the positive control sample that was purified pANDA vector including the transgene. The 636 bp probe sequence detected its complementary sequence on pANDA vector. The circular vector was separately linearized by *Hind*III and *Bam*HI restriction enzymes. Using *Hind*III restriction enzyme, an expected band, 14.8 Kb, was observed on the

nylon membrane after the detection step. While an expected band, 926 bp, was obtained on the nylon membrane for the sample that was cut by *Bam*HI. No band was detected for the control sample of non-transformed plants (Figure 4.7.4).

Although the southern blotting process was optimized with a positive control, the continuous attempts with different conditions and variables failed to obtain a reproducible result for transgenic plant samples. Only one experiment led to an observable band after detection this indicated two bands of approximately 3 Kb and 4 Kb for T2 plant line A1.1 as shown in Figure 4.7.5. This event is expounded in the section 5.6 (Southern analysis discussion). The condition of this successful experiment was applied for the other experiments; however no positive result was observed for the other samples.

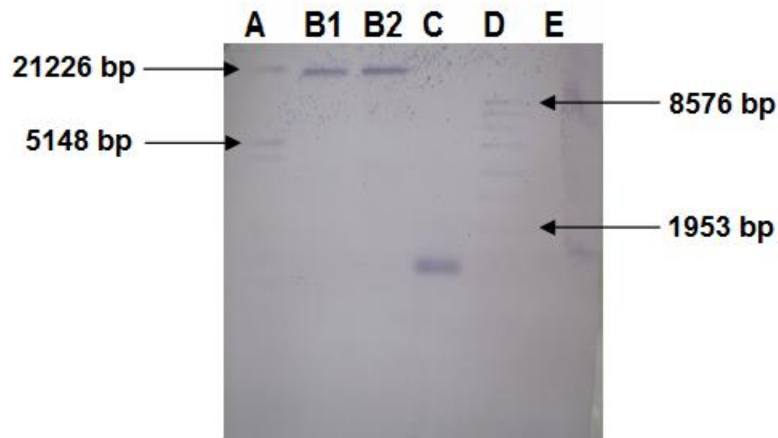

Figure 4.7.4: Southern-blot analysis for the detection of *Gus* linker sequence of pANDA vector to use as control positive of southern blotting of transgenic samples. Lane A: DIG-labelled DNA Molecular Weight Marker III (Roche, Germany); Lane B1: 14.8 Kb detected fragment of *Gus* linker sequence in the pANDA vector genome that was cut by *Hind*III restriction enzyme; Lane B1 and B2 are two replicates; Lane C: 926 bp detected fragment of *Gus* linker sequence in the pANDA vector genome that was cut by *Bam*HI during restriction digestion step; Lane D: DIG-labelled DNA Molecular Weight Marker VII (Roche, Germany); Lane E: -ve control.

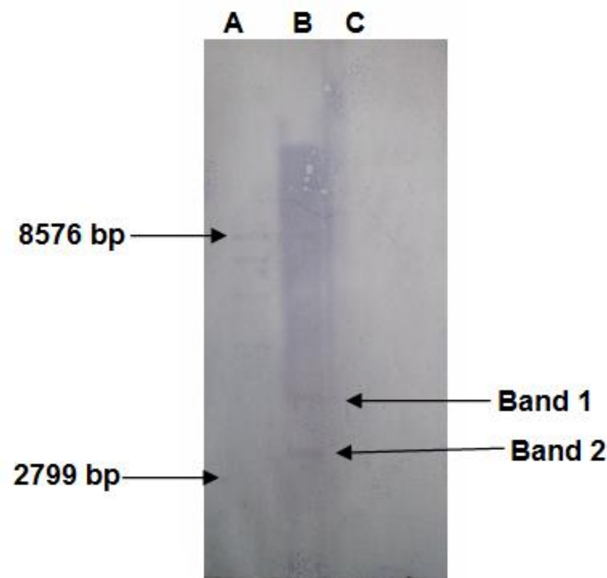

Figure 4.7.5: Southern-blot analysis of A1.1 DNA sample while *Hind*III was used to cut. Lane A: DIG-labelled DNA Molecular Weight Marker VII (Roche, Germany); Lane B: *Hind*III digested DNA from A1.1 sample; Lane C: -ve control-untransformed plant.

4.8 Pollen viability tests

4.8.1 Iodine-potassium iodide staining

The microscopic observation of pollen after I_2-KI staining was done successfully for each sample in three different fields while the number of stained round fertile (SRF) pollen grains and the total number of pollen grains were counted for each sample in three random different fields. The Figure 4.8.1 shows the pollen grains after I_2-KI staining under the microscope and the average number of SRF and total pollen grains was calculated for each sample is shown in Table 4.8.1. Finally, the fertility percentage was calculated through the ratio of fertile pollen to total pollen in three random microscopic fields. A pollen grain was considered fertile when its colour was totally black and the shape was round. The fertility percentage of transgenic and non-transgenic plants is shown in Table 4.8.1. There was no meaningful difference between fertility percentage of pollen form transgenic plants and pollen from non-transgenic plants.

Table 4.8.1: Fertility percentage of pollen from transgenic and non-transgenic plants subjected to I_2-KI staining.

Plant	Total number of pollen Average of three fields	Total SRF Average of three fields	Fertility percentage
A1.1 Transgenic	98.4	85.7	87.09±3.28
A2.4 Transgenic	100.7	85.4	84.80±4.09
A3.7 Transgenic	103.7	94	90.64±2.18
Ci3 Non-transgenic	106	85.4	80.56±1.52
Ci6 Non-transgenic	99.4	85	85.51±5.78
Ci8 Non-transgenic	101.4	86.4	85.20±5.78

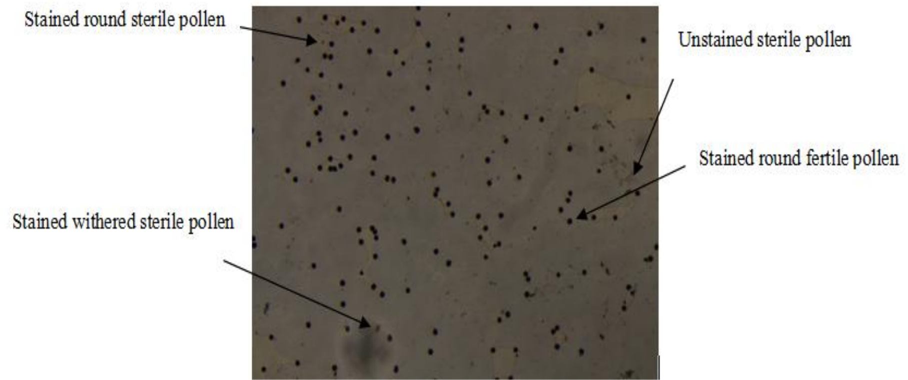

Figure 4.8.1: Pollen grains of rice stained by I_2-KI under light microscope (40X).

4.8.2 *In vitro* pollen tube germination

Pollen grains from three transgenic plants and three control un-transformed plants were observed at different time intervals after their anther dehiscence. Figure 4.8.2.1 shows the anther dehiscence of transgenic *Oryza rufipogon* rice (IRGC105491).

Figure 4.8.2.1: Anther dehiscence of a transgenic *Oryza refipogon* (IRGC105491) rice plant.

The number of germinated pollen tubes was scored after observation under the microscope after one hour after incubation at room temperature. The pollen was considered germinated when the pollen tube length was longer than the diameter of the pollen grain (Wang *et al*., 2004). The germination percentage was calculated from three observation fields under the microscope while the test was done with three replicates for each sample. Appendix F shows the germination percentage of pollen tubes for each plant at different time intervals after anther dehiscence. Three graphs were depicted to compare its specifications shown in Figure 4.8.2.2.

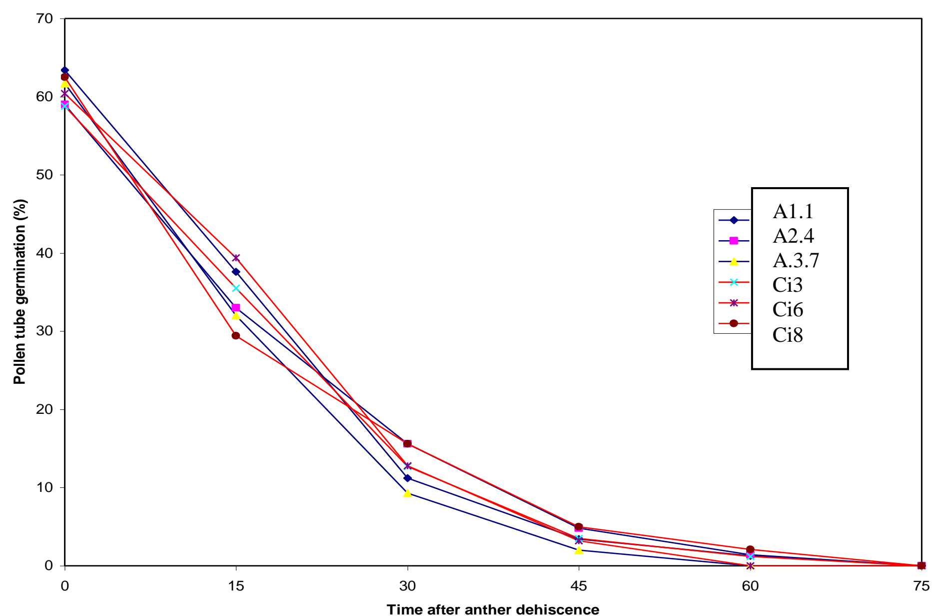

Figure 4.8.2.3: Pollen tube germination of transgenic plants (A1.1, A2.4 and A3.7) and non-transgenic plants (Ci3, Ci6 and Ci8).

CHAPTER 5

DISCUSSION

5.1 Plant growth

Germination initiates with the uptake of water by absorption by the dry seed which leads to embryo expansion (Finch-Savage and Leubner-Metzger, 2006). There are some basic factors for germination of seeds such as oxygen, water and appropriate temperature. On the other hand, the seeds can be sensitive to other important factors like light as it has been considered both to stimulate germination and to terminate dormancy (Baskin and Baskin, 2001; Finch-Savage and Leubner-Metzger, 2006). Most of the effective factors on germination rate were optimal in the greenhouse, so the only different effective factor on germination could be seed quality from the content and genetic points of view. As seen in appendix F, there was no meaningful difference between average germination rate of transgenic lines and non-transgenic control line (Ci) suggesting that the transgene construct had no effect on the germination rate of transgenic lines.

5.2 DNA extraction

After DNA isolation, electrophoresis on 1% agarose gel, Figure 4.1, shows an intact single band for each DNA sample that proved the successful extraction of total genomic DNA from the leaves of plants without any degradation. The absorbance at 260 nm reflects DNA concentration and the ratio of A260/A280 reflects the ratio of nucleic acids to proteins in the sample while a ratio of A260/A280 > 1.8 indicates low protein contamination in a DNA sample (Sambrook *et al.*, 1989). Most of the DNA samples exhibited high purity with an A260/A280 ratio above 1.8 while some samples exhibited lower purity with an A260/A280 ratio as small as 0.99. Any way, because the

reliable extracted method (kit isolation) was used, the extracted DNA samples showed a high level of quality that was proved by positive result of PCR amplification on them.

5.3 PCR screening of plants in two generations

The functional identification of genes has become increasingly important after obtaining the full sequence of rice genome in 2005. Different tagged lines have been produced although the available tagged genes are not enough for comprehensive study of gene function. To assist functional genomic studies in rice, Miki and Shimamoto (2004) developed the pANDA vector as a gateway vector for RNA interference of rice genes (Sato, 2005).

pANDA vectors were transformed into *A. tumefaciens* strain LBA4404 prior to the plant transformation in a research project at University of Malaya (Law, 2011), and the T0 transgenic plants were created via *A. tumefaciens* mediated transformation. In that project, the pANDA vector carrying a sequence of rice *CLAVATA1 (CLV1*) receptor kinase-like protein gene, was successfully introduced into the transgenic T0 plants. The transgene construct comprised of the maize ubiquitin promoter with an intron (1.9 Kb), a 926 bp fragment of the coding region of the *E. coli* GUS gene (*Gus* linker), which is the most known used reporter gene in GM plants (Gilissen *et al.*, 1998), flanked on either side by inverted repeats (IR) of the gene of interest (Appendix E). In this study, a one hundred base pair fragment encoding a putative *CLAVATA1* (*CLV1*) receptor kinase-like protein was located in the sense and antisense orientation to form inverted repeat sequence either side of the *Gus* linker. The inverted repeats (IR) have the main role in the formation of RNAi hairpin construct after transcription in the host aimed at RNAi silencing of the *CLV1* in transgenic rice plants transformed by this vector (Law, 2011).

The presence of the transgene construct including the *Gus* linker sequence was confirmed in T0, T1 and T2 transgenic plants by PCR amplification with the observation of a 636 bp band after gel electrophoresis (Figures 4.3.1, 4.5.1 and appendix B). The plants were considered transgenic if the PCR amplification of their DNA samples generated a 636 bp band while there was no band for non-transgenic control plant after gel electrophoresis on 1% agarose gel. As it is shown in Figures 4.3.1 and 4.5.1, the transgenic samples produced a 636 bp band. The length of band was estimated to be 636 bp because it was located between 600 and 700 bp bands of marker. The presence of control negative sample in each run of PCR amplification showed the lack of contamination while no band was observed for negative control in 1% agarose gels after electrophoresis.

The control-untransformed plants were grown to prove the accuracy of experiment including the absence of sample and reaction contamination. The results in this study showed that there was no contamination. All control-untransformed plants that were grown among T1 and T2 plants were confirmed to be non-transgenic after PCR amplification of their DNA, supporting the accuracy of experiments as shown in Figure 4.3.2.

5.4 Segregation analysis of T2 plants

Determination of homozygosity of transgenic plants is important for molecular breeding and transgenic studies (Honda *et al.*, 2002). T0 transgenic plants are normally heterozygous for their transgene. Homozygous transgenic plants are usually found among T1 plants that are the descendants of each independent plant. For this propose, T1 transgenic plants, achieved after self-fertilization of the primary transgenic plants

(T0 plants), grow until seed production that finally leads to determining the segregation analysis in the T2 generation (Bhat and Srinivasan, 2002; Honda *et al.*, 2002).

Detection of transgenic plants harbouring a single copy of the introduced transgene is crucial in transgenic studies. In this case, the segregation test is helpful for determination of transgenic plants with single-locus DNA (Kihara *et al*., 2006).

Peterson's chi-square ($\chi 2$) test, invented by Peterson in 1900, can be applied to determine whether there is a meaningful difference between the observed frequencies and the expected frequencies while population distribution estimates by a single random sample containing n independent observation (Kirk, 2007). In Mendelian inheritance, the chi-square test provides an appropriate way of determining when aberration from expected Mendelian ratios is significant enough to reject the particular Mendelian hypothesis under the considered test (Gigerenzer *et al.*, 1990).

Mendelian inheritance patterns are normally reported in transgenic lines, and the presence of this pattern, in transgenic lines, is not related to the method used for transgene transformation (Pinheiro *et al.*, 2009). Agrobacterium-mediated transformation method normally generates low copy number transgenic plants, and the transgenes are transmitted to the next generation according to Mendelian and in some cases non-Mendelian inheritance (Yin *et al.*, 2004). At least three factors can affect the stable inheritance pattern of a transgene: the transgene, the nature of the recipient genome and their interaction (Pinheiro *et al.*, 2009).

Non-Mendelian segregation has been reported with a frequency between 10% and 50% of independent transgenic lines produced by Agrobacterium-mediated transformation (Yin *et al.*, 2004). This phenomenon can arise because of various mechanisms including transgene silencing, transgene elimination, lower viability and fertilization ability of transgenic pollen and gamete selection (Kiani *et al.*, 2009; Pinheiro, *et al.*, 2009). Non-Mendelian segregation also can occur due to either poor expression or unstable transmission of the transgene (Yin *et al.*, 2004).

In the current study, the segregation of the transgenes in each line was compared to the expected 3:1 ratio of Mendelian inheritance ratio by applying the chi-square test, and it was revealed that the transgenic lines A1.1, A2.2, A5.2, A7.4, A8.5 and A9.2 had approximately the best fit of 3:1. As shown in Table 4.4.2, all $\chi 2$ values of indicated lines showed significant fit to a 3:1 ratio suggesting the transgene was integrated as a single locus and inherited following a Mendelian pattern (Shown in Table 4.5). On the other hand, the A3.2 line presented deviation from the expected Mendelian ration when the T2 plants derived from a T1 transgenic plant display a segregation ratio that is significantly different from 3:1 ratio suggests the presence of two or more unlinked copies of the transgene, all in a heterozygous state (German *et al.*, 2003).

5.5 Southern blotting analysis

Although the segregation analysis is useful for identification of transgenic plants with single locus DNA integration, it doesn't determine the copy number at the locus. Southern blotting is a standard method to determine the copy number of the transgene within the genome of transgenic plants (Kihara *et al.*, 2006).

The optimization of the whole procedure was performed successfully for the positive control however this was not reproducible for the plant samples with only one time that two bands were observed on the nylon membrane after detection step. These two bands showed the presence of two copy of transgene in the genome of relevant sample. Although different changes were applied in the conditions of the procedure even in different steps, no band was obtained during repeated attempts. After several attempts, the southern blotting procedure was not attempted further because of the time limitation of the project and also the lack of support for the necessary extra expenditure for supplying new materials.

The probe was produced by amplification of a 636 bp fragment of the *Gus* linker sequence of the transgene construct located in the centre of the *Gus* linker sequence. Consequently, it was attached to the complementary part of transgene during the hybridization step that led to the detection of transgene into the genome of control samples and also the genome of transgenic plant. There was no restriction site in the selected fragment of *Gus* linker sequence for the restriction enzymes that were used in the digestion step of southern blotting procedure otherwise the enzymes would cut the transgenes within the transgenic genomes resulting no detection of transgenes. The lack of restriction site for the digestive enzymes was proved by presenting the fragment sequence to the Restriction Summary software of Sequence Manipulation Suit server (http://www.bioinformatics.org/sms2/rest_summary.htm) to analyze it regarding the lack of the restriction sites. As it is shown in the Appendix D, there was no restriction site, in the complement fragment of probe, for restriction enzymes that were selected to cut the total genomic DNA of transgenic samples in digestion step. In this case, the 636 bp fragment of *Gus* linker sequence, selected as complementary fragment of probe, was

intact after digestion while the whole genome was exposed to digestion by the restriction enzymes.

Different factors and procedures can affect southern blotting result including quality and quantity of DNA, restriction digestion with proper restriction enzyme, electrophoresis of digested DNA, blotting of digested DNA from gel to nylon membrane, hybridization and detection of the interest gene (Tumolo *et al.*, 1995; Sambrook and Russell, 2001; Erlandson and Theilmann, 2009).

One factor that could influence the result of southern blotting is quality and quantity of DNA. Good quality of DNA was important because the restriction enzymes needed to digest DNA completely without the interference of inhibitors. Besides, huge amount of intact DNA was needed for detection of transgene in the last step (Hey-Chi *et al.*, 1991; Hames, 1995; Tumolo *et al.*, 1995). As it is mentioned in the section the single intact DNA with standard quality was obtained from all of the samples while the quantity of DNA proved to be enough because of the obtained bands that observed in the only successful attempt. In the only successful attempt, 30µg of DNA was successfully digested that finally led to obtain a transgene band as it is shown in Figure 4.7.5. Although the higher concentration of DNA, 40µg and 50µg, was used for digestion step of southern blotting, the detection of transgene was failed in the last step. In this case, the DNA quality and quantity couldn’t be a reason for failed experiments.

Complete digestion of DNA is also very critical point of southern blotting process while improper digestion leads to failure in the blotting and hybridization steps. The electrophoresis of digested DNA can give the best evidence of the digestion quality where the undigested DNA sample is run in the same gel to compare with digested

DNA sample (Hames, 1995; Amiss and Prenell, 2006). The digestion step was optimized successfully, and only complete digested samples were preceded to the next step. Therefore, as only complete digestion of DNA samples were investigated by gel electrophoresis of them, the digestion couldn't be the reason of failure in the whole procedure of southern blotting. The Figure 4.7.3 is shown the complete digestion of DNA for one of the samples.

Electrophoresis of digested DNA was another important step that could be a reason of failure for the southern blotting experiments. On the one hand, short time of electrophoresis can impede the complete separation of digested fragments of DNA that leads to incomplete transfer of fragments from gel to nylon membrane in the blotting step. On the other hand, long periods of electrophoresis can lead to smaller fragments running off the gel (Snowdon and Langsdorf, 1998; Amiss and Prenell, 2006). The most time was recorded 8 hours when the digested fragments were still in the gel. After all, the electrophoresis of digested DNA was optimized, and it couldn't be the reason of failure.

The transfer of digested DNA to nylon membrane was also optimized successfully while the gel was checked under UV light to prove the lack of DNA fragments on it. Besides the observation of control positive band could be a good reason for successful blotting process (Sambrook and Russell, 2001).

The hybridization step was also optimized where the hybridization of control positive sample with probe was led to observation of band after detection in all of the experiments after control positive optimization (Hames, 1995; Sambrook and Russell, 2001). Besides, the only successful experiment could be an approval of optimized

hybridization process. Therefore, the probe was worked successfully where it was hybridized to the interest fragment of control positive genome. The hybridization temperature was proved to be proper for the attachment of the probe to the complementary fragment of the control positive genome and also for the attachment of probe to the complementary fragments of DNA sample in the only successful experiments. Although these evidence indicated the proper hybridization temperature, two different temperature, two degrees below and up of the previous applied temperature, were selected as new hybridization temperatures that led to failure even about control positive sample (Sambrook and Russell, 2001; Smith-Zagone *et al.*, 2007).

Detection step was optimized properly where all of the control positive samples gave appropriate band after detection step (Hames, 1995; Jungkind and Kessler, 2002). Therefore, the failure of experiments was not related to the detection step also.

Generally, these critical points were investigated during the whole procedure of southern blotting, but no logical reason was found to solve the unsuccessful attempts.

5.6 Pollen fertility tests for biosafety considerations

Pollen has a crucial role as a vector for the flow the genes in plants (Wang *et al.*, 2004). In this case, when transgenic plants are produced, the pollen must be investigated and also compare with that of control-untransformed plants. One of the methods that is usually applied for these investigations and comparison is pollen viability test, and it is to get an estimation of the ability of the pollen to carry out the function of delivering the sperm cell to the embryo during pollination (Bengtsson, 2006b).

The objective of the pollen viability tests was to investigate if there were any differences between control-untransformed plants and transgenic plants concerning pollen physiology, viability and pollen growth. As a matter of fact, it was crucial to ensure that the insertion of transgene into the genome of the plants had not influenced the pollen viability.

In the pollen viability tests, three transgenic plants from three different lines and three plants from one transgenic line were tested to get an indication of the viability between transgenic and control-untransformed plants. The plants A1.1, A2.4 and A3.7 were tested as transgenic plants while the plants A4.3, A4.6 and A4.8 were tested as control-untransformed plants.

5.6.1 Pollen staining for viability

In rice pollen, starch is preferentially accumulated and metabolized upon germination to provide energy and carbon skeleton for the growing pollen tube. Therefore, starch has been considered as an important indicator for the pollen viability (Tao *et al.*, 2007). The reaction between starch and iodine leads to formation of color that is the most useful and characteristics reactions of polysaccharides (Bailey and Whelan, 1961). Iodine-potassium iodide solution commonly applies to detect starch content of the pollen as an evidence of pollen fertility. After staining, fertile pollen grains are black stained and round while sterile pollen grains include unstained withered, unstained spherical and partially stained round pollen. The fertile and sterile pollen grains are shown in Figure 4.8.1. Pollen fertility was also calculated as ratio of fertile pollen to the total number of pollen grains.

The fertility percentage of transgenic and control-untransformed plants ranged from 80.64% to 90.16% while there was no meaningful difference in the level of starch staining between transgenic and control-untransformed plants. Therefore, pollen staining assay revealed that transgene had not influenced the pollen viability of transgenic plants although it gave an indirect estimation of pollen viability.

5.6.2 Pollen tube germination

As pollen staining test gives an indirect estimation of pollen viability, *in vitro* pollen germination experiment was necessary to examine pollen viability directly (Tao *et al.*, 2007). Pollen from both transgenic and control-untransformed plants lost their viability after anther dehiscence over time that as shown in appendix F. In detail, the pollen, transgenic and control-untransformed, showed the highest germination rate immediately after dehiscence and they lost completely their viability 60 to 75 minutes after anther dehiscence.

Although in some species pollen remains viable for several hours e.g. up to 34 to 38 hours in intact flower of *Fagopyrum esculentum Moench*, pollen lost their viability in less than an hour at room temperature (Adhikari and Campbell, 1998). The results of that study showed that experimentally genetically modified plants have the same reaction of wild plants in the nature. Similarly, in this thesis research, the pollen tube germination rate declined with the same slope for transgenic and control-untransformed plants (shown in Figure 4.8.2.3). This event indicates that the transgene has not affected the viability of pollen because there is no meaningful difference between transgenic and control-untransformed plants in the field of pollen viability.

CHAPTER 6

CONCLUSION

The main aims of this project were focused on two areas. The first was the determination of the segregation analysis in T1 plants, and the second was the assessment of pollen viability towards establishment of biosafety data for these plants. The segregation analysis of T1 plants was performed successfully.

The segregation analysis revealed that most of the transgenic lines showed 3:1 Mendel's segregation ratio while there was only one line that deviated from this ratio. Segregation analysis led to the conclusion that most of the lines had a single locus insertion of the transgene according to their 3:1 Mendel's segregation ratio, but Southern blotting was applied to determine copy number. Unfortunately, this method did not lead to any certain conclusion because no reproducible result was obtained for the experiment although for one replicate two bands were observed on the membrane, suggesting the presence of two copy number of transgene into the genome of the plant.

On the other hand, the presence of the transgene, within the genome of rice plants, could change the pollen viability. This study concluded that pollen viability did not vary with the presence of transgene, as the control untransformed plants and transgenic plants showed the same attributes from the pollen viability point of view.

This thesis has established the groundwork for further studies with transgenic *O. rufipogon*, an important wild rice variety in Malaysia.