STUDIES ON TRANSFORMATION OF THE THAI RICE, KDML105 BY *Agrobacterium tumefaciens*

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หัวข้อวิทยานิพนธ์	การศึกษาการถ่ายฝากยีนในข้าวขาวดอกมะลิ 105 โดยใช้ *Agrobacterium tumefaciens*
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บทคัดย่อ

จากการศึกษาการปรับปรุงความถี่ของการพัฒนาไปเป็นต้นพืช โดยผ่านขบวนการ somatic embryogenesis ในข้าวขาวดอกมะลิ 105 พบว่าทำได้โดย นำคัพภะมาเลี้ยงบนอาหาร NB ซึ่งเป็นอาหารที่เหมาะสมต่อการชักนำให้เกิดแคลลัส, ต่อ somatic embryogenesis, และต่อการพัฒนาไปเป็นต้นพืช โดยพบว่าประมาณ 73.45-82.22 เปอร์เซ็นต์ของแคลลัสที่เลี้ยงบนอาหารนี้ มีการพัฒนาไปเป็นต้นพืช ส่วนอาหารที่ใช้ในการชักนำให้แคลลัสพัฒนาไปเป็นต้นพืชที่ประกอบด้วย phytagel 6 กรัมต่อลิตร และ 7.5 กรัมต่อลิตร สามารถชักนำให้แคลลัสพัฒนาไปเป็นต้นพืชได้ดีที่สุด (82.22 เปอร์เซ็นต์ และ 86.67 เปอร์เซ็นต์ ตามลำดับ)

จากการศึกษาปัจจัยต่าง ๆ ที่มีผลต่อการถ่ายฝากยีนของข้าวขาวดอกมะลิ 105 โดยใช้ *Agrobacterium tumefaciens* พบว่าการเติม acetosyringone 50 ไมโครโมลาร์ ลงในอาหารที่ใช้เลี้ยงแคลลัสร่วมกับ *Agrobacterium tumefaciens* มีความสำคัญต่อการเพิ่มความถี่ของการถ่ายฝากยีนแบบชั่วคราว สำหรับ embryogenic callus ที่เจริญมาจาก scutellum ซึ่งมีอายุประมาณ 6-8 สัปดาห์ เป็นชิ้นส่วนที่เหมาะสมที่สุดที่ใช้ในการถ่ายฝากยีน และอาหาร NB ที่ประกอบด้วย hygromycin 40 มิลลิกรัมต่อลิตร เป็นอาหารที่เหมาะสมต่อการคัดเลือกแคลลัสที่มีการถ่ายฝากยีน เมื่อทำการทดลองถ่ายฝากยีนเข้าสู่แคลลัสโดยใช้ *Agrobacterium tumefaciens* ที่มีพลาสมิดซึ่งประกอบด้วยยีนต่าง ๆ ที่กำหนดการสร้างเอนไซม์ β-D-glucuronidase, โปรตีน green fluorescent, และเอนไซม์ hygromycin phosphotransferase โดยทำตามสภาวะที่อธิบายไว้ข้างต้น พบว่าประมาณ 15.00-18.37 เปอร์เซ็นต์ของแคลลัสที่ใช้ในการทดลอง มีการพัฒนาไปเป็นต้นพืชที่มีการถ่ายฝากยีน

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ABSTRACT

A simple method for improving the frequency of plant regeneration in KDML105 through somatic embryogenesis has been established. NB medium was an optimized medium for promoting callus induction, embryogenesis and plant regeneration. The frequencies of regeneration between 73.45 and 82.22% have been obtained from calli cultured on this medium. The high regeneration frequencies, 82.22% and 86.67%, were obtained from calli cultured on regeneration medium containing 6 g/l and 7.5 g/l phytagel, respectively.

Various factors were studied in order to establish a procedure for the transformation of KDML105 by *Agrobacterium tumefaciens*. Supplementation of 50 µM acetosyringone to the medium during cocultivation was important for enhancing the frequency of transient transformation. The 6-8 weeks-old scutellum-derived embryogenic calli were excellent starting materials. Selection medium based on NB medium supplemented with 40 mg/l hygromycin was an optimized medium for selection of transformed rice cells. When calli were infected with *Agrobacterium tumefaciens* carrying a plasmid containing genes for β-D-glucuronidase, green fluorescent protein and hygromycin phosphotransferase and using the conditions described above, the percentages of transformation between 15.00 and 18.37 were obtained.

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CHAPTER 1

INTRODUCTION

Rice is the staple food and the principle crop in humid and subhumid Asia. The monsoon climate and the high temperature during periods of heavy rainfall favor rice cultivation in this region. From the Philippines in the east to eastern India in the west and from southern China in the north to Indonesia in the south, rice accounts for 30-50% of agricultural incomes and provides 50-80% of the calories consumed. Because of its importance in providing national food security and generating employment and incomes for the low-income people in society, rice is regarded as a strategic commodity and an important component of culture in many Asian countries. More than 90% of all rice is produced and consumed in Asia.

Since 1966, the Asian population has increased by 85% but rice production only has doubled. The race between rice production and population growth is far from over. Asian population is still growing at 1.7% per year, and is projected to increase by another 85% before stabilizing by the end of the 21st century. Rice supplies must increase at the rate at which population grows. It is projected that during the 1990-2025 period, the demand for rice may increase by another 70%, which will require an increase in production from 480 million tons of unmilled rice at present to 810 million tons by 2025 (International Rice Research Institute, 1995; Rosegrant *et al.*, 1995). And this addition of rice will come from reducing the present 40% harvest losses attributed to biotic and abiotic stresses, and have to be grown with considerably fewer inputs of agrochemical and under sustainable conditions (International Rice Research Institute, 1992). This immense task will require that tradition rice breeding and production be supported by every possible contribution from science, especially genetic engineering by adding alien genes to the rice gene pool.

Genetic engineering also allows the re-introduction of rice genes that have been extracted and modified to give altered properties. Such gene transfers are impossible with conventional breeding methods. Moreover, genetic engineering allows the introduction of one or two well-characterized genes at a time. There is no need for the extensive backcrossing done in conventional hybridization to remove undesirable genes.

Several methods are now available to transfer genes into rice. So far, the protoplast system, including biolistic and *Agrobacterium*-mediated gene transfers are noteworthy. All these three methods have produced fertile transgenic rice. Protoplasts are plant cells freed of their cell wall by enzymatic digestion. Protoplasts can uptake foreign DNA after treatment with polyethyleneglycol, a neutral polymer, or by application of an electric current in a process known as electroporation. In the biolistic method, also known as particle bombardment, DNA associated with tiny gold particles is shot into immature embryos or embryogenic calli with a burst of high pressure. *Agrobacterium*-mediated transformation makes use of a species of plant parasitic bacteria, *Agrobacterium tumefaciens*, which harbors a Ti plasmid that is capable of inserting its T-DNA into plant chromosomes. However, transformation methods based on the use of *Agrobacterium tumefaciens* are still preferred in many instances. The advantages of *Agrobacterium*-mediated gene transfer over other methods are the high efficiency of transformation, the transfer of pieces of DNA with defined ends, the transfer of relatively large segments of DNA, the more predictable pattern of foreign DNA integration than other transformation techniques, and the absence of a requirement for protoplast-culture techniques (Chan *et al.*, 1993; Hiei *et al.*, 1994; Rashid *et al.*, 1996).

In recent years, the successful transformation by *Agrobacterium* with subsequent integration into DNA has been restricted to japonica rice (Chan *et al.*, 1993; Hiei *et al.*, 1994). Hiei *et al.* (1994) obtained transformation frequency of japonica rice as high as that of dicotyledonous plants. The genetic transformation of indica rice employing *Agrobacterium* has been restricted to a few reports without an established transformation procedure and only transgenic calli with a low frequency have been obtained (Li *et al.*, 1992). Recently, Rashid *et al.* (1996) obtained transformation frequency of indica rice as high as reported in japonica cultivars (Hiei *et al.*, 1994).

There are about 3,500 varieties including wild and indigenous rice in Thailand. Many indigenous varieties are considered to be high-quality and promoted by the government, particularly white jasmine rice or Khao Dawk Mali 105 (KDML105), the most popular of all among consumers. KDML105 has a very good grain quality, pleasant aroma, and tolerance to stresses such as drought, salty soil, and acid soil. KDML105 is also photoperiod sensitive and susceptible to many insect pests such as rice brown planthopper, rice green leafhopper, rice gall midge, and diseases such as blast, brown spot, bacteria leaf blight, yellow orange leaf virus, and ragged stunt virus. Improvement of KDML105 resistant to insect pests and diseases through the use of genetic engineering instead of the use of traditional breeding is an attractive alternative.

The objectives of the present study were to improve the frequency of plant regeneration in KDML105 through somatic embryogenesis, to establish a procedure for KDML105 transformation by *Agrobacterium*, and to investigate the use of the β-D-glucuronidase gene and the green fluorescent protein gene to optimize the transformation protocol.

CHAPTER 2

LITERATURE REVIEWS

Rice has been cultivated for more than 7,000 years as a major crop and currently supports more than 50% of the world population. Cultivated rice consists of two species, *Oryza sativa* and *Oryza glaberrima*. *Oryza sativa* is diploid (2n = 24), annual (potentially perennial), short-day, with a determinate inflorescence and is normally self-fertilized. There are two major subspecies of *Oryza sativa* grown in different regions of the world, *japonica* and *indica*. The *japonica* varieties are planted in temperate areas such as China, Japan, the Republic of Korea, and also to a small extent in the United States. The *indica* varieties are widely grown in tropical areas such as China, Vietnam, the Philippines, Thailand, Indonesia, Sri Lanka, as well as in southern parts of the United States.

2.1 Somatic embryogenesis in plants

Cell totipotency is the development of a whole organism starting from a single asexual cell via embryogenesis. Somatic tissues of a plant represent products of mitotic divisions and each cell possessed the built-in capacity to regenerate the phenotype of the whole organism from which it is derived through embryogenesis when grown under appropriate culture conditions. The phenomenon is designated 'somatic embryogenesis' which occurs both in diploid and haploid cells without the intervention of a sexual fusion. Embryogenesis in both somatic and reproductive cells corresponds at least in early stages to cell divisions characteristic of zygotic development, though with differing chromosome numbers and occurs in the absence of an auxin. The adventive embryos are bipolar in organization with an integrated root-shoot axis arising in culture, unlike the development of shoots or roots (organogenesis) which are monopolar, originating independently and later integrating into one axis. The morphogenetic events of the somatic cell showed a striking resemblance to developmental sequences occurring in the fertilized egg cell, acquiring early bipolarity passing through proembryonal, globular, heart- and torpedo-stages. The cell totipotency concept implies that somatic cells retain their functional nature freed from neighboring tissues and behave as so many zygotes and the DNA is conserved during differentiation processes (Narayanaswamy, 1994).

2.2 Factors influencing the plant regeneration frequency in rice through somatic embryogenesis

Strategies to improve plant regeneration frequencies in cereals, including rice, have been steadily evolving during the last decade (Grimes and Hodges, 1990; Peterson and Smith, 1991; Rance *et al.*, 1994; Jain *et al.*, 1996; Sivamani *et al.*, 1996). While it has been possible to obtain high plant regeneration frequencies in *japonica* rice varieties, the success for reproducible fertile plant regeneration has limited in *indica* rice varieties.

There are various factors affecting plant regeneration frequency in rice. Genotype, callus size, hormonal composition of the medium, partial desiccation or water stress, and other medium supplements are shown to be important.

Grimes and Hodges (1990) investigated whether the inorganic NO_3^- : NH_4^+ ratio can influence *indica* rice plant regeneration from primary callus and the response of these calli to an exogenous auxin source, 2,4-D. Various NO_3^- : NH_4^+ ratios were tested in order to find out whether it was related to the NO_3^- : NH_4^+ ratios commonly used for rice cell culture. A broad range of different NO_3^- : NH_4^+ ratios (50 : 50 to 80 : 20) supported plant regeneration equally well. The morphology of the regenerated plants was strongly influenced by the nitrogen ratio. However, lowering the NO_3^- : NH_4^+ ratio from 80 : 20 (the original basal medium composition) to 75 : 25 resulted in a three-fold increase in plant height and root growth. As the NO_3^- : NH_4^+ ratio was lowered even further, the shoots became shorter and thicker while the roots became more truncated and formed fewer secondary roots. Addition of tryptophan (250 μM) inhibited regeneration at all ratios of NO_3^- : NH_4^+. The addition of casein hydrolysate significantly affected the optimal NO_3^- : NH_4^+ ratio for regeneration. When casein hydrolysate was present in the medium, optimal regeneration was achieved using a NO_3^- : NH_4^+ ratio of 50 : 50. These plants appeared quite normal, with none of the abnormal morphologies observed on plants regenerated at the ratio of NO_3^- : NH_4^+ of 50 : 50 but without casein hydrolysate. The NO_3^- : NH_4^+ ratio also affected the response of these calli to different concentrations of 2,4-D. At a NO_3^- : NH_4^+ ratio of 80 : 20, there was a strong dose-response curve to 2,4-D while at a NO_3^- : NH_4^+ ratio of 50 : 50, the dose-response curve was much flatter.

Peterson and Smith (1991) described the effect of various hormones and callus sizes on plant regeneration of American and international rice cultivars. Ten 10 mg-callus pieces on regeneration medium resulted in a 2- to 10-fold increase in plant over than those of single 100 mg pieces. Plant regeneration of Texas rice cultivars and Taipei 309 was enhanced by the use of ABA in the subculture medium with a 2-fold and a 3- to 10-fold increase in plant regeneration with 2.6 mg/l and 26 mg/l ABA in the subculture media, respectively. Regeneration of plants from callus of IR36 and IR64 was not enhanced by ABA but by the use of BAP and Trp in the subculture medium or by 2,4-D alone. The subculture medium containing BAP and Trp produced a 5-fold increase in plant regeneration rate in IR64 callus and was equal to subculture medium containing only 2,4-D for IR36 callus.

Rance *et al.* (1994) described a simple method to enhance the regeneration frequency from mature embryo derived calli and subcultured calli of three *indica* rice breeding lines: TN1, IR72 and IR64. Partial desiccation of *indica* rice calli dramatically promotes organogenesis and leads to high regeneration ability. Partial desiccation increased the regeneration of *indica* rice 2-4 folds. The better regeneration frequencies were obtained for desiccation treatments for 40%, this desiccation rate corresponded to 21-23 hours of treatment. By this method, Rance *et al.* were able to transfer the first group of plantlets from Magenta boxes to soil at day 48-50. Short period of time *in vitro* culture leads to very few changes in the phenotypic and fertility characteristics of the regenerated plants.

Jain *et al.* (1996) reported that the frequency of regeneration of fertile plants from cell suspensions was significantly increased using water stress treatments. Jain *et al.* studied the effects of partial desiccation, mannitol, and increasing agarose concentrations on plant regeneration from cell suspension-derived calli of three *indica* rice varieties (IR43, Pusa Basmati 1, and Basmati 385). The addition of mannitol to the regeneration medium reduced the callus growth and water content, making the tissue more compact and embryogenic. These calli, at lower mannitol concentrations (0.1 M, 0.2 M) formed shoots with greater frequencies compared to the calli without mannitol treatment. Mannitol, at 0.4 M concentration completely inhibited shoot regeneration but promoted embryogenesis. These calli regenerated shoots with greater frequencies when transferred to mannitol-free medium. Partial desiccation also increased shoot regeneration of *indica* rice varieties up to 3 folds. The best shoot regeneration frequency, 63%, was obtained after 24 h desiccation in Pusa Basmati 1 calli. When the agarose concentration of the regeneration medium was increased from 0.5% to 1%, shoot regeneration frequencies increased significantly in all the rice varieties tested.

Sivamani *et al.* (1996) reported the successful development of a procedure for selectively propagating large quantities of highly embryogenic calli derived from mature *indica* rice (TN1) seeds. Dehusked and sterilized TN1 seeds were placed on medium for 6 days for inducing primary calli, following by subculturing on the same fresh medium three times at 2-week intervals. At this point, many loosely attached small globular calli appeared on top of each compact primary callus, which were gently removed with a pair of forceps. The small calli of 1-3 mm in diameter were transferred to plates containing fresh medium. After 2 weeks, a cluster of small globular calli had emerged on most calli. Regeneration tests with these calli at this stage showed that 60-70% of the calli were able to regenerate and produce highly fertile plants. Sivamani *et al.* recommended that this procedure can be used as a starting point for most *indica* rice varieties. For callus induction and propagation, one should vary the concentrations or types of cytokinins in the callus induction medium. Likewise, regeneration conditions should also be adjusted. The calli usually regenerate better if they are grown on an optimized callus propagation medium containing proper concentration of certain type(s) of cytokinin before being transferred to regeneration medium.

2.3 *Agrobacterium tumefaciens*

Agrobacterium tumefaciens (*Agrobacterium*) is a soil phytopathogen that genetically transforms host cells, causing crown gall tumors, an agronomically important disease that affects most dicotyledonous plants. In nature, these tumors are formed at the soil-air junction, the so-called crown of the plant. The *Agrobacterium*-plant cell interaction is the only known natural example of DNA transport between kingdoms. In this process, DNA is transported from wild-type *Agrobacterium* into the plant cell nucleus. Expression of this transferred DNA (T-DNA) results in neoplastic growths (tumors) on the host plant. The wild-type T-DNA carries genes involved in the synthesis of plant growth hormones and the production of opines, tumor-specific compounds formed by the condensation of an amino acid with a keto acid or a sugar. It is the production of growth hormones in the transformed host cells that includes the formation of tumors. These tumors then synthesize opines, a major carbon and nitrogen source for *Agrobacterium*. Agrobacteria are usually classified based on the type of opines specified by the bacterial T-DNA, the most common strains being octopine or nopaline specific. Opine imports into the bacterial cell and the subsequent catabolism within the bacterial cell requires specialized enzymes. Because these enzymes are encoded by the *Agrobacterium* tumor-inducing (Ti) plasmid, practically no other soil microorganism can metabolize opines, creating a favorable biological niche for *Agrobacterium*.

Three genetic components of *Agrobacterium* are required for plant cell transformation (Figure 2.1). The first component is the T-DNA, which is actually transported from the bacterium to the plant cell. The T-DNA is a discrete segment of DNA located on the 200-kb Ti plasmid of *Agrobacterium*; it is delineated by 25-bp imperfect direct repeats known as the T-DNA borders. The second component is the 35-kb *virulence* (*vir*) region, also located on the Ti plasmid, which is composed of seven major loci (*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, and *virH*). The protein products of these genes, termed virulence (Vir) proteins, respond to the specific compounds secreted by the wounded plant to generate a copy of the T-DNA and mediate its transfer into the host cell. The third component is the suite of *chromosomal virulence* (*chv*) genes, located on the *Agrobacterium* chromosome. The *chv* genes are involved in bacterial chemotaxis toward and attachment to the wounded plant cell.

Because the T-DNA element is defined by its borders, the coding region of the wild-type T-DNA can be replaced by any DNA sequence without any effect on its transfer from *Agrobacterium* to the plant. Thus, *Agrobacterium* is often used to produce transgenic plants expressing genes of interest (Sheng and Citovsky, 1996).

2.4 Cellular processes involved in *Agrobacterium*-plant interactions

Agrobacterium-plant cell interaction was divided into seven steps, each of which represents a fundamental aspect of prokaryotic as well as eukaryotic cellular biology (Figure 2.1 and Table 2.1). Steps including *Agrobacterium*-host cell recognition, transduction of plant signals, activation of *vir* genes, and production of a transferable copy of the T-DNA are characteristics of bacterial conjugation. Transport of the T-DNA from the bacteria into the host plant cell may combine the hallmarks of both bacterial conjugation and viral infection, whereas T-DNA nuclear transport and integration probably occur by typical eukaryotic mechanisms.

2.4.1 Cell-cell recognition

Cell-cell contact is required for the onset of many intercellular interactions, including host-pathogen recognition. Attachment of *Agrobacterium* to host plant cells is a prerequisite for DNA transfer. *Agrobacterium*-host cell recognition is a two-step process. Firstly, the bacteria loosely bind to the host cell surface, and secondly, the bound bacteria synthesize cellulose filaments that stabilize the initial binding, resulting in a tight association between *Agrobacterium* and the host cell.

Because each plant cell binds a finite number of bacteria, saturable surface receptors are thought to be involved. In animals, vitronectin, an important component of the extracellular matrix, is reported to function as a receptor for several bacterial strains. Vitronectin-like molecules, which have been found on the cell surface of many plant species, may mediate *Agrobacterium*-plant cell binding. Human vitronectin as well as anti-vitronectin antibodies block attachment of *Agrobacterium* to cultured plant cells. Further, *Agrobacterium* strains that are unable to bind plant cells due to mutations in their chromosomal *chvB*, *pscA*, or *att* loci also show reduced binding to vitronectin. Potentially, a plant vitromectin-like protein (PVN) may represent one of the receptors responsible for the specific interaction between *Agrobacterium* and plant cells (Figure 2.1, step 1). In addition to PVNs, other plant cell surface proteins and carbohydrates are likely to be involved in the interaction with *Agrobacterium*. However, the identity of these molecules remains to be determined.

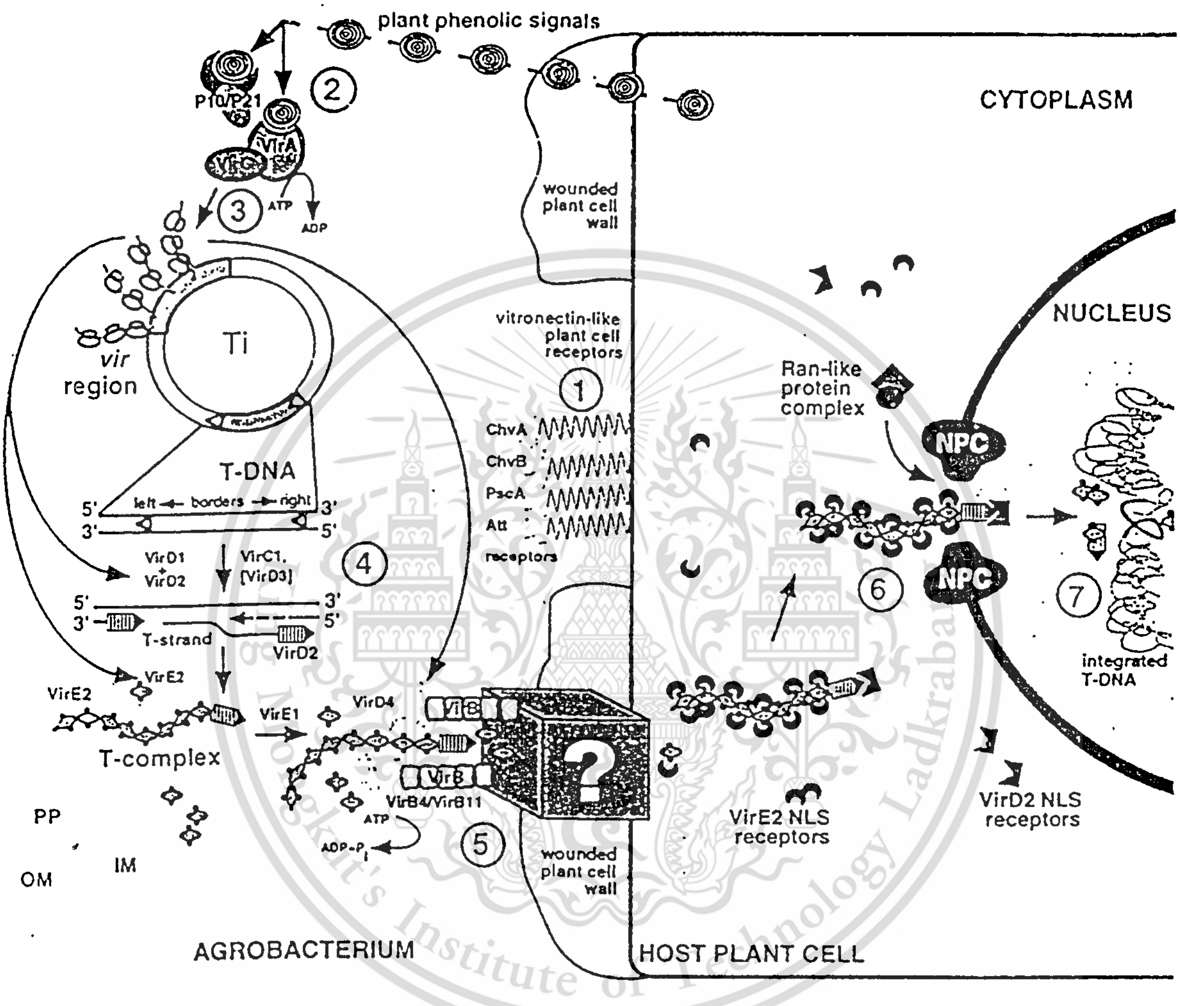

Figure 2.1 *Agrobacterium*-Plant Cell Interactions. (Sheng and Citovsky, 1996) This diagram summarizes all major cellular reactions involved in T-DNA transport. Step 1 through 7 indicate sequential processes that occur during *Agrobacterium* infection. Step 1, binding of *Agrobacterium* to the host cell surface receptors; step 2, recognition of plant signal molecules by the bacterial VirA/VirG sensor-transducer system; step 3, activation of the bacterial *vir* genes; step 4, production of the transferable T-strand; step 5, formation of the T-complex and its transport into the host plant cell; step 6, nuclear import of the T-complex; and step 7, T-DNA integration. IM, bacterial inner membrane; NPC, nuclear pore complex; OM, bacterial outer membrane; PP, bacterial periplasm.

Table 2.1 Summary of cellular processes involved in *Agrobacterium*-plant interactions. (Sheng and Citovsky, 1996)

Cellular process	Specific step in *Agrobacterium*-plant cell interaction	*Agrobacterium* proteins involved in the process
Cell-cell recognition	Binding of *Agrobacterium* to the host cell surface receptors	ChvA, ChvB, PscA, Att
Signal transduction	Recognition of plant signal molecules and activation of the T-DNA transport pathway	ChvE, VirA, VirG, [p21, p10][a]
Transcriptional activation	Expression of vir genes after phosphorylation of the transcriptional activator	VirG
Conjugal DNA metabolism	Nicking at the T-DNA borders and mobilization of the transferable single-stranded copy of the T-DNA (T-strand)	VirD1, VirD2, VirC1, [VirD3]
Intercellular transport[b]	Formation of protein-DNA T-complex; formation of a transmembrane channel; export of the T-complex into the cytoplasm of the host plant cell	VirE2, VirE1, VirD2, VirD4, VirB4, VirB7, VirB9, VirB10, VirB11, [VirB1, other VirB proteins]
Nuclear import	Interaction with the host cell NLS receptors and transport of the T-complex through the nuclear pore	VirD2, VirE2
T-DNA integration	Integration into the plant cell genome; synthesis of the second strand of the T-DNA	[VirD2, VirE2]

[a] The proteins with a proposed but not proven function are shown in brackets.

[b] VirB proteins are involved in the formation of a transmembrane channel and possibly in export of the T-complex. VirE and VirD proteins are involved in the formation of the T-complex and probably its transport through the VirB channel.

2.4.2 Signal transduction and transcriptional activation of *vir* genes

To regulate infection of plants, *Agrobacterium* has evolved a two component signal transduction system composed of the virulent proteins VirA and VirG. Together, these proteins sense signal molecules secreted by wounded plant cells and activate the expression of other *vir* genes, thereby initiating the process of T-DNA transport.

2.4.2.1 Plant signals

Wounded plants secrete sap with a characteristic acidic pH (5.0 to 5.8) and a high content of various phenolic compounds, such as lignin and flavonoid precursors. These conditions specifically stimulate *Agrobacterium vir* gene expression. The best characterized and most effective *vir* gene inducers are monocyclic phenolics such as acetosyringone (AS). These molecules are not detected, or are detected at low levels, in uninjured plants, but their amounts significantly increase in wounded plant cells. The specific composition of phenolic compounds in plant exudates is thought to underlie the host specificity shown by many *Agrobacterium* strains. Interestingly, many other plant-microbe interactions are initiated by specific phenolic compounds in host plant exudates.

When only small amounts of phenolics are secreted from the damaged plant cell, the release of sugars may help to activate the major phenolic-mediated wound signaling pathway. Monosaccharides such as glucose and galactose significantly increase *vir* gene expression only when AS is limited or absent. This enhancement does not result from nutritional benefits to *Agrobacterium*, because nonmetabolizable sugars also increase *vir* gene induction. Conversely, low opine levels further enhance *vir* gene expression in the presence of AS.

As a general rule, conditions that promote *vir* gene induction are poor for bacterial growth, whereas conditions that support vegetative bacterial growth are unsuitable for *vir* gene induction. Therefore, it has been proposed that in the plant wound environment containing low opine concentrations, *Agrobacterium* stays in an optimal state for *vir* gene induction and T-DNA transfer. The high opine concentrations produced in mature tumors restore *Agrobacterium* to vegetative growth and allow the opines to be used as carbon and nitrogen sources.

Although phenolic inducer molecules are required for the initiation of T-DNA transport, most of these compounds are bacteriostatic at high concentrations. A plant-inducible locus in the *vir* region, termed *virH* or *pinF*, may be involved in the detoxification of the harmful phenolics secreted by the wounded plant.

2.4.2.2 *vir* gene induction

Signal molecules released by the wounded plant cell are recognized by the VirA/VirG two-component regulatory system. It is not clear whether the phenolic signals are sensed directly by the sensor component. VirA, or by another receptor protein that then interacts with VirA (Figure 2.1, step 2). Some data suggest that plant phenolics initially interact with the chromosomally encoded proteins P10 and P21. However, more recent genetic data indicates that VirA senses plant signals directly. Direct interaction between VirA and AS, however, requires that AS diffuse through the outer membrane of *Agrobacterium* to reach VirA, which associated with the bacterial inner membrane and lacks extracellular domains. Unlike phenolics, sugar enhancers have been shown conclusively to interact with chromosomally encoded glucose/galactose binding protein, ChvE, which in turn interacts with VirA.

VirA functions as a protein kinase and phosphotransferase. In the presence of plant signals, VirA autophosphorylates at its His-474 residue. The phosphohistidine high energy phosphate bond is then transferred to the Asp-52 or possibly the Asp-8 residue in VirG. Phosphorylated VirG is very stable. The stability of VirG is thought to facilitate maximal levels of *vir* gene induction.

To activate the expression of the other *vir* genes, VirG interacts with the *vir* box, a conserved 12-bp sequence (TNCAATTGAAAPy) in the promoter regions of highly inducible *vir* genes. The incomplete dyad symmetry of the *vir* box suggests that VirG may bind as a dimer. *In vivo*, VirG phosphorylation is required for the activation of *vir* gene expression, although the actual role of phosphorylation has not yet been determined (Figure 2.1, step 3). After VirG binds to the *vir* box-containing promoter, phosphorylation may allow interactions with other proteins, such as RNA polymerase. In addition, although unphosphorylated VirG can specifically bind *vir* promoters, phosphorylation of VirG may increase its DNA binding affinity. It is still unclear whether VirG phosphorylation occurs before or after its binding to the *vir* box.

2.4.3 Conjugal DNA metabolism

2.4.3.1 The T-DNA element

Induction of *vir* gene expression ultimately results in the production of a T-DNA copy that is capable of genetically transforming plant cells. Different types of Ti plasmids carry T-DNA elements of different composition. For example, the T-DNA in the nopaline Ti plasmid is a contiguous stretch of 22 kb (Figure 2.1, step 4), whereas the octopine-specific T-DNA is composed of three independently transported adjacent T-DNAs: left (13 kb), central (1.5 kb), and right (7.8 kb). The borders of a T-DNA element are defined as conserved 25-bp sequences that delimit the transferred segments. Genetic studies using deletion mutants show that the right border is absolutely required for *Agrobacterium* pathogenicity, whereas the left border is not. Furthermore, inversion of the right border leads to reduced virulence and transfer of nearly the entired Ti plasmid instead of the T-DNA region. These results suggest that transfer of the T-DNA is polar from right to left, as determined by the orientation of the T-DNA border repeats.

Interestingly, the T-DNA itself has no effect on the efficiency of transfer. Consequently, nononcogenic ("disarmed") Ti plasmids, with most of the internal sequences of the T-DNA replaced by the DNA of interest, are widely used as vectors for genetic transformation of plants.

2.4.3.2 Production of the transferable T-strand

vir-induced *Agrobacterium* cells generate a linear single-stranded copy of the T-DNA region, designated the T-strand. The T-strand is found at approximately one copy per induced *Agrobacterium* cell and is derived from the coding strand of the T-DNA element. T-strand production is thought to occur in a 5' to 3' direction, initiating at the right T-DNA border and terminating at the left border (Figure 2.1, step 4). VirD1 and VirD2 proteins are thought to function together as an endonuclease that carries out site- and strand-specific nicks between the third and fourth base pair of the bottom strand of the T-DNA borders (Figure 2.1, step 4). *In vitro* studies demonstrate that purified VirD1 and VirD2 indeed act as a site-specific endonuclease on a supercoiled plasmid containing a 25-bp border repeat. Following cleavage, VirD2 covalently attaches to the 5' end of the T-strand at the right border nick and to the 5' end of the remaining bottom strand of the Ti plasmid at the left border nick (Figure 2.1, step 4). The excised T-strand is removed, and the resulting single-stranded gap is repaired, most likely by replacement of DNA strand synthesis. The replacement reaction presumably removes the VirD2 molecule attached to the 5' end of the left border, restoring the circular DNA molecule of the Ti plasmid. Recent data indicates that VirD2 also may participate in ligating the left border nick. Finally, another virulence protein, VirC1, can enhance T-strand production from the octopine Ti plasmid when VirD1 and VirD2 are limiting. Because so few plant-induced *Agrobacterium* proteins are necessary for T-strand production, bacterial housekeeping enzymes of DNA repair and metabolism, such as helicases, also may be involved in this process.

2.4.3.3 Structural model of the T-DNA transfer intermediate, the T-complex

It is thought that the T-strand is transferred out of the bacterium and into the plant cell as a protein-nucleic acid complex. This T-DNA transport intermediate, designated the T-complex, is composed of at least three components (Figure 2.1, step 5). The T-strand DNA molecule, which carries the genetic information, and its cognate VirD2 and VirE2 proteins, which protect the T-strand, shape it into a transferable (thin and unfolded) form and supply specific targeting signals. As mentioned previously, one molecule of VirD2 is covalently attached to each T-strand. Although both VirD1 and VirD2 are involved in border specific nicking, there is no experimental evidence that VirD1 remains bound to the generated T-strand. Also associated with the T-strand is VirE2, an ssDNA binding protein (SSB). Binding of VirE2 to ssDNA *in vitro* is strong and cooperative, leading to the formation of very stable unfolded VirE2-ssDNA complexes that are largely inaccessible to external nucleolytic activity. Based on electron microscopy data and *in vitro* VirE2-ssDNA binding kinetics, the nopaline-specific T-complex is proposed to be 3600 nm long and 2 nm wide. As such, it would contain 600 molecules of VirE2 and one molecule of VirD2 and have a predicted molecule mass of 50,000 kD.

This structural and functional model of the T-complex implies that both VirD2 and VirE2 proteins are transported into the recipient plant cell together with the T-strand (Figure 2.1, step 5). This idea makes biological sense. It is likely that an SSB with as high an affinity for ssDNA as VirE2 will form a complex with the T-strand already inside *Agrobacterium*, especially if both VirE2 and the T-strand are transported into plant cell through the same channel. Formation of T-complexes is also supported by the observation that in extracts from *vir*-induced *Agrobacterium*, T-strands and VirE2 are coimmunoprecipitated by anti-VirE2 antibodies. However, coinoculation of the same plant with *Agrobacterium* carrying a T-DNA but lacking VirE2 and a strain producing VirE2 but lacking T-DNA resulted in a productive infection by these separately nonpathogenic bacteria. There are two interpretations of this intercellular complementation. Firstly, VirE2 and T-strands can be transported independently from the *Agrobacterium* into the plant cell, and secondly, VirE2 functions primarily inside the plant cell. The latter conclusion is supported by the observation that *virE2* expression in transgenic plants restores infectivity of a VirE2 deficient *Agrobacterium*.

2.4.4 Intercellular transport

Intercellular DNA transport requires a direct passageway between donor and recipient cells. Thus, *Agrobacterium* is predicted to form a channel through which T-complexes are transferred into the cytoplasm of the host plant cell. The molecular mechanism by which this passageway is formed and functions is still a biological black box (Figure 2.1, step 5). However, it seems likely that the *Agrobacterium*-plant cell channel is encoded by the *virB* locus, most of which is required for bacterial virulence but not for T-strand production.

The *virB* operon contains 11 open reading frames, nine of which encode proteins shown to associate with bacterial membranes. Recent molecular studies have focused on several individual VirB proteins. Specifically, VirB9 and VirB10 were each shown to form separate membrane-associated high molecular weight complexes. Although not in the same complex with VirB10, VirB9 was absolutely required for VirB10 complex formation, possibly serving to stabilize this protein or facilitate its insertion into the bacterial membrane. VirB9, in turn, was stabilized by VirB7 after the formation of disulfide cross-linked VirB9-VirB7 heterodimers. In addition to its dependence on VirB7, VirB9 accumulation at least partly required the production of VirB8. Thus, the accumulation of some *virB* gene products depends on the presence of other VirB proteins. These data suggest that coordinate protein synthesis stabilizes individual VirB polypeptides, perhaps allowing them to form a multiprotein channel structure (Figure 1, step 5).

Agrobacterium-plant cell transport of T-complexes through the VirB channel is most likely an energy-dependent process. Two VirB proteins, VirB4 and VirB11, are the better candidates to provide energy for this translocation. VirB4 has a nucleotide binding site, whereas VirB11 is both an ATPase and a protein kinase, and both proteins localize to the inner bacterial membrane. Furthermore, a recent study indicates that in AS-induced *Agrobacterium*, VirB1 is processed to a lower molecular weight that partially exported into the surrounding medium. It is possible that the secreted form of VirB1 interacts with the recipient plant cell, where it may mediate the docking of the VirB channel at the infection site on the cell surface.

2.4.5 Nuclear import

2.4.5.1 VirD2 and VirE2 probably mediate nuclear import of the T-complex

Unlike bacterial conjugation, the recipient cell in *Agrobacterium* T-DNA transport is an eukaryote. Therefore, although the generation of a transferable T-strand molecule may have parallels with bacterial conjugation, the delivery of the T-complex into the target cell does not. Instead, the later steps of T-DNA transfer, penetration into the host cell nucleus (Figure 2.1, step 6) and integration into the nuclear genome (Figure 2.1, step 7), are more related to virus infection. The T-complex thus resembles a viral (or subviral) particle that is capable of plant cell transformation.

Because pathogenic microorganisms often use existing cellular machinery for their own needs, *Agrobacterium* probably employs an endogenous cellular pathway for transport of the invading T-complex into the plant cell nucleus. Consequently, several recent studies have used the *Agrobacterium* T-complex as a model system to examine the general process of nuclear import of nucleic acids in plant cells. The estimated size of the T-complex (50,000 kD) far exceeds the size-exclusion limit of the nuclear pore (60 kD), suggesting a requirement for active transport processes. Because the T-strand presumably does not itself carry targeting signals, T-complex nuclear import is most likely mediated by the VirD2 and VirE2 proteins (Figure 2.1, step 6).

As a rule, active nuclear import of proteins requires a specific nuclear localization signal (NLS). The most common type of NLS is the bipartite signal. The first active domain of a bipartite NLS consists of two adjacent basic residues and is followed by a variable-length linker and the second active domain, which contains at least three out of the five basic amino acids. The functional VirD2 NLS was found to reside in the C terminus of the protein and to conform to the bipartite consensus motif. The biological relevance of this NLS was confirmed by the observations that *Agrobacterium* T-DNA expression and tumorigenicity are reduced in NLS deletion mutants of VirD2. Collectively, these results suggest that the VirD2 protein, which is attached to the 5' end of the T-strand, acts in directing the T-complex to the host cell nucleus.

2.4.5.2 VirD2 and VirE2 NLSs represent two functional types of plant nuclear targeting signals

In *Agrobacterium* harboring the nopaline Ti plasmid, the 22-kb T-DNA region is predicted to produce a T-strand associated with one molecule of VirD2 and 600 molecules of VirE2, assuming the average VirE2 binding capacity of 36 nucleotides per protein monomer. Because each VirE2 molecule carries two NLSs, whereas VirD2 contains one functional NLS, the nopaline-specific T-complex is predicted to have a

VirD2 NLS:VirE2 NLS ratio of 1:1200. Why would *Agrobacterium* need an NLS on VirD2? One possibility is that VirD2 and VirE2 are imported into the plant cell nucleus by functionally different targeting sequences and that both signals are required for optimal nuclear import of the T-complex.

2.4.5.3 Implications for nuclear import of T-DNA and nucleic acids in general

The functional variations in the NLS sequence may reflect cellular regulation of nuclear import of proteins and/or protein-nucleic acid complexes. For example, the nuclear import of the *Agrobacterium* T-complex may occur in a polar and linear fashion potentially important for the subsequent integration of the T-strand into the plant cell genome. The T-complex model proposes that the 5' end of the T-strand is associated with the VirD2 molecule, whereas the 3' end probably has a VirE2 molecule attached in its proximity; the functional variation between the NLS signals of these proteins may specify the ends of the T-strand and determine the polarity of its transport and integration (Figure 2.1, step 6).

2.4.6 T-DNA integration

Nuclear import of the *Agrobacterium* T-complex culminates with the integration of the transports T-strand into the host plant cell chromosome (Figure 2.1, step 7). The molecular mechanisms by which this is achieved are largely unknown. Unlike other mobile DNA elements such as transposons and retroviruses, T-DNA does not encode enzymatic activities enabling integration. Thus, T-DNA insertion into the plant genome must be mediated by proteins transported from the infecting bacterium and/or by host cell factors.

Recently, both T-strand-associated proteins, VirD2 and VirE2, have been implicated in the integration process (Figure 2.1, step 7). An *Agrobacterium* mutant carrying an Arg to Gly substitution at position 129 in VirD2 produced T-DNA insertion junctions in which the normally conserved 5' end of the integrated DNA was truncated or significantly rearranged. These data suggest that integration may precede second-strand synthesis, which would be performed by the plant cell DNA repair machinery following T-strand integration. However, a second study of T-DNA integration patterns led to the suggestion that T-strands are converted into a double-stranded form before integration. Both models propose that T-DNA integration initiates at the left border region and that VirD2 ligates the right border end of the T-DNA to the genomic plant DNA, thus completing the integration process.

2.5 Transformation of monocotyledonous plants with *Agrobacterium tumefaciens*

Transformation of dicotyledonous plants with *Agrobacterium tumefaciens* is well established and widely used. A number of foreign genes carried between the T-DNA borders of the Ti plasmid in *Agrobacterium* have been delivered to plant cells, integrated into the chromosome, and stably inherited by subsequent generations. This has not been the case for monocotyledonous plants in general. In the past, the monocots and particularly the graminaceous crop species have been considered to be outside the *Agrobacterium* host range (Decleene, 1985). Gene transfer methods developed for economically important monocotyledonous species have been restricted to the direct transfer of DNA into protoplasts, or particle discharge methods of direct DNA transfer into intact cells. Transformation methods based on the used of *Agrobacterium tumefaciens* are still preferred in many instances.

The advantages of *Agrobacterium*-mediated gene transfer over other methods that can be used for the transformation of higher plants include the high efficiency of transformation, the transfer of pieces of DNA with defined ends, the transfer of relatively large segments of DNA, and the absence of a requirement for protoplast-culture techniques (Chan *et al.*, 1993; Hiei *et al.*, 1994; Rashid *et al.*, 1996).

Li *et al.* (1992) studied factors influencing the transient expression of GUS gene under 35S promoter following *Agrobacterium*-mediated infection. Several parameters were found to be important for obtaining efficient transient GUS expression after cocultivation of rice tissues with *Agrobacterium*. The growth regulator, 2,4-D, inhibited

GUS expression if present during the seed germination period, but the presence of 6 mg/L 2,4-D during cocultivation of the explants with *A. tumefaciens* slightly enhanced GUS expression efficiency. All 21 rice cultivars tested expressed GUS after cocultivation with *A. tumefaciens.* The GUS expression frequency was highest amongst the *indica* cultivars. The frequencies of GUS expression in *japonica* cultivars and in *Oryza glaberrima* cultivars were generally one-half to one-third the level found for *indica* varieties. Leaf explants were more susceptible to *A. tumefaciens*-facilitated GUS expression than were roots or seed remnants. The *vir* genes of an agropine-type Ti-plasmid of *A. tumefaciens* were found to be more effective than those of a nopaline- or octopine-type Ti plasmid.

Liu *et al.* (1992) found that multiple copies of octopine- and agropine-type *virG* in *Agrobacterium tumefaciens* strains containing an agropine-type Ti-plasmid enhanced the frequency of transient transformation of rice. This enhancement is influenced by the type of Ti plasmid resident within a given *A. tumefaciens* strain, the species of plant infected, and the particular tissue (seedling section or callus) infected. No enhancement of the transient transformation frequency was obtained when multiple copies of octopine- and agropine-type *virG* gene were present a nopaline-type *A. tumefaciens* strain.

Chan *et al.* (1993) indicated that there are two factors which contribute the success of rice transformation and regeneration. The first factor could be the addition of potato suspension culture (PSC) during co-culture of *Agrobacterium* with the immature rice embryos significantly improved the transformation efficiency. PSC was rich in acetosyringone and sinapinic acid, which were generally believed to enhance transformation of various plant species (Stachel *et al.*, 1985). The second factor for successful transformation and regeneration could be the use of immature rice embryos (10 to 12 days after pollination) as the transformation material.

Hiei *et al.* (1994) reported that various factors affected the efficiency of transformation. Selection of a suitable starting material and optimization of the conditions of cocultivation are shown to be important. Callus cultures initiated from scutella were excellent starting materials for transformation of rice by *Agrobacterium*. Scutellum-derived calli maintained on medium containing 2,4-D for 25 days were transformed very efficiently, but scutella excised from seedlings after 5 days of germination on medium containing 2,4-D were transformed at a very low efficiency. Pre-culture of calli in fresh medium for 4 days before infection was an important step for *japonica* rice transformation. The addition of acetosyringone and glucose to the medium, pH of the medium (pH 5.2), and a temperature between 22°C and 28°C during cocultivation were important for enhancing transformation in *japonica* rice. Transformation was not successful when acetosyringone was omitted or cocultivation was carried out at a temperature higher or lower than that range. A super binary vector, in which a DNA fragment from the virulence region was introduced into a binary vector, was more effective for transforming rice.

Vijayachandra *et al.* (1995) studied the ability of rice plants to induce or inhibit *vir* genes of *Agrobacterium*. An analysis of rice leaf segments revealed that they neither induced *vir* genes nor inhibited *vir* gene induction. Scutellum from four-day old seedlings is the only part that induced *vir* genes of *Agrobacterium* quite efficiently. Freshly excised scutellum did not induce *virE* whereas scutellum pre-incubated for 72 h in the induction medium induced *virE* very well. Pre-incubation is likely to help in the transformation of rice scutellum. The scutellum-derived calli induced *virE* to an appreciable level but the level of induction of *virE* by the scutella excised from the seedlings germinated on medium containing 2,4-D was much lower. The scutellum-derived calli which induced *virE* more efficiently were also found to be transformed more efficiently by *Agrobacterium*. The scutellum-derived calli induced *virE*, and the level of induction was slightly lower than that of induction achieved by using scutella from seeds germinated on hormone-free medium.

Aldemita and Hodges (1996) reported *Agrobacterium tumefaciens*-mediated transformation of immature embryos of rice with LBA4404 (pTOK233) which contained the *virB*, *virC*, and *virG* genes derived from the supervirulent Ti-plasmid pTio542, and *hpt*, *nptII*, and *gusA* with an intron within the T-DNA region, resulted in efficient (about 27%) production of transgenic plants of the *japonica* variety, Radon, and an acceptable efficiency (from 1-5%) for the *indica* varieties, IR72 and TCS10. Key factors facilitating the transformation of rice by *Agrobacterium tumefaciens* appeared to be the use of embryos as the explant, the use of hygromycin as the selection agent does not interfere with rice regeneration, the presence of extra copies of certain *vir* genes on the binary vector of pTOK233, and maintaining high concentrations of acetosyringone for inducing the *vir* genes during cocultivation of embryos with *Agrobacterium*.

Rashid *et al.* (1996) found that inclusion of acetosyringone (50 μM) during co-culture is vital for Basmati rice transformation. Three-week-old scutella calli served as an excellent starting material. Calli infected for 2-3 days showed GUS activity. It yield the maximum transformation efficiency and further transgenic plant production.

2.6 Use of reporter genes in transformed plant cells

Once foreign DNA has been integrated into plant genomic DNA, it is essential to be able to detect the foreign DNA so that those cells that have been transformed can be identified. Furthermore, in studies of plant transcriptional regulatory signals and the functioning of these signals in specific plant tissues (such as in leaf, root, and flowers), it is often important to be able to quantify the level of expression of a gene with a readily identified product. Quantification and other applications require the use of reporter genes that either permit transformed cells to be selected or encode an activity that can be assayed. To these ends, researchers have tested a number of different genes as reporters for transformation, including both genes that can be used as dominant selectable markers and genes whose proteins produce a detectable response to a specific assay (Table 2.2). Many of these reporter genes are from bacteria and have been equipped with plant-specific regulatory sequences for expression in plant cells. Dominant marker selection provides a direct means of obtaining only transformed cells in culture. For example, in the presence of the antibiotic kanamycin, only plant cells with a function neomycin phosphotransferase gene can grow.

Table 2.2 Plant cell reporter gene system (Glick and Pasternak, 1994)

Enzyme activity	Dominant selection	Readily assayed
Neomycin phosphotransferase (Kanamycin kinase)	Yes	Yes
Hygromycin phosphotransferase	Yes	Yes
Dihydrofolate reductase (Tetrahydrofolate dehydrogenase)	Yes	Yes
Chloramphenical acetyltransferase	Yes	Yes
Gentamycin acetyltransferase	Yes	Yes
Nopaline synthase	No	Yes
Octopine synthase	No	Yes
β-D-glucuronidase	No	Yes
Streptomycin phosphotransferase	Yes	Yes
Firefly luciferase	No	Yes
Bacterial luciferase	No	Yes
Threonine dehydratase	Yes	Yes
5-Enolpyruvylshikimate-3-phosphate synthase	Yes	No
Phosphinothricin acetyltransferase	Yes	Yes
Acetolactate synthase	Yes	No
Bromoxynil nitrilase	Yes	No

Some reporter gene products (e.g., β-D-glucuronidase and green fluorescent protein) can be detected in intact plant tissues. The *Escherichia coli* β-D-glucuronidase (GUS) gene encodes a stable enzyme that is not normally present in plants and that catalyzes the cleavage of a range of β-D-glucuronides. The GUS activity in transformed plant tissues can be localized by observing the blue color that is formed after the hydrolysis of the uncolored substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid. Alternatively, GUS activity in plant extracts can be more quantitatively and sensitively assayed by a fluorometric analysis that involves the hydrolysis of a 4- methylumbelliferyl β-D-glucuronide substrate to form a fluorescent product (Glick and Pasternak, 1994).

The green fluorescent protein (GFP) gene isolated from the bioluminescent jellyfish (*Aequorea victoria*) encodes a protein that emits green fluorescence at 508 nm when excited with blue or UV light. The advantage of this gene as a reporter is that expression can be detected in living cells and no exogenous substrate and cofactors are required. Fluorescence results from an internal ρ-hydroxybenzylidene-imidazolidinone chromophore generated by cyclization and oxidation of a Ser-Tyr-Gly sequence at amino acid residues 65 to 67 (Anderson *et al.*, 1996; Pang *et al.*, 1996; Tian *et al.*, 1997; van der Geest and Petolino, 1998).

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant material

Rice seeds (*Oryza sativa* L., *indica* variety KDML105) used in this experiment were provided by the National Rice Research Institute (Pathumthani, Thailand).

3.2 *Agrobacterium* strains and transformation vectors

The strains of *Agrobacterium tumefaciens* used were as follows: EHA105 (pCAMBIA1301), EHA105(pCAMBIA1301/30063), AGL1(pCAMBIA1301) and LBA4404 (pCAMBIA1301).

pCAMBIA1301 was provided by R. A. Jefferson (CAMBIA, Australia). pCAMBIA1301 is a binary vector that contains genes for hygromycin resistance and GUS in the T-DNA region, as well as kanamycin resistance (Jefferson *et al.*, unpublished data) (Figure 3.1). The gene for GUS has an intron in the 5' end of the coding sequences and is connected to the 35S promoter of cauliflower mosaic virus. This intron-GUS reporter gene expresses GUS activity in plant cells but not in the cells of *Agrobacterium tumefaciens*.

pCAMBIA1301/30063 contains genes for hygromycin resistance, GFP and GUS in the T-DNA region, as well as kanamycin resistance (Figure 3.2). pCAMBIA1301/30063 was constructed by P. Ouwerkerk (Laiden University, the Netherlands), by cloning the *s65Tpgfp*-intron from pMon30063 (Pang *et al.*, 1996), into the *BamHI/PstI* site of the plasmid pCAMBIA1301 (CAMBIA, Australia). The *s65Tpgfp*-intron has the synthetic *gfp* gene, which contained the ST-LS1 intron from potato and was placed under the control of an enhanced 35S promoter of cauliflower mosaic virus and located between the soybean heat-shock protein 17.9 leader and the nopaline synthase 3' region (Pang *et al.*, 1996). The *gus* gene containing catalase intron was placed under the control of a

35Spromoter of cauliflower mosaic virus (CAMBIA, Australia). The *gfp* gene and *gus* gene express their activity in plant cells but not in the cells of *Agrobacterium tumefaciens*.

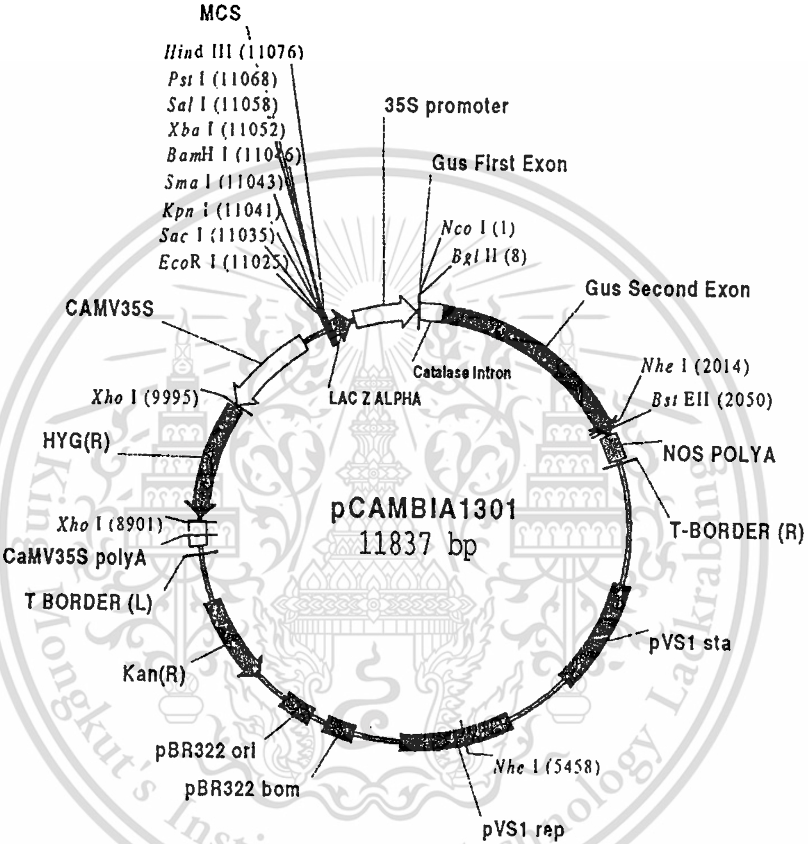

Figure 3.1 Transformation vector; pCAMBIA1301

PCAMBIA1301	E35s pro	GmHSP 17.9 leader	*S65Tpgfp*-intron	Nos ter

Figure 3.2 Transformation vector; pCAMBIA1301/30063

3.3 Media

The media used in this experiment are summarized in Table 3.1 and Table 3.2.

Table 3.1 Plant media

Media	Composition	pH	Remark
Basal NB medium	N6 macro elements, B5 micro elements and vitamins, 500 mg/l L-proline, 500 mg/l L-glutamine, 300 mg/l casein hydrolysate	5.8	
Basal R_2 medium	R_2 macro and micro elements, LS vitamins		
Callus induction medium (NB)	Basal NB medium supplemented with 30 g/l sucrose, 2 mg/l 2,4-D, 3 g/l phytagel	5.8	
Pre-regeneration medium (PRN)	Basal NB medium supplemented with 30 g/l sucrose, 1 mg/l NAA, 2 mg/l BAP, 5 mg/l ABA, 4.5 g/l phytagel	5.8	
Regeneration medium (RN)	Basal NB medium supplemented with 30 g/l sucrose, 0.5 mg/l NAA, 3 mg/l BAP, 1 mg/l kinetin, 6 g/l phytagel	5.8	
Cocultivation liquid medium (R_2L)	Basal R_2 medium supplemented with 10 g/l glucose, 2.5 mg/l 2,4-D, 100 μM acetosyringone	5.2	filtration
Cocultivation medium solidified (R_2S)	Cocultivation medium liquid supplemented with 7 g/l agarose type I	5.2	filtration
Selection medium	Basal NB medium (or basal R_2 medium) supplemented with 30 g/l sucrose, 2.5 mg/l 2,4-D, 400 mg/l cefotaxime, 100 mg/l vancomycin, 7 g/l agarose type I, 40 mg/l hygromycin	6.0	

Table 3.2 *Agrobacterium* media

Media	Composition	pH
AB media	AB buffer, AB salt, 5 g/l glucose, 15 g/l agar	
AAM liquid media	AA macro and micro elements, AA amino acids, MS vitamins, 68.5 g/l sucrose, 35 g/l glucose, 500 mg/l casamino acids, 100 µM acetosyringone	5.2

3.4 Chemical substances

- Agarose type I: low EEO, Sigma
- Ammonium chloride, Merck
- Ammonium nitrate, Fluka
- Ammonium sulfate, Merck
- Arginine, Fluka
- N^6-Benzylaminopurine, Fluka
- Boric acid, Merck
- 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, Duchefa
- Casamino acids, Difco
- Casein hydrolysate, Fluka
- Cefotaxime, Hoechst AG
- Copper (II) sulfate pentahydrate, Merck
- Cobaltous chloride hexahydrate, Fluka
- Calcium chloride dihydrate, Merck
- 2,4-Dichlorophenoxy acetic acid, Fluka
- EDTA, Disodium salt dihydrate, J.T.Baker
- Ferrous sulfate heptahydrate, Fluka
- Glucose, Fluka
- Glutamine, Fluka
- Glycine, Fluka
- Hygromycin B, Sigma
- Kinetin, Sigma

- Magnesium sulfate heptahydrate, J.T.Baker
- Manganese sulfate monohydrate, J.T.Baker
- Manganese sulfate tetrahydrate, J.T.Baker
- Myo-inositol, Sigma
- α-Naphthalene acetic acid, Sigma
- Nicotinic acid, Fluka
- Phytagel, Sigma
- Potassium iodide, BDH
- Potassium nitrate, Univar
- Potassium hydroxide, BDH
- Potassium dihydrogen phosphate, Merck
- Proline, Fluka
- Pyridoxine HCl, Merck
- Thiamine HCl, Fluka
- Tween 20, Merck
- Sodium molybdate dihydrate, Fluka
- Sucrose
- Vancomycine HCl, Duchefa
- Zinc sulfate heptahydrate, Fluka

3.5 Equipments

- Autoclave
- Balance model PG803, Mettler
- Balance model PS5002, Mettler
- Laminar flow cabinet model HS123, ISSCO
- Hot-air oven
- Hot plates with regulators and magnetic stirrers
- Incubator
- Millipore filters with accessories
- pH meter

- Plant growth chamber
- Stereo microscope
- Shaker model KS501, KIKA-Labortechnik
- UV-visible spectrophotometer model UV-1601, Shimadzu
- Water bath
- Glassware (erlenmeyer flasks of assorted sizes; volumetric flasks; measuring cylinders; beakers; graduated pipettes; glass petri plates; glass rods; wide-mouthed screw cap bottles; cell wells™, Corning; disposable sterile tissue culture dishes 100 x 20 mm, Corning)
- Miscellaneous (filter paper-Whatmann; parafilm; forceps-stainless steel fine-pointed; scapels; spatulas)

3.6 Experimental methods

3.6.1 Study on the effect of cytokinin concentration (in the callus induction medium) on the regeneration frequency of KDML105

3.6.1.1 Dehusked mature rice seeds were surface-sterilized in 70% ethanol for 1 min. followed by 30% commercial bleach with few drops of tween 20 for 30 min. The seeds were then rinsed four times with copious amounts of sterile distilled water.

3.6.1.2 The seeds were placed on sterile petri dishes containing NB medium supplemented with cytokinins, BAP and kinetin, at various concentrations. The concentrations were as follows: 0.25 mg/l BAP plus 0.25 mg/l kinetin, 0.5 mg/l BAP plus 0.5 mg/l kinetin and 1 mg/l BAP and 1 mg/l kinetin. The seeds were allowed to dry by leaving the petri dishes opened in the air flow of the sterile laminar flow hood until the seeds were dry. The petri dishes were then closed and sealed with parafilm and incubated in the dark at 28°C for two weeks to induce primary calli. The calli were subcultured on the same fresh medium three times at two weeks interval.

3.6.1.3 The calli were then transferred to RN regeneration medium containing 6 g/l phytagel. The cultures were incubated in the dark at 28°C for one week, followed by being incubated in the light at 28°C for three weeks, to induce organogenesis.

3.6.1.4 The percentages of regenerated plantlets were determined. This percentage corresponds to the ratio of the calli regenerated plants (with shoots and a developed root system) to the initial number of calli, and multiplied by 100.

3.6.2 Study on the effect of phytagel concentration (in the regeneration medium) on the regeneration frequency of KDML105

3.6.2.1 Dehusked mature rice seeds were surface-sterilized according to the method in 3.6.1.1.

3.6.2.2 The seeds were placed on sterile petri dishes containing NB medium and allowed to dry. The petri dishes were then closed and sealed with parafilm and incubated in the dark at 28°C for two weeks. The calli were subcultured on the same fresh medium three times at two weeks interval.

3.6.2.3 The calli were then transferred to RN regeneration medium containing phytagel at various concentrations (3, 4.5, 6, and 7.5 g/l). The conditions of the culture incubation were the same as those in 3.6.1.3.

3.6.2.4 The percentages of regenerated plantlets were determined according to the method in 3.6.1.4.

3.6.3 Study on the effect of partial desiccation on the regeneration frequency of KDML105

3.6.3.1 The calli used in this study were prepared according to the methods in 3.6.1.1 and 3.6.2.2.

3.6.3.2 The calli were transferred to an empty sterile petri dish containing two sterile disks of Whatmann paper. The petri dishes were sealed with parafilm and stored in the dark at 28°C for 24 hours.

3.6.3.3 The partial desiccated calli were then transferred to RN regeneration medium containing 3 or 6 g/l phytagel. The conditions of the culture incubation were the same as those in 3.6.1.3.

3.6.3.4 The percentages of regenerated plantlets were determined according to the method in 3.6.1.4.

3.6.4 Study on the effect of abscisic acid (in the pre-regeneration medium) on the regeneration frequency of KDML105

3.6.4.1 The calli used in this study were prepared according to the methods in 3.6.1.1 and 3.6.2.2.

3.6.4.2 The calli were transferred to the pre-regeneration medium containing 5 mg/l ABA, and incubated in the dark at 28°C for 7 days.

3.6.4.3 After the pre-regeneration stage, the calli were then transferred to RN regeneration medium containing 6 g/l phytagel and the method was the same as that of 3.6.1.3.

3.6.4.4 The percentages of regenerated plantlets were determined according to the method in 3.6.1.4.

3.6.5 Study on the effect of *Agrobacterium* concentration on the frequency of transient transformation of KDML105

3.6.5.1 Dehusked mature rice seeds were surface-sterilized according to the 3.6.1.1.

3.6.5.2 The seeds were placed on sterile petri dishes containing NB medium and allowed to dry. The petri dishes were then closed and sealed with parafilm and incubated in the dark at 28°C for 7 days.

3.6.5.3 *Agrobacterium tumefaciens* strain EHA105(pCAMBIA1301) was grown on AB medium supplemented with 50 mg/l kanamycin at 28°C for 3 days. The bacteria were collected with a flat spatula and resuspended in AAM medium to give an absorbance at 600 nm of 0.01, 0.1, and 1. The *Agrobacterium* suspension were incubated at 28°C for 1 hour.

3.6.5.4 The 7 days old calli were placed on petri dishes containing NB medium supplemented with 50 μM acetosyringone.

3.6.5.5 Fifteen microliters of the *Agrobacterium* suspension were dropped on each callus. The petri dishes were sealed with parafilm and incubated in the dark at 28°C for 3 days.

3.6.5.6 After cocultivation, the infected calli were washed with sterile distilled water containing 250 mg/l cefotaxime. They were then transferred to X-gluc solution, and incubated at 37°C overnight (14-16 hours) and scored for evidence and extent of GUS activity.

3.6.5.7 The frequencies of transient transformation were determined. This frequency was calculated as the ratio of the number of GUS-positive calli to the number of calli stained, and multiplied by 100. The calli were scored as GUS-positive (GUS^+) if any blue spot or streak was present, or as GUS-negative (GUS^-) if no blue-stained was detectable.

3.6.6 Study on the effect of acetosyringone concentration (in the cocultivation medium) on the frequency of transient transformation of KDML105

3.6.6.1 The calli used in this study were prepared according to the methods in 3.6.1.1 and 3.6.5.2.

3.6.6.2 *Agrobacterium tumefaciens* strain EHA105(pCAMBIA1301) was grown on AB medium supplemented with 50 mg/l kanamycin at 28°C for 3 days. The bacteria were collected with a flat spatula and resuspended in AAM medium to give an absorbance at 600 nm of 0.01. The *Agrobacterium* suspension was incubated at 28°C for 1 hour.

3.6.6.3 The 7 days old calli were placed on petri dishes containing NB medium supplemented with acetosyringone at various concentrations (50, 100, 250, and 500 μM).

3.6.6.4 The experiment was carried out according to the methods in 3.6.5.6, and 3.6.5.7.

3.6.7 Study on the effect of age of calli on the frequency of transient transformation of KDML105

3.6.7.1 Dehusked mature rice seeds were surface-sterilized according to the method in 3.6.1.1.

3.6.7.2 The seeds were placed on sterile petri dishes containing NB medium and allowed to dry. The petri dishes were then closed and sealed with parafilm and incubated in the dark at 28°C for 5, 7, 10, 12, 14, 17, 19, and 21 days.

3.6.7.3 The *Agrobacterium* suspension was prepared according to the method in 3.6.6.2.

3.6.7.4 The 5, 7, 10, 12, 14, 17, 19, and 21 days old calli were placed on petri dishes containing NB medium supplemented with 50 μM acetosyringone.

3.6.7.5 The experiment was carried out according to the method in 3.6.5.6, and 3.6.5.7.

3.6.8 Study on the effect of immersion period of calli in the *Agrobacterium* suspension on the frequency of transient transformation of KDML105

3.6.8.1 Dehusked mature rice seeds were surface-sterilized according to the method in 3.6.1.1.

3.6.8.2 The seeds were placed on disposable sterile petri dishes (100 x 20 mm, Corning) containing NB medium (10 seeds/petri dish) and allowed to dry. The petri dishes were then closed and sealed with parafilm and incubated in the dark at 28°C for four weeks. At this point, many loosely attached small globular calli appeared on top of each compact primary callus, which were gently removed with a pair of forceps. The small globular calli were subcultured in fresh NB medium, and incubated in the dark at 28°C for two weeks. One more round of subcultured were carried out in the same manner. After two 2-week subculture cycles, the small globular calli (about 3 mm. in diameter) were selected for transformtaion.

3.6.8.3 *Agrobacterium tumefaciens* strain EHA105(pCAMBIA1301/30063) was grown on AB medium supplemented with 50 mg/l kanamycin at 28°C for 3 days. The bacteria were collected with a flat spatula and resuspended in R_2L cocultivation liquid medium to give an absorbance at 600 nm of 1.

3.6.8.4 The calli were immersed in the *Agrobacterium* suspension for 10, 12.5, and 15 min., blotted dry on sterile filter paper. Each individual piece of callus was gently rolled over the sterile filter paper until it had acquired a dry appearance.

3.6.8.5 The calli were placed on disposable sterile petri dishes (100 x 20 mm, Corning) containing R_2S cocultivation medium solidified (10 calli/petri dish). The petri dishes were sealed with parafilm and incubated in the dark at 25°C for 3 days.

3.6.8.6 The cocultivated calli were transferred to disposable sterile petri dishes (100 x 20 mm, Corning) containing selection medium (based on NB medium) and incubated in the dark at 28°C for three weeks.

3.6.8.7 GFP gene expression was observed under a fluorescence microscope. The frequencies of transient transformation were determined. This frequency was calculated as the ratio of the number of GFP-positive calli to the total number of calli, and multiplied by 100. The calli were scored as GFP-positive (GFP^+) if the calli emitted green fluorescence, or as GFP-negative (GFP^-) if the calli did not emit green fluorescence.

3.6.9 Study on the effect of basal medium (in the selection medium) on the frequency of transient transformation of KDML105

3.6.9.1 The calli used in this study were prepared according to the methods in 3.6.1.1 and 3.6.8.2.

3.6.9.2 The *Agrobacterium* suspension was prepared according to the method in 3.6.8.3.

3.6.9.3 The calli were immersed in the *Agrobacterium* suspension for 10-15 min., blotted dry on sterile filter paper according to the method in 3.6.8.4.

3.6.9.4 The calli were cocultivated with *Agrobacterium* according to the method in 3.6.8.5.

3.6.9.5 The cocultivated calli were transferred to disposable sterile petri dishes (100 x 20 mm, Corning) containing selection medium (based on NB medium or R_2 medium) supplemented with 25 or 40 mg/l hygromycin. The conditions of the culture incubation were the same as those in 3.6.8.6.

3.6.9.6 The frequencies of transient transformation were determined according to the method in 3.6.8.7.

3.6.9.7 The GFP-positive/hygromycin resistant calli were subcultured on selection medium based on NB medium supplemented with 40 mg/l hygromycin and incubated in the dark at 28°C for two weeks.

3.6.9.8 The hygromycin resistant clones were then transferred to RN regeneration medium containing 50 mg/l hygromycin. The conditions of the culture incubation were the same as those in 3.6.1.3.

3.6.9.9 The percentages of transformation were determined. This percentage corresponds to the ratio of the GFP-positive/hygromycin resistant clones regenerated plants (with shoots and a developed root system) to the initial number of cocultivated calli, and multiplied by 100.

3.6.10 Study on the effect of hygromycin concentration (in the selection medium) on the frequency of transient transformation of KDML105

3.6.10.1 The calli used in this study were prepared according to the methods in 3.6.1.1 and 3.6.8.2.

3.6.10.2 The *Agrobacterium* suspension was prepared according to the method in 3.6.8.3.

3.6.10.3 The calli were immersed in the *Agrobacterium* suspension for 10 min., blotted dry on sterile filter paper according to the method in 3.6.8.4.

3.6.10.4 The calli were cocultivated with *Agrobacterium* according to the method in 3.6.8.5

3.6.10.5 The cocultivated calli were transferred to disposable sterile petri dishes (100 x 20 mm, Corning) containing selection medium (based on NB medium) supplemented with hygromycin at various concentrations (25, 30, 40, and 50 mg/l). The conditions of the culture incubation were the same as those in 3.6.8.6.

3.6.10.6 A representative piece of each callus was transferred to X-gluc solution, and incubated at 37°C overnight (14-16 hours) and scored for evidence and extent of GUS activity.

3.6.10.7 The frequencies of transient transformation were determined according to the method in 3.6.5.7.

3.6.10.8 The GUS-positive/hygromycin resistant calli were subcultured on selection medium based on NB medium supplemented with 40 or 50 mg/l hygromycin and incubated in the dark at 28°C for two weeks.

3.6.10.9 The hygromycin resistant clones were then transferred to RN regeneration medium containing 50 mg/l hygromycin. The conditions of the culture incubation were the same as those in 3.6.1.3.

3.6.10.10 The percentages of transformation were determined. This percentage corresponds to the ratio of the GUS-positive/hygromycin resistant clones regenerated plants (with shoots and a developed root system) to the initial number of cocultivated calli, and multiplied by 100.

3.6.11 Study on the effect of *Agrobacterium* strains on the frequency of transient transformation of KDML105

3.6.11.1 The calli used in this study were prepared according to the methods in 3.6.1.1 and 3.6.8.2.

3.6.11.2 Various strains of *Agrobacterium tumefaciens*, EHA105 (pCAMBIA1301), AGL1(pCAMBIA1301) and LBA4404(pCAMBIA1301), were grown on AB medium supplemented with 50 mg/l kanamycin at 28°C for 3 days. The *Agrobacterium* suspension was prepared according to the method in 3.6.8.3.

3.6.11.3 The calli were immersed in the *Agrobacterium* suspension for 10 min., blotted dry on sterile filter paper according to the method in 3.6.8.4.

3.6.11.4 The calli were cocultivated with *Agrobacterium* according to the method in 3.6.8.5

3.6.11.5 The cocultivated calli were transferred to disposable sterile petri dishes (100 x 20 mm, Corning) containing selection medium (based on NB medium) supplemented with 40 mg/l hygromycin. The conditions of the culture incubation were the same as those in 3.6.8.6.

3.6.11.6 The experiment was carried out according to the methods in 3.6.10.6, 3.6.10.7, 3.6.10.8, 3.6.10.9, and 3.6.10.10.

CHAPTER 4

RESULTS

4.1 Effect of cytokinin concentration (in the callus induction medium) on the regeneration frequency of KDML105

To establish an optimized medium for promoting callus induction, embryogenesis, and plant regeneration, the concentrations of cytokinins in the callus induction medium were studied. The dehusked and sterilized KDML105 seeds were placed on NB medium supplemented with cytokinins, BAP and kinetin, at various concentrations (Table 4.1) for two weeks to induce primary calli, followed by subculturing on the same fresh medium three times at two weeks interval. They were then transferred to RN regeneration medium containing 6 g/l phytagel, incubated in the dark for one week, followed by being incubated in the light for three weeks to induce organogenesis. After four weeks, the percentages of regenerated plantlets were determined. This percentage corresponds to the ratio of the number of calli regenerated plants (with shoots and a developed root system) to the initial number of calli, and multiplied by 100. The results obtained are presented in Table 4.1. The addition of BAP and kinetin to NB medium did not enhance the percentage of regeneration. The highest percentage of regeneration, 73.45%, was obtained from calli cultured on NB medium, while 43.85%, 67.18%, and 46.71% were obtained from calli cultured on media containing cytokinins. Calli grown on NB medium and NB medium supplemented with 0.5 mg/l BAP plus 0.5 mg/l kinetin appeared to be more embryogenic than calli grown on NB medium supplemented with 0.25 mg/l BAP plus 0.25 mg/l kinetin and NB medium supplemented with 1 mg/l BAP plus 1 mg/l kinetin. Embryogenic calli appeared to be dry and compact with globular structures and a yellowish color (Figure 4.1) while non-embryogenic calli appeared to be wet and friable with a whitish color.

Table 4.1 Effect of cytokinin concentration (in the callus induction medium) on plant regeneration from scutellum-derived calli in KDML105.

Cytokinin concentration in callus induction medium BAP – kinetin (mg/l)	The initial Number of calli	The number of calli regenerated plants[1]	Percentage of regeneration
0 – 0	452	332	73.45
0.25 – 0.25	260	114	43.85
0.5 – 0.5	262	176	67.18
1 - 1	334	156	46.71

[1] The data were obtained in four weeks after transferring calli to regeneration medium.

Figure 4.1 Embryogenic callus, 8 weeks after culturing on NB medium.

4.2 Effect of phytagel concentration (in the regeneration medium) on the regeneration frequency of KDML105

To study the effect of phytagel concentration on plant regeneration, calli produced on NB medium were transferred to RN regeneration medium supplemented with phytagel at various concentrations (3, 4.5, 6, and 7.5 g/l), incubated in the dark for one week, followed by being incubated in the light for three weeks to induce organogenesis. The percentages of regeneration were recorded. This percentage corresponds to the ratio of the number of calli regenerated plants (with shoots and a developed root system) to the number of calli treated, and multiplied by 100. The results obtained are presented in Table 4.2. When the phytagel concentration of the regeneration medium was increased from 3 g/l to 7.5 g/l, the percentage of regeneration increased considerably (Figure 4.2). Calli grown on regeneration medium containing 3 g/l phytagel regenerated into few plantlets. The higher percentages of regeneration, 82.22% and 86.67%, were obtained from calli cultured on regeneration medium containing 6 g/l and 7.5 g/l phytagel, respectively. Calli cultured on these two media generally resulted in the development of green spots within 14 days (Figure 4.3), shoot formation within 19-21 days (Figure 4.4) and plantlets within 24-28 days after transferring to regeneration medium (Figure 4.5 and 4.6).

Table 4.2 Effect of phytagel concentration (in the regeneration medium) on plant regeneration from scutellum-derived calli in KDML105.

Phytagel concentration in regeneration medium (g/l)	The initial Number of calli	The number of calli regenerated plants[1]	Percentage of regeneration[2]
3	90	5	5.56 ± 5.09
4.5	90	29	32.22 ± 1.92
6	90	74	82.22 ± 1.92
7.5	90	78	86.67 ± 3.33

[1] The data were obtained in four weeks after transferring calli to regeneration medium.

[2] Values represent the averages of three replications ± standard deviation.

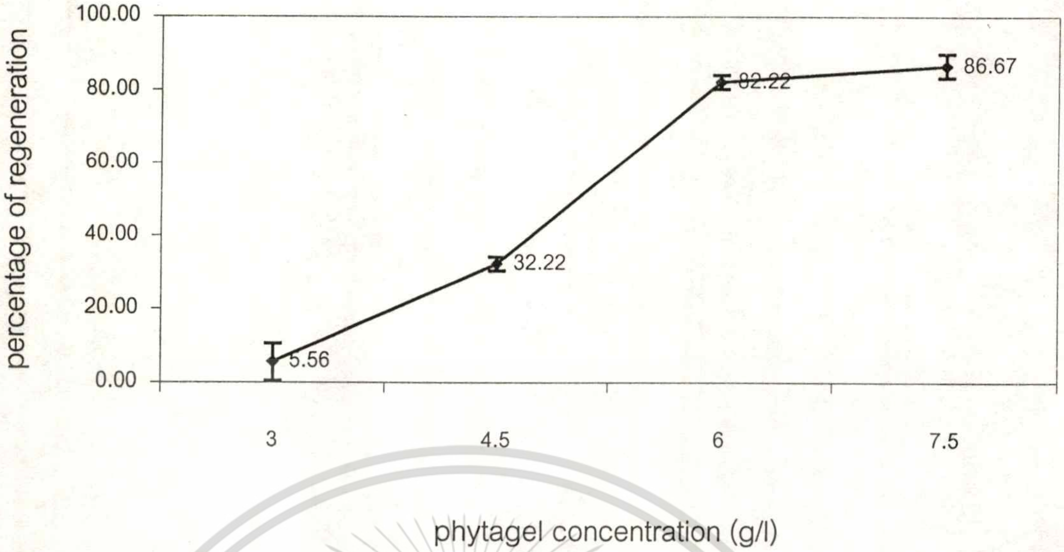

Figure 4.2 Effect of phytagel concentration (in the regeneration medium) on plant regeneration from scutellum-derived calli in KDML105.

Figure 4.3 Development of green spots, 2 weeks after transferring to regeneration medium containing 6 g/l phytagel.

Figure 4.4 Shoot formation of calli.

Figure 4.5 Regeneration of multiple shoots and roots, 4 weeks after transferring to regeneration medium containing 6 g/l phytagel.

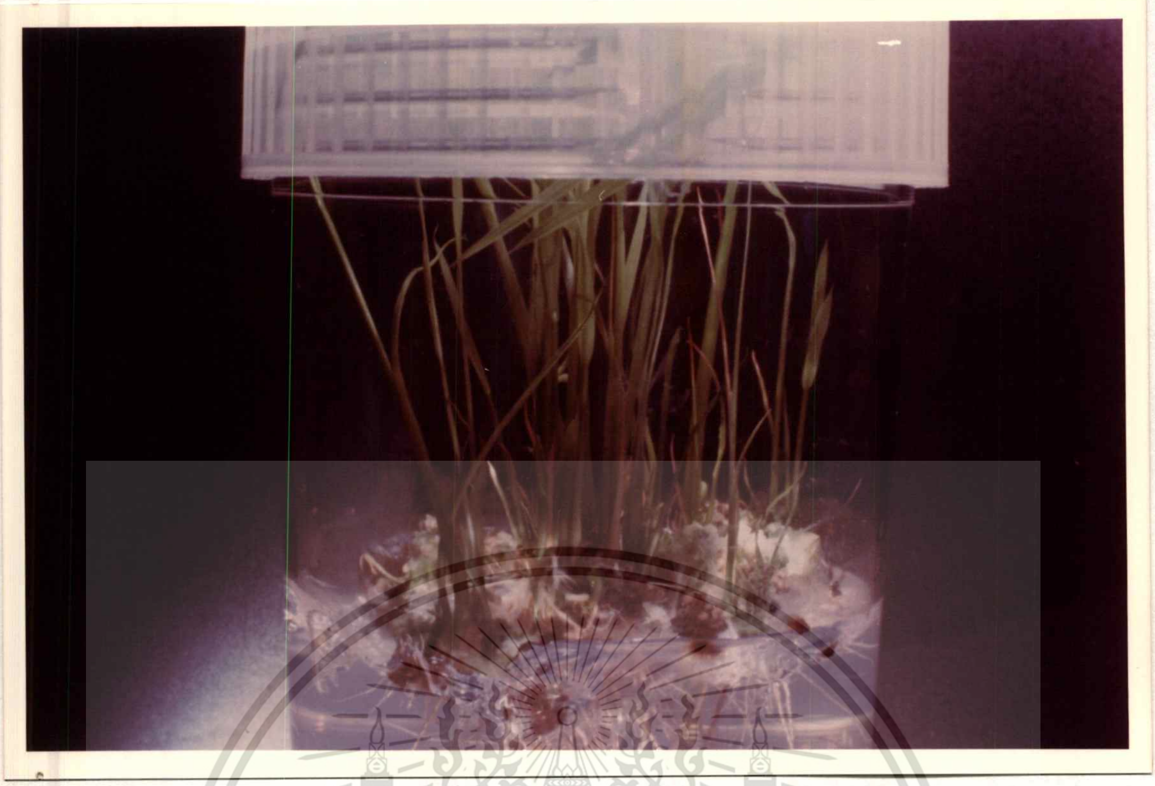

Figure 4.6 Plants with multiple tillers.

4.3 Effect of partial desiccation on the regeneration frequency of KDML105

Calli produced on NB medium were transferred to an empty sterile Petri dish containing two sterile disks of Whatmann paper. The Petri dishes were sealed with parafilm and stored at 28°C in the dark for 24 hours. The partial desiccated calli were transferred to RN regeneration medium containing 3 or 6 g/l phytagel, incubated in the dark for one week, followed by being incubated in the light for three weeks to induce organogenesis. After four weeks, the percentages of regenerated plantlets were determined. This percentage corresponds to the ratio of the number of calli regenerated plants to the number of calli treated, and multiplied by 100. Table 4.3 summarizes the results obtained from the experiments for the partial desiccation versus the control. Partial desiccation stimulated the percentage of regeneration in calli regenerated on regeneration medium containing 3 g/l phytagel by 2.88 folds. The high percentages of regeneration obtained from calli regenerated on regeneration medium containing 6 g/l phytagel were as follows: 74% for partial desiccation and 82.22% for control. The first set of green spots was observed as early as 11 days after desiccation whereas for controlled calli, the first set of green spots appeared in 14 days after transferring to the regeneration medium. Plants with shoots and a developed root system were obtained on the 19-22 days post desiccation compared to 24-28 days for the controlled calli. This indicated that partial desiccation did not enhance the percentage of regeneration in calli regenerated on regeneration medium containing 6 g/l phytagel but shortened the time in tissue culture.

Table 4.3 Effect of partial desiccation on plant regeneration from scutellum-derived calli in KDML105.

Desiccation treatment (hours)	Phytagel concentration in regeneration medium (g/l)	The initial number of calli	The number of calli regenerated plants[1]	Percentage of regeneration[2]
0	3	90	5	5.56 ± 5.09
24	3	150	24	16 ± 3.46
0	6	90	74	82.22 ± 1.92
24	6	150	111	74 ± 7.21

[1] The data were obtained in four weeks after transferring to regeneration medium.

[2] Values represent the averages of three replications ± standard deviation.

4.4 Effect of abscisic acid (in the pre-regeneration medium) on the regeneration frequency of KDML105

To study the effect of ABA (in the pre-regeneration medium) on the plant regeneration, calli produced on NB medium were transferred to pre-regeneration medium containing 5 mg/l ABA, and incubated in the dark for 7 days. After the pre-regeneration stage, the calli were transferred to RN regeneration medium containing 6 g/l phytagel, and incubated in the dark for one week, followed by being incubated in the light for three weeks to induce organogenesis. After four weeks, the percentages of regenerated plantlets were determined. This percentage corresponds to the ratio of the number of calli regenerated plants to the number of calli treated, multiplied by 100.

Controlled calli and calli on pre-regeneration media showed the embryogenic appearance (dry and compact with globular structures and a yellowish color). The percentages of regeneration obtained were as follows: 70.67% for the calli pre-regenerated on medium containing 5 mg/l ABA and 78% for the control calli (Table 4.4). ABA in the pre-regeneration medium did not enhance the percentage of regeneration and did not shorten the period of time *in vitro* culture.

Table 4.4 Effect of abscisic acid in the pre-regeneration medium on plant regeneration from scutellum-derived calli in KDML105.

Abscisic concentration in pre-regeneration medium (mg/l)	The initial Number of calli	The number of calli regenerated plants[1]	Percentage of regeneration[2]
0	150	117	78 ± 4
5	150	106	70.67 ± 3.06

[1] The data were obtained in four weeks after transferring to regeneration medium.

[2] Values represent the averages of three replications ± standard deviation.

4.5 Effect of *Agrobacterium* concentration on the frequency of transient transformation of KDML105

To study the effect of *Agrobacterium* concentration on transformation, various concentrations of suspensions of *Agrobacterium tumefaciens* strain EHA105 (pCAMBIA1301) (Table 4.5) were studied. Fifteen microliters of each *Agrobacterium* suspension were dropped on the seven days old calli placed on NB medium supplemented with 50 µM acetosyringone. These calli were incubated in the dark at 28°C for 3 days. After cocultivation, the frequencies of transient transformation were determined. This frequency corresponds to the ratio of the number of GUS-positive calli to the number of calli stained, and multiplied by 100. The results obtained are presented in Table 4.5 and Figure 4.7. The overgrowth of bacteria occurred on calli inoculated with *Agrobacterium* suspension which gives an absorbance at 600 nm of 1 but did not occur on the others. The higher frequency of transient transformation, 86.67% and 83.33%

were obtained from calli inoculated with *Agrobacterium* suspension which gives an absorbance at 600 nm of 0.01 and 0.1, respectively. These calli exhibited a large part of blue staining in comparison to the calli inoculated with *Agrobacterium* suspension which gives an absorbance at 600 nm of 1.

Table 4.5 Effect of *Agrobacterium* concentration on the frequency of transient transformation of KDML105.

An absorbance at 600 nm	The initial Number of calli	The number of GUS-positive calli	Frequency of transient transformation (%)^2
0.01	30	26	86.67 ± 5.77
0.1	30	25	83.33 ± 20.82
1	30	21	70 ± 10

[1] The data were obtained after cocultivation with *Agrobacterium tumefaciens*.

[2] Values represent the averages of three replications ± standard deviation.

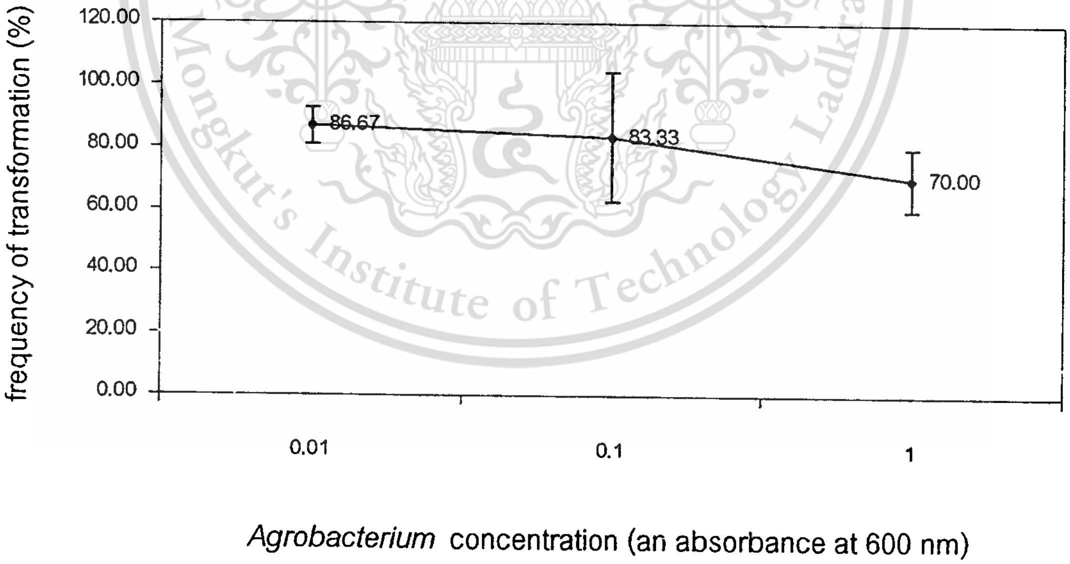

Figure 4.7 Effect of *Agrobacterium* concentration on the frequency of transient transformation of KDML105.

4.6 Effect of acetosyringone on the frequency of transient transformation of KDML105

To establish an optimized cocultivation medium for efficient transformation, the concentrations of acetosyringone were studied. The seven days old calli were cocultivated with *Agrobacterium tumefaciens* strain EHA105(pCAMBIA1301) on NB medium supplemented with acetosyringone at various concentrations (50, 100, 250, and 500 μM), and incubated in the dark at 28°C for 3 days. After cocultivation, the frequencies of transient transformation were determined. This frequency corresponds to the ratio of the number of GUS-positive calli to the number of calli stained, and multiplied by 100. The results obtained are presented in Table 4.6 and Figure 4.8. The additions of 50, 100, and 250 μM acetosyringone to NB medium did stimulate the frequency of transient transformation. The highest frequency, 86.67%, was obtained from the calli cocultivated on NB medium supplemented with 50 μM acetosyringone, while 70% and 80% were obtained from the calli cocultivated on NB medium supplemented with 100 μM and 250 μM acetosyringone, respectively. Calli cocultivated on NB medium supplemented with 50 μM showed a large portion of intense blue spots whereas the others showed a smaller portion of intense blue spots (Figure 4.9). The addition of 500 μM acetosyringone to NB medium inhibited the transformation, only 6.67% of 30 calli cocultivated on this medium exhibited blue staining for GUS.

Table 4.6 Effect of acetosyringone on the frequency of transient transformation of KDML105.

Acetosyringone concentration in cocultivation medium (μM)	The initial number of calli	The number of GUS-positive calli[1]	Frequency of transient transformation (%)[2]
0	30	17	56.67 ± 20.82
50	30	26	86.67 ± 11.55
100	30	21	70 ± 10
250	30	24	80 ± 10
500	30	2	6.67 ± 5.77

[1] The data were obtained after cocultivation with *Agrobacterium tumefaciens*.

[2] Values represent the averages of three replications ± standard deviation.

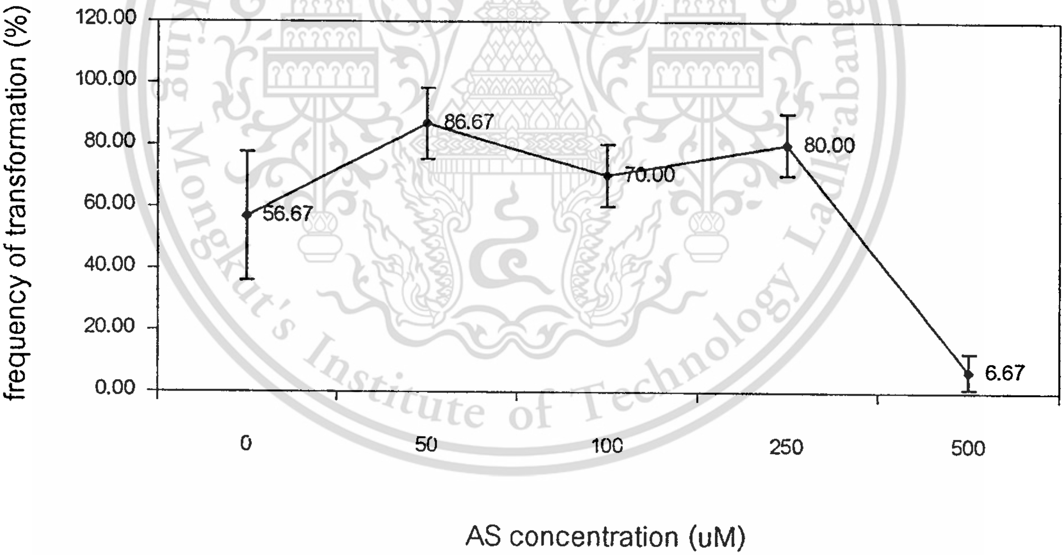

Figure 4.8 Effect of acetosyringone in the cocultivation medium on the frequency of transient transformation of KDML105.

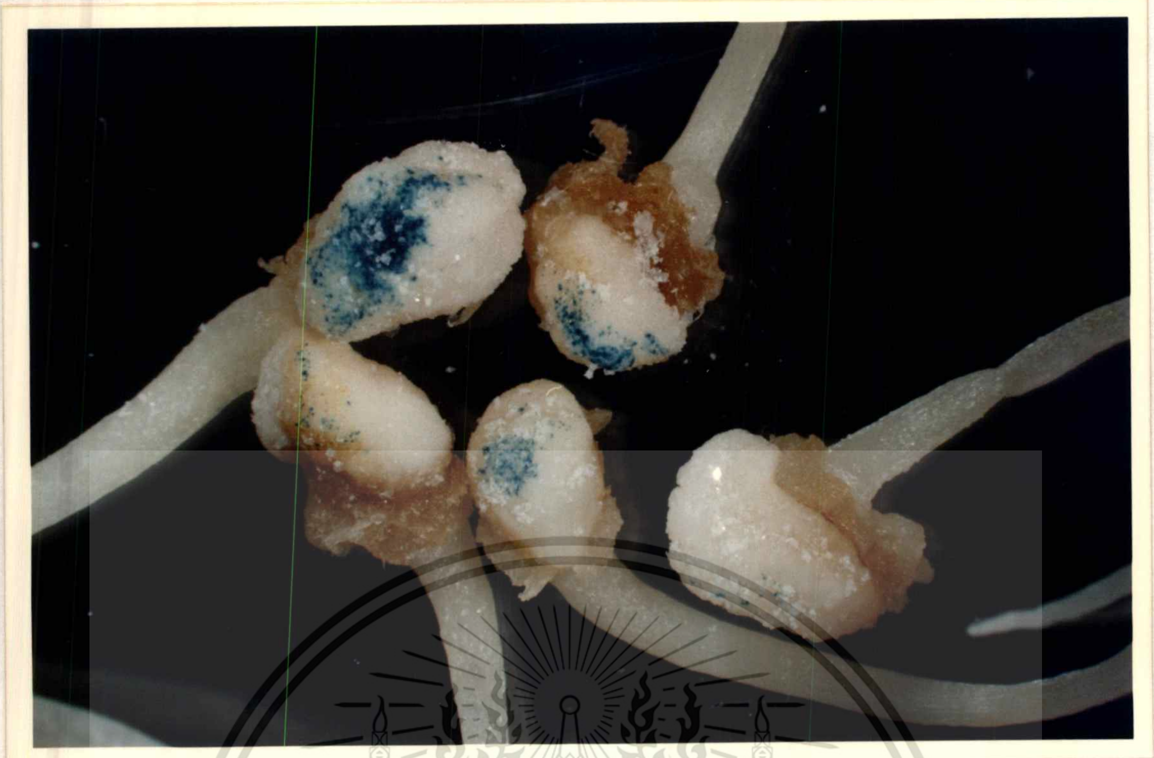

Figure 4.9 Expression of GUS in 7 days old calli after cocultivation with *Agrobacterium tumefaciens*.

4.7 Effect of age of calli on the frequency of transient transformation of KDML105

Different ages of calli, 5-21 days old, were cocultivated with *Agrobacterium tumefaciens* strain EHA105(pCAMBIA1301) on NB medium supplemented with 50 μM acetosyringone, and incubated in the dark at 28°C for 3 days. After cocultivation, the frequencies of transient transformation were determined. This frequency corresponds to the ratio of the number of GUS-positive calli to the number of calli stained, and multiplied by 100. The results obtained are presented in Table 4.7 and Figure 4.10. The high frequencies of transient transformation were as follows: 83.33% for 7 and 10 days old calli and 80% for 14 days old calli. The acceptable frequencies of transformation, from 63.33% to 73.33%, were obtained from the 12, 17, and 19 days old calli. The results of this study indicated that the 7 days old calli are the good choice as starting material for transformation of rice by *Agrobacterium*. These calli cocultivated with *Agrobacterium* were further cultured on NB medium containing 400 mg/l cefotaxime and 50 mg/l hygromycin for selecting transformed cells. During the selection, all of the calli turned brown and died. None of hygromycin resistant calli was obtained.

Table 4.7 Effect of age of calli on the frequency of transient transformation of KDML105.

Age of calli (days)	The initial number of calli	The number of GUS-positive calli	Frequency of transient transformation (%)[2]
5	30	14	46.67 ± 11.55
7	30	25	83.33 ± 15.28
10	30	25	83.33 ± 5.77
12	30	22	73.33 ± 5.77
14	30	24	80 ± 17.32
17	30	21	70 ± 10
19	30	19	63.33 ± 15.28
21	30	12	40 ± 17.32

[1] The data were obtained after cocultivation with *Agrobacterium tumefaciens*.

[2] Values represent the averages of three replications ± standard deviation.

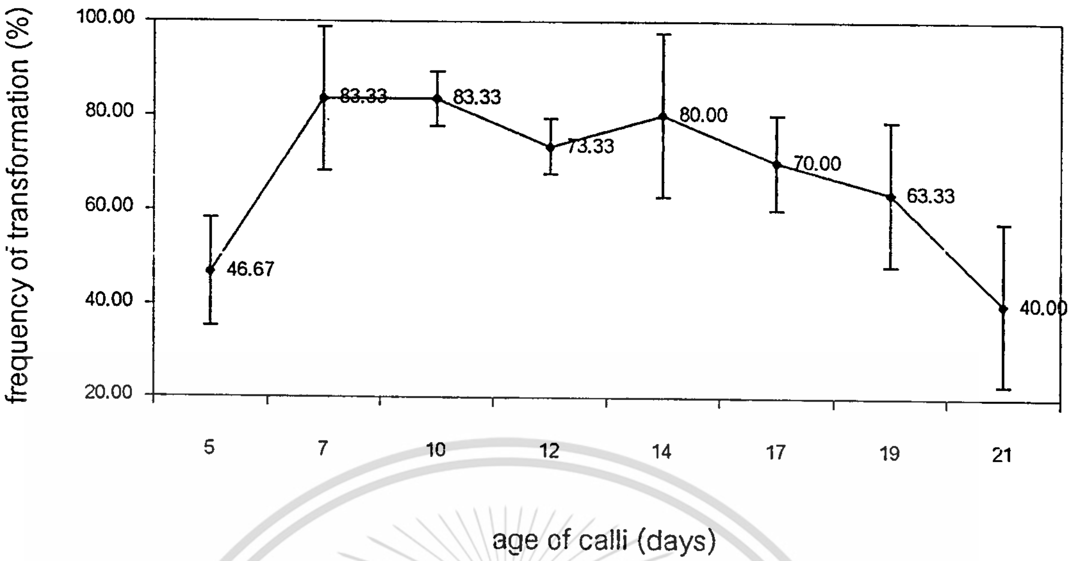

Figure 4.10 Effect of age of calli on the frequency of transient transformation of KDML105.

4.8 Effect of immersion period of calli in the *Agrobacterium* suspension on the frequency of transient transformation of KDML105

The small globular calli were immersed in the suspension of *Agrobacterium* strain EHA105(pCAMBIA1301/30063), for 10, 12.5, and 15 min., blotted dry and placed on R_2S cocultivation medium sollidified in the dark at 25°C for 3 days. After cocultivation, the calli were transferred to selection medium (based on NB medium) and incubated in the dark for three weeks. The frequencies of transient transformation were determined. This frequency corresponds to the ratio of the number of GFP-positive calli to the initial number of cocultivated calli, and multiplied by 100. The results obtained are presented in Table 4.8. When the immersion period of calli in the *Agrobacterium* suspension was increased from 10 min. to 15 min., the frequency of transient transformation decreased considerably (Table 4.8 and Figure 4.11). The highest transformation frequency, 23%, was obtained from 10 min. immersion of calli in the *Agrobacterium* suspension while 19.58% and 13% were obtained from 12.5 min. and 15 min. immersion, respectively.

Table 4.8 Effect of immersion period of calli in the *Agrobacterium* suspension on the frequency of transient of transformation of KDML105.

Immersion period of calli in the *Agrobacterium* suspension (min.)	The initial number of calli	The number of GFP-positive calli[1]	Frequency of Transient transformation (%)
10	100	23	23
12.5	97	19	19.58
15	100	13	13

[1] The data were obtained in three weeks after cocultivation with *Agrobacterium tumefaciens*.

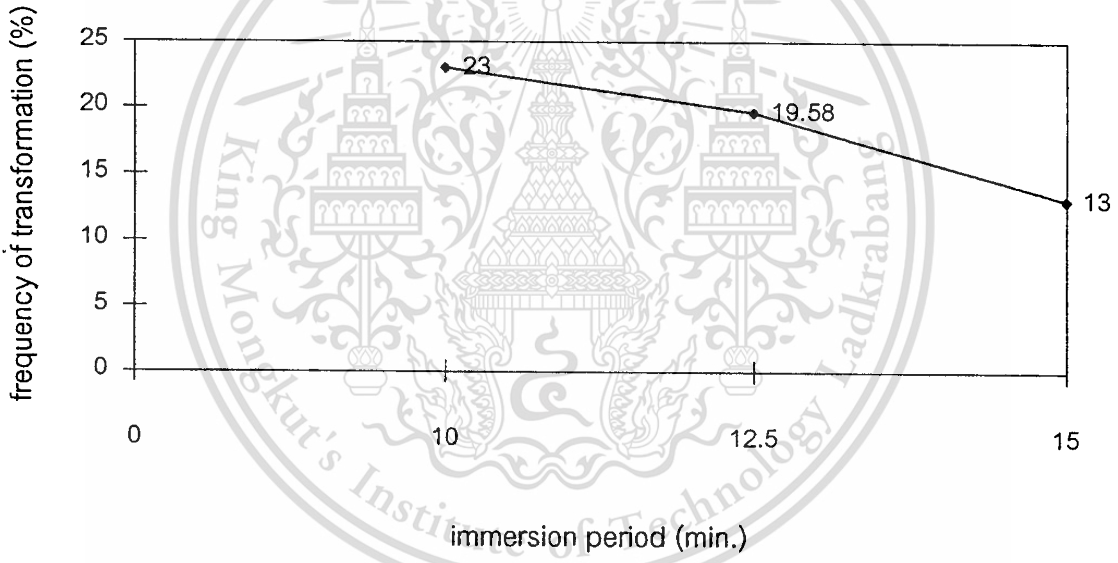

Figure 4.11 Effect of immersion period of calli in the *Agrobacterium* suspension on the frequency of transient transformation of KDML105.

4.9 Effect of basal medium (in the selection medium) on the frequency of transient transformation of KDML105

The small globular calli were cocultivated with *Agrobacterium tumefaciens* strain EHA105(pCAMBIA1301/30063) on R_2S cocultivation medium solidified in the dark at 25° C for 3 days. After cocultivation. the calli were transferred to selection medium, based on NB medium or R_2 medium, supplemented with 25 or 40 mg/l hygromycin and incubated in the dark for three weeks. The frequencies of transient transformation were determined. These frequencies corresponded to the ratio of the number of GFP-positive calli to the initial number of cocultivated calli, and multiplied by 100. The results obtained are presented in Table 4.9. Most of the calli grown on selection medium based on R_2 medium supplemented with 25 mg/l hygromycin turned brown and died, only 8.16% of 98 calli emitted green fluorescence. The higher frequencies transient transformation, 25.25% and 26% were obtained from calli grown on selection medium based on NB medium supplemented with 25 mg/l and 40 mg/l hygromycin (Figure 4.12), respectively. The overgrowth of GFP-negative/hygromycin resistant calli was found in the calli grown on selection medium based on NB medium supplemented with 25 mg/l hygromycin. These GFP-negative/hygromycin resistant calli did not exhibit blue staining for GUS.

Table 4.9 Effect of basal medium (in the selection medium) on the frequency of transient transformation of KDML105.

Basal medium	Hygromycin concentration (mg/l)	The initial number of calli	The number of GFP-positive calli[1]	Frequency of transient transformation (%)
NB	0[2]	100	3	3
NB	25	99	25	25.25
NB	40	100	26	26
R_2	0[2]	90	5	5.55
R_2	25	98	8	8.16

[1] The data were obtained in three weeks after cocultivation with *Agrobacterium tumefaciens*.

[2] After cocultivation, the calli were transferred to selection medium without hygromycin for one week then the calli were transferred to selection medium supplemented with 25 mg/l hygromycin for two weeks.

The GFP-positive/hygromycin resistant calli were subcultured on selection medium based on NB medium supplemented with 40 mg/l hygromycin (Figure 4.13 and 4.15). After two weeks, 19 clones from calli grown on selection medium based on NB medium supplemented with 25 mg/l hygromycin turned brown and did not proliferate any hygromycin resistant embryogenic calli. These calli were discarded from the experiment. Only 2 of 6 clones emitted green fluorescence (Table 4.10). All of the hygromycin resistant calli were then transferred to RN regeneration medium containing 6 g/l phytagel and 50 mg/l hygromycin, incubated in the dark for one week, followed by being incubated in the light for three weeks to induce organogenesis (Figure 4.16). The percentages of transformation were determined. This percentage corresponds to the ratio of the number of hygromycin resistant plant to the initial number of cocultivated calli, and multiplied by 100. None of hygromycin resistant plant was obtained from calli grown on selection medium based on NB medium supplemented with 25 mg/l hygromycin whereas calli grown on selection medium based on NB medium supplemented with 40 mg/l hygromycin showed a transformation efficiency of 15%

(Table 4.10). The regenerated shoots were further transferred to rooting medium for full plant formation with extensive root system (Figure 4.17). The percentages of 90 and 73 of 26 hygromycin resistant plants have shown GFP and GUS activity in roots and leaves (Figure 4.18 and 4.19). Ten transgenic plants from 6 hygromycin resistant clones were transferred to the nursery and grown to flowering and seed set (Figure 4.20-4.23). All of these plants had no phenotypic changes. Most of them exhibited blue staining for GUS in roots and leaves. However, 10% and 20% of them exhibited no GUS activity in anther and stigma, respectively (Table 4.11).

Table 4.10 Effect of basal medium (in the selection medium) on the percentage of transformation of KDML105.

Medium/ Hyg conc. (mg/l)	Number of calli						Percentage of transformation[1]
	Cocultivated calli	GFP^+/HygR 3 weeks after cocultivation	HygR calli 5 weeks after cocultivation	GFP^+/HygR 5 weeks after cocultivation	HygR calli on regeneration medium	Calli produced HygR plants	
NB/25	99	25	6	2	6	0	0
NB/40	100	26	26	25	26	15	15

[1] The data were obtained in four weeks after transferring to regeneration medium.

Table 4.11 Expression of GUS gene in the transgenic KDML105 (R_0).

Transformant[1]	Expression of GUS gene				Number of R_1 seeds
	Roots	Leaves	Anther	Stigma	
1	+	+	+	+	41
2a	+	+	+	+	-
2b	+	+	+	+	-
3a	+	+	+	+	-
3b	+	+	+	+	17
4a	+	+	+	-	24
4b	+	+	+	+	20
5	+	+	-	-	-
6a	+	+	+	+	-
6b	+	+	+	+	50

[1] Transformants with designations that differ only in the letters of the alphabet were regenerated from a single hygromycin resistant clone.

Figure 4.12 Calli grown on selection medium based on NB medium supplemented with 40 mg/l hygromycin.

Figure 4.13 Hygromycin resistant calli culturing on selection medium based on NB medium supplemented with 40 mg/l hygromycin.

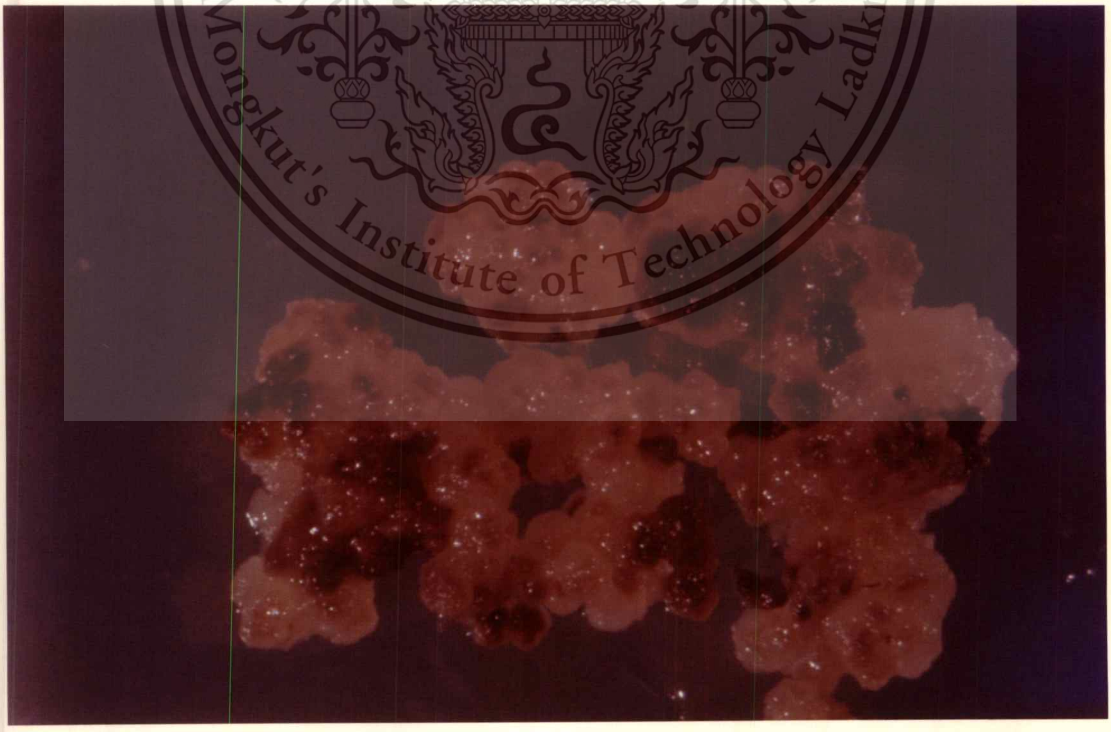

Figure 4.14 Hygromycin sensitive calli culturing on selection medium based on NB medium supplemented with 40 mg/l hygromycin.

Figure 4.15 Expression of GFP in hygromycin resistant calli.

Figure 4.16 Regeneration of hygromycin resistant plants 4 weeks after transferring to regeneration medium containing 6 g/l phytagel and 50 mg/l hygromycin.

Figure 4.17 Transgenic plants with multiple tillers.

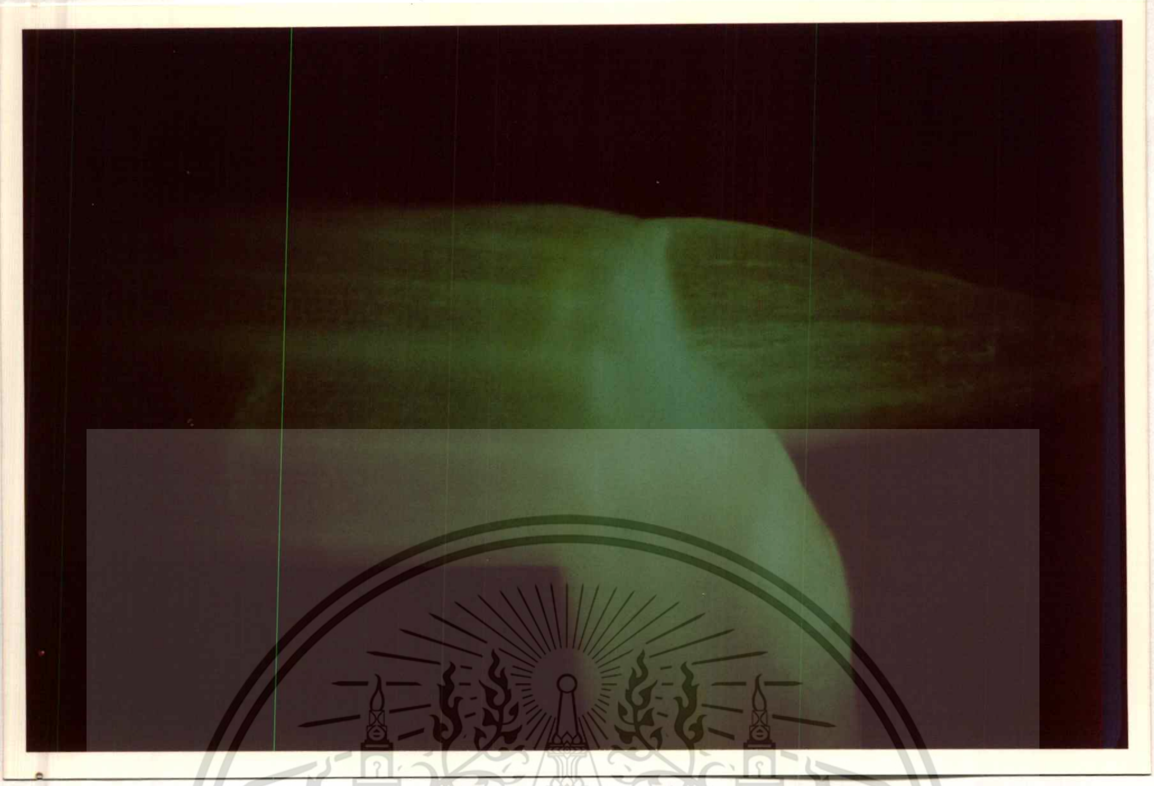

Figure 4.18 Expression of GFP in leaf of transgenic plant.

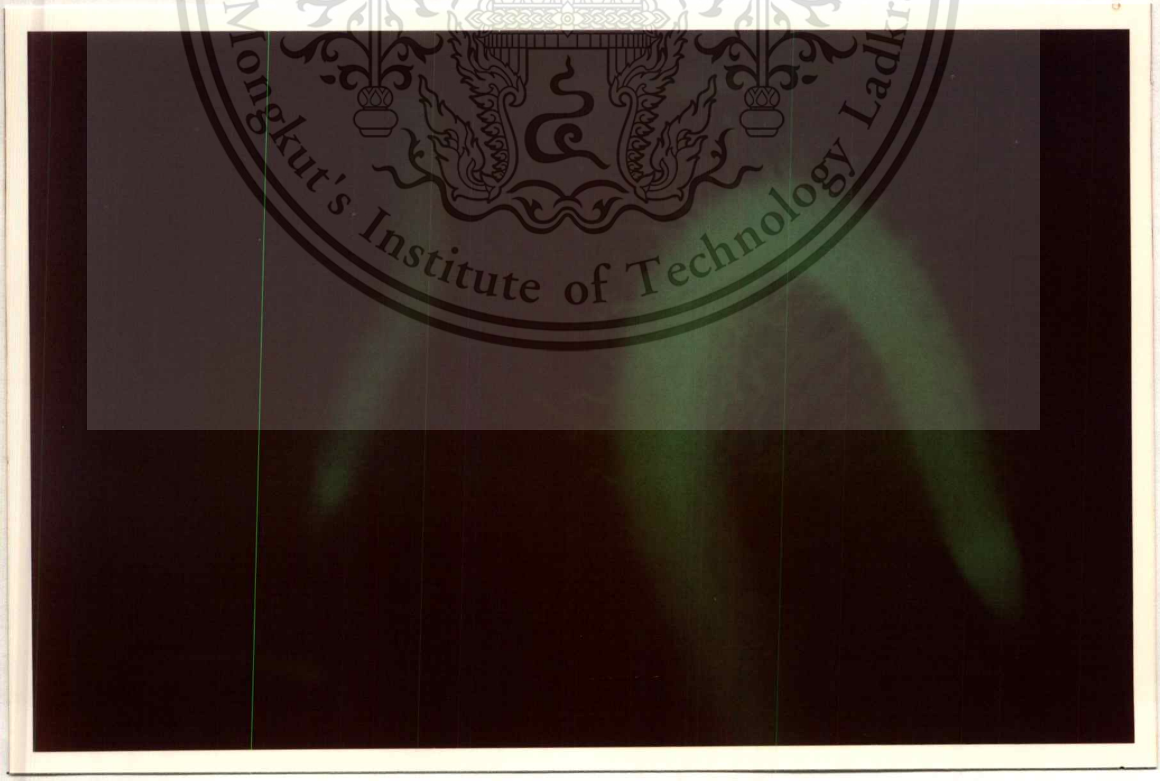

Figure 4.19 Expression of GFP in root of transgenic plant.

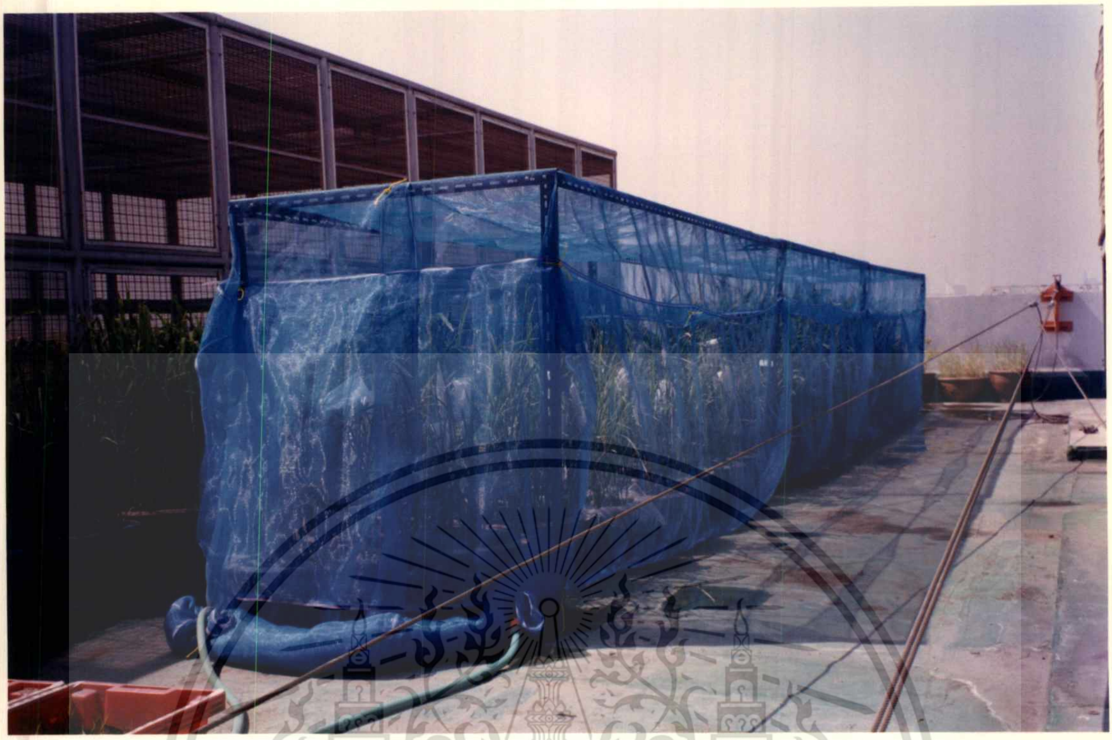

Figure 4.20 Transgenic plants in the nursery.

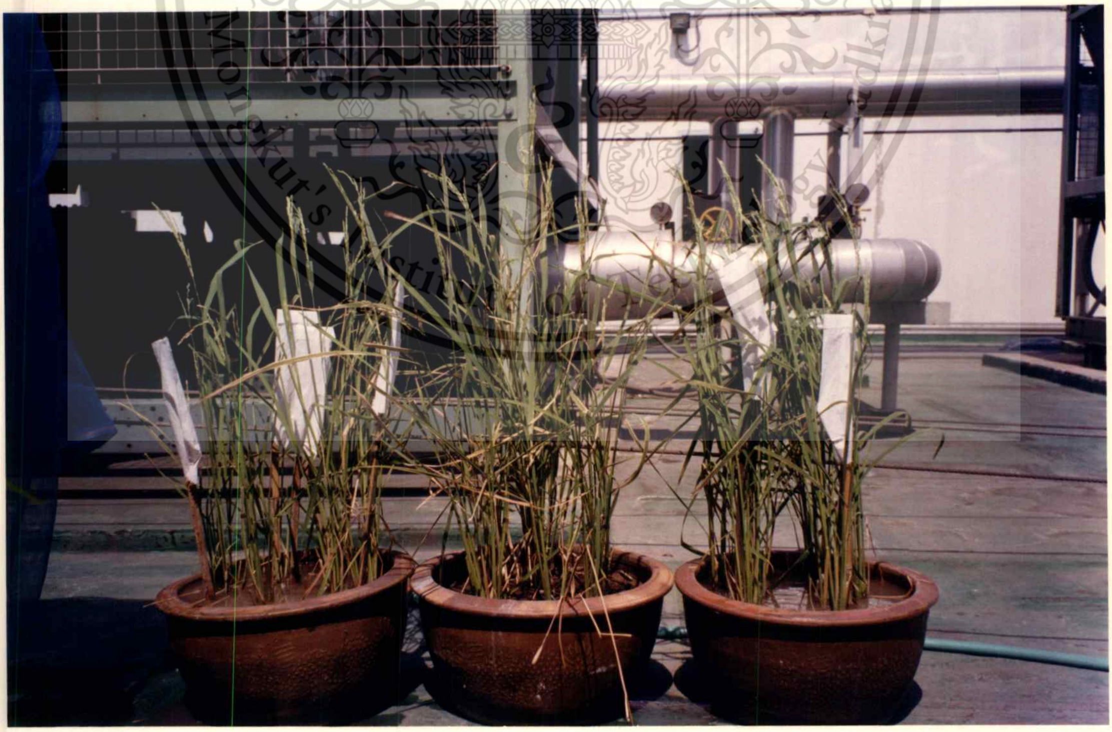

Figure 4.21 Transgenic plants (left and right) and controlled plant regenerated from subcultured calli (middle).

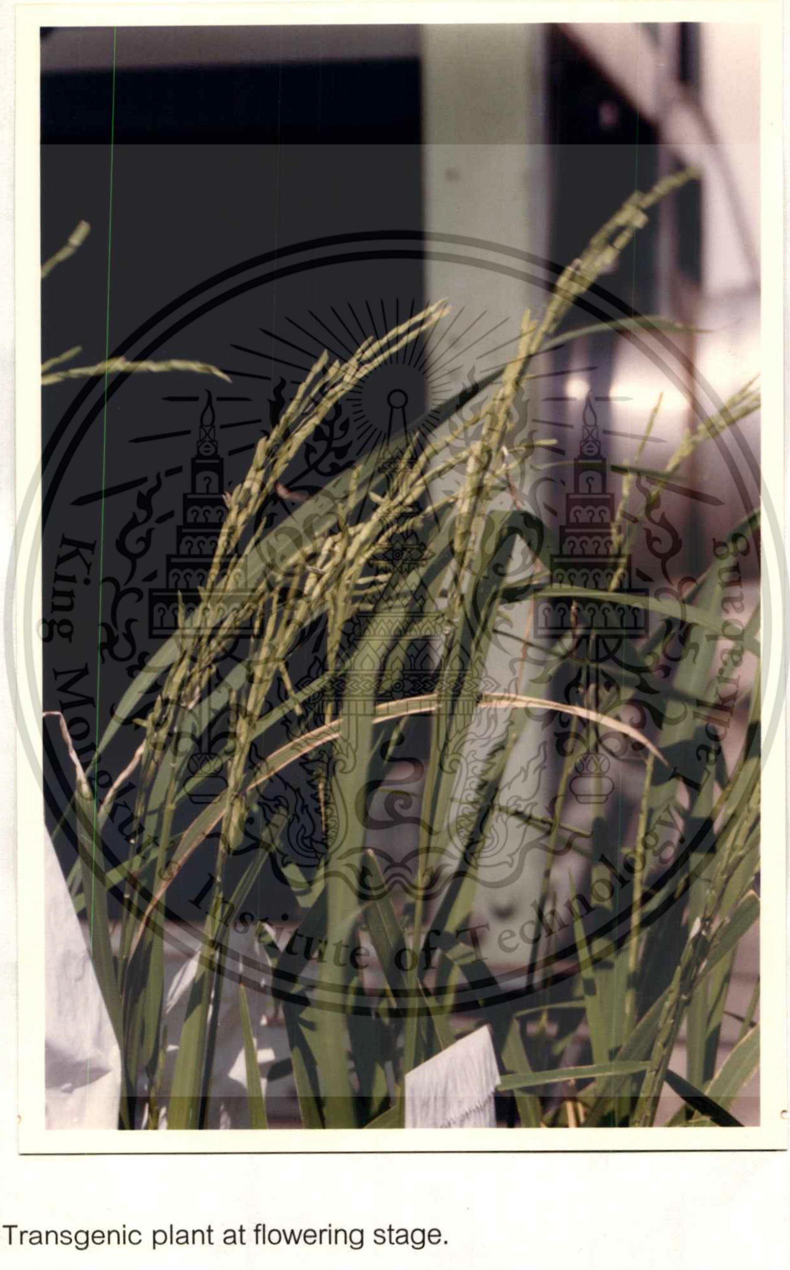

Figure 4.22 Transgenic plant at flowering stage.

Figure 4.23 Transgenic plant at maturity stage.

4.10 Effect of hygromycin concentration (in the selection medium) on the frequency of transient transformation of KDML105

The small globular calli were cocultivated with *Agrobacterium tumefaciens* strain EHA105(pCAMBIA1301/30063) on R_2S cocultivation medium solidified in the dark at 25° C for 3 days. After cocultivation, the calli were transferred to selection medium, based on NB medium supplemented with hygromycin at various concentrations (25, 30, 40, and 50 mg/l) and incubated in the dark for three weeks. The frequencies of transient transformation were determined. This frequency corresponds to the ratio of the number of GUS-positive calli to the initial number of cocultivated calli, and multiplied by 100. The results obtained are presented in Table 4.12. The higher frequencies of transient transformation, 27.55% and 24% were obtained from calli grown on selection medium based on NB medium supplemented with 40 and 50 mg/l hygromycin, respectively. The GUS-negative calli turned brown and died during the selection on these two media. The overgrowth of GUS-negative/hygromycin resistant calli was found in the calli grown on selection medium based on NB medium supplemented with 25 and 30 mg/l hygromycin. The results of this study indicated that the hygromycin concentrations at 25 and 30 mg/l are not strong enough to inhibit the growth of the untransformed cells.

Table 4.12 Effect of hygromycin concentration (in the selection medium) on the frequency of transient transformation of KDML105.

Hygromycin concentration (mg/l)	The initial number of calli	The number of GUS-positive calli[1]	Frequency of Transient transformation (%)
25	97	17	17.53
30	100	18	18
40	98	27	27.55
50	100	24	24

[1] The data were obtained in three weeks after cocultivation with *Agrobacterium tumefaciens*.

The GUS-positive/hygromycin resistant calli were subcultured on selection medium based on NB medium supplemented with 40 or 50 mg/l hygromycin (Figure 4.24). After two weeks, 11 and 9 clones from calli grown on selection medium based on NB medium supplemented with 25 and 30 mg/l hygromycin, respectively turned brown and did not proliferate any hygromycin resistant embryogenic calli. These calli were discarded from the experiment (Table 4.13). All of the hygromycin resistant calli were then transferred to RN regeneration medium containing 6 g/l phytagel and 50 mg/l hygromycin, incubated in the dark for one week, followed by being incubated in the light for three weeks to induce organogenesis. The percentages of transformation were determined. This percentage corresponds to the ratio of the number of hygromycin resistant plant to the initial number of cocultivated calli, and multiplied by 100. None of hygromycin resistant plant was obtained from calli grown on selection medium based on NB medium supplemented with 25 mg/l hygromycin while calli grown on selection medium based on NB medium supplemented with 30 and 50 mg/l hygromycin showed a transformation efficiency of 2% and 14%, respectively. The highest percentage of transformation, 18.37% was obtained from calli grown on selection medium based on NB medium supplemented with 40 mg/l hygromycin (Table 4.13). The percentages of 88.24 and 70.59 of 34 hygromycin resistant plants showed blue staining in roots and leaves, respectively (Table 4.14).

Table 4.13 Effect of hygromycin concentration (in the selection medium) on the percentage of transformation of KDML105.

Hygromycin Concentration (mg/l)	Number of calli				Percentage of transformation[1]
	Cocultivated calli	GUS$^+$/HygR 3 weeks after cocultivation	GUS$^+$/HygR 5 weeks after cocultivation	Calli produced HygR plants	
25	97	17	6	0	0
30	100	18	9	2	2
40	98	27	27	18	18.37
50	100	24	24	14	14

[1] The data were obtained in four weeks after transferring to regeneration medium.

Table 4.14 Expression of GUS gene in roots and leaves of hygromycin resistant plants.

Hygromycin Conc. (mg/l)	Number of transformants	Expression of GUS gene		Percentage of GUS$^+$	
		Roots	Leaves	Roots	Leaves
30	2	2	2	100	100
40	18	16	12	88.89	66.67
50	14	12	10	85.71	71.43
Total	34	30	24	88.24	70.59

Figure 4.24 Expression of GUS hygromycin resistant calli.

4.11 Effect of *Agrobacterium* strain on the frequency of transient transformation of KDML105

The small globular calli were cocultivated with various strains of *Agrobacterium tumefaciens*, EHA105(pCAMBIA1301), AGL1(pCAMBIA1301) or LBA4404 (pCAMBIA1301) on R_2S cocultivation medium solidified in the dark at 25°C for 3 days. After cocultivation, the calli were transferred to selection medium, based on NB medium supplemented with 40 mg/l hygromycin and incubated in the dark for three weeks. The frequencies of transient transformation were determined. This frequency corresponds to the ratio of the number of GUS-positive calli to the initial number of cocultivated calli, and multiplied by 100. The results obtained are presented in Table 4.15. The results of this study indicated that the *Agrobacterium tumefaciens* strains EHA105 (pCAMBIA1301), AGL1(pCAMBIA1301), and LBA4404(pCAMBIA1301) are able to transform calli of KDML105, but EHA105(pCAMBIA1301) was definitely the most effective for transformation of KDML105 in comparison to the others.

Table 4.15 Effect of *Agrobacterium* strain on the frequency of transient transformation of KDML105.

Agrobacterium strain	The initial Number of calli	The number of GUS-positive calli[1]	Frequency of transient transformation (%)
EHA105(pCAMBIA1301)	98	30	30.61
AGL1(pCAMBIA1301)	100	20	20
LBA4404(pCAMBIA1301)	100	20	20

[1] The data were obtained in three weeks after cocultivation with *Agrobacterium tumefaciens*.

The GUS-positive/hygromycin resistant calli were subcultured on selection medium based on NB medium supplemented with 40 mg/l hygromycin for two weeks. These calli were then transferred to RN regeneration medium containing 6 g/l phytagel and 50 mg/l hygromycin, incubated in the dark for one week, followed by being incubated in the light for three weeks to induce organogenesis. The percentages of transformation were determined. This percentage corresponds to the ratio of the number of hygromycin resistant plant to the initial number of cocultivated calli, and multiplied by 100. The results obtained are presented in Table 4.16. The highest percentage of transformation, 17.35%, was obtained from calli cocultivated with *Agrobacterium tumefaciens* strain EHA105(pCAMBIA1301) while 9% and 10% were obtained from calli cocultivated with *Agrobacterium tumefaciens* strains AGL1(pCAMBIA1301) and

LBA4404(pCAMBIA1301), respectively. The percentages of 80.56 and 63.89 of 36 hygromycin resistant plants showed blue staining in roots and leaves, respectively (Table 4.17).

Table 4.16 Effect of *Agrobacterium* strain on the percentage of transformation of KDML105.

Agrobacterium strain	Number of calli				Percentage of transformation[1]
	Cocultivated calli	GUS^+/HygR 3 weeks after cocultivation	GUS^+/HygR 5 weeks after cocultivation	Calli produced HygR plants	
EHA105 (pCAMBIA1301)	98	30	30	17	17.35
AGL1 (pCAMBIA1301)	100	20	20	9	9
LBA4404 (pCAMBIA1301)	100	20	20	10	10

[1] The data were obtained in four weeks after transferring to regeneration medium.

Table 4.17 Expression of GUS gene in roots and leaves of hygromycin resistant plants.

Agrobacterium Strain	Number of Transformants	Expression of GUS gene		Percentage of GUS^+	
		Roots	Leaves	Roots	Leaves
EHA105 (pCAMBIA1301)	17	14	12	82.35	70.59
AGL1 (pCAMBIA1301)	9	7	6	77.78	66.67
LBA4404 (pCAMBIA1301)	10	8	5	80	50
Total	36	29	23	80.56	63.89

CHAPTER 5

DISCUSSION

A number of factors have been examined to improve the frequency of somatic embryogenesis and plant regeneration in rice. Different reports have shown the followings to affect plant regeneration frequency in rice: genotype, callus size, hormonal composition of the medium, partial desiccation or water stress, and other medium supplements. Addition of cytokinins in both callus induction and regeneration medium has been reported to be beneficial for shoot regeneration in *indica* rice varieties TN1, IR72 and IR64 (Rance *et al.*, 1994; Sivamani *et al.*, 1996). Lee *et al.* (1989) showed that addition of BAP in the regeneration medium had a positive effect on regeneration frequencies and number of plant produced. It was found in this experiment that the calli regenerated well when they were grown on NB medium but not on media containing cytokinins, suggesting that NB medium is an optimized callus induction and propagation medium for KDML105. The difference seen between cultivars, TN1, IR72, IR64 and KDML105, in response to cytokinins in the callus induction medium might be due to variations in the endogenous levels of hormones.

In the regeneration experiments, another important factor was the phytagel concentration used for medium solidification. When the phytagel concentrations of the regeneration medium were increased from 3 g/l to 6 g/l and 7.5 g/l, the frequency of shoot formation increased considerably. The shoot regeneration frequency of KDML105 was over 15 folds in the 6 g/l and 7.5 g/l phytagel containing media compared to the medium with 3 g/l phytagel. Phytagel, at higher concentrations, by virtue of its solidifying effect of the medium, may limit water uptake by tissues. Consequently, the tissues growing on higher concentrations of phytagel were drier. This condition may also improve the oxygen supplied to the embryogenic cells. There are reports of an increase in the growth of cells and protoplasts of several plant species, including rice, in oxygen-enriched atmospheres (d'Utra Vaz *et al.*, 1992; Anthony *et al.*, 1994). A similar stimulatory effect of solidifying a gent on plant regeneration from calli of *indica* rice varieties has been reported by Jain *et al.* (1996). They reported that when the agarose concentration of the regeneration medium was increased from 0.5% to 1%, shoot regeneration from cell suspension-derived calli of IR43 and Pusa Basmati 1 increased by over 8 folds, from 6% and 11% to 53% and 86%, respectively.

Tsukahara and Hirosawa (1992) reported that a 24 hours dehydration of suspension culture-derived calli of the japonica variety "Sasanishiki" enhanced their regeneration frequency from 5% up to 47%. Rance *et al.*, (1994) reported that partial desiccation (21-23 hours dehydration) of mature embryo-derived calli dramatically promotes organogenesis and leads to high regeneration ability. They were able to obtain 66.5%, 61.1% and 73.7% of calli that regenerated into plants for the *indica* varieties TN1, IR72 and IR64 whereas in non-desiccated controls only 30%, 15.5% and 18.7% of calli regenerated, respectively. In a recent study, Jain *et al.* (1996) reported that dehydration of the callus for 24 hours enhanced shoot regeneration in *indica* varieties, Basmati 385, IR43 and Pusa Basmati 1. The best regeneration frequencies, 54-98%, were obtained when 24 hours desiccated calli were grown on regeneration medium with 1% (w/v) agarose. In the present study, partial desiccation stimulated the percentage of regeneration in calli regenerated on regeneration medium containing 3 g/l phytagel, from 5.56% to 16%, but not in calli regenerated on regeneration medium containing 6 g/l phytagel.

It has been well established that water stressed plants show higher abscisic acid (ABA) biosynthetic activity and ABA contents. However, it is not clear how the ABA levels of the cells or tissues influence the morphogenic potential (Jain *et al.*, 1996). Peterson and Smith (1991) reported that the plant regenerations of Texas rice cultivars (Lemont, Rico 1, Rexmont, and Skybonnet) and of Taipei 309 were enhanced by 2-10 folds when the calli were cultured on pre-culture medium containing 2.6-26 mg/l ABA prior to being placed on the regeneration medium. Tsukahara and Hirosawa (1992) reported that addition of 0.1-100 mg/l ABA to the regeneration medium did not increase the regeneration frequency of *japonica* rice variety Sasanishiki. In this study, effect of ABA in the pre-regeneration medium on plant regeneration was tested, and no effects were observed within the concentration tested, 5 mg/l. Although it has been reported that ABA and NaCl treatments of rice suspension cells provoked the expression of similar genes, this drying treatment could not be substituted by exogenous applied ABA (Sinozaki *et al.*, 1989).

In the present study, there were at least three key factors affected the efficiency of transformation of KDML105. Firstly, the addition of acetosyringone to the media during cocultivation of calli with *Agrobacterium*, secondly, the choice of explant as starting material (6-8 weeks old scutellum derived, embryogenic calli) and finally the addition of a suitable concentration of hygromycin to the selection medium.

Monocyclic phenolic compounds, such as acetosyringone has been found to stimulate *Agrobacterium vir* gene expression and to extend the host range of some *Agrobacterium tumefaciens* strains (Boulton *et al.*, 1989; Godwin *et al.*, 1991; Sheng and Citovsky, 1996). Acetosyringone was also found to be non-lethal to *Agrobacterium* at concentration greater than 200 µM (Stachel *et al.*, 1985). Previously, Hiei *et al.* (1994) indicated that addition of acetosyringone to the medium during cocultivation was important for enhancing transfomation on *japonica* rice. Rashid *et al.* (1996) reported that the presence of acetosyringone during cocultivation has played a vital role for transformation efficiency of *indica* rice, Basmati 370. Transformation was not successful when acetosyringone was omitted from the medium. In this study, inclusion of acetosyringone at concentrations ranging from 50 to 250 µM to the medium during cocultivation resulted in increased the frequency of transient transformation. Transformation was not successful when 500 µM of acetosyringone was added to the medium. Acetosyringone, at high concentration, may inactivate the growth of the calli, making the calli not suitable for transformation.

The choice of explants as starting material was one of the most important factors. Scutellum and scutellum-derived calli were shown to be excellent starting materials for transformation of rice by *Agrobacterium* in many studies (Hiei *et al.*, 1994; Vijayachandra *et al.*, 1995; Rashid *et al.*, 1996). In the transformation experiments, different ages of calli were analyzed for their responses to cocultivation with *Agrobacterium tumefaciens*. The variation found between different ages of calli in their responses to *Agrobacterium*-mediated transformation may be due to differences in their ability to induce *vir* gene or to produce conditions, which increase the success of T-DNA transfer. The 7 days-old scutellum-derived calli showed high frequency of transformation immediately after cocultivation, but all of them died during the selection. When the 6-8 weeks-old scutellum-derived embryogenic calli were used for transformation experiments, approximately 18% of the cocultivated calli could grow during the selection and could regenerate into the hygromycin resistant plants. Hiei *et al.* (1994) also described that prior to infection, pre-culturing of calli in a fresh medium for 4 days was an important step for *japonica* rice transformation. Recently, Vijayachandra *et al.* (1995) showed that pre-incubation of scutella for 72 hours is required for efficient *vir* induction. Pre-incubation is likely to help in the transformation of rice scutellum. Pretreatment of tissues, for example, by wounding or enzymatic digestion of cell walls were also found to be essential in other studies (Raineri *et al.*, 1990; Mooney *et al.*, 1991; Chan *et al.*, 1993; Park *et al.*, 1996). Such pre-treatments were not necessary in the present transformation experiments.

A key step in obtaining transgenic plants is the selection of transformed calli or plants. The hygromycin phosphotransferase gene was widely used in many rice transformation experiments to confer resistance to the amino-glycoside antibiotic hygromycin B. Hygromycin allows good discrimination between transformed and untransformed tissues and does not seem to cause albinos or fertility problems (Ayres and Park, 1994; Hiei *et al.*, 1994; Rashid *et al.*, 1996). It was found in this experiment that it was important to add the suitable concentrations of hygromycin (40 or 50 mg/l) to the selection medium immediately after the cocultivation in order to obtain the efficient transformation of KDML105, and the hygromycin concentration at 25 and 30 mg/l were not strong enough to inhibit the growth of the untransformed cells.

When calli were infected with *Agrobacterium tumefaciens* using the conditions described above, high percentage of transformation of KDML105 (between 15 and 18.37%) was obtained. The percentage of transformation of KDML105 was as high as reported in other *japonica* and *indica* cultivars (Hiei *et al.*, 1994; Aldemita and Hodges, 1996; Rashid *et al.*, 1996)

The β-D-glucuronidase (GUS) gene has become the most commonly and routinely used reporter gene system for several reasons such as the enzyme is very stable under different physiological conditions and the GUS activity can also be detected *in situ* by histochemical analysis; nevertheless, the assay of GUS expression is cytotoxic (Jefferson *et al.* 1987; Thomasset *et al.*, 1996). In contrast, the green fluorescent protein (GFP) gene has recently been shown to have the characteristics of a reporter and selection marker (Chalfie *et al.*, 1994). The advantage of this gene as a reporter is that expression can be detected in living cells and no exogenous substrate and cofactors are required. The present study demonstrated that GFP gene from jelly fish could be expressed in calli, leaves, and roots of KDML105. This suggests that the GFP gene may be a useful reporter/marker for genetic transformation in rice. GFP could also be useful in developing and optimizing transformation methods by continuously monitoring each transformation event at different stages (Pang *et al.*, 1996). Combination of a selection marker gene (such as the hygromycin phosphotransferase gene) with GFP might facilitate the recovery of GFP expressing cells and the identification of transformed cells.

CHAPTER 6

CONCLUSION AND SUGGESTION

In conclusion, the results in the present study showed that NB medium is an optimized medium for promoting callus induction, embryogenesis and plant regeneration of KDML105. A shoot regeneration frequency of between 73.45 and 82.22% has been obtained from calli cultured on this medium. The phytagel concentration of the regeneration medium is another important factor for the induction of somatic embryogenesis and high frequency plant regeneration from scutellum-derived calli of KDML105. When the phytagel concentration of the regeneration medium was increased from 3 g/l to 6 or 7.5 g/l, the frequency of shoot formation increased by over 15 folds, from 5.56% to 82.22 or 86.67%, respectively.

From the present study, it was demonstrated that inclusion of acetosyringone to the medium during cocultivation is important for increasing the frequency of transient transformation. The highest frequency, 86.67%, has been obtained from calli cocultivated on NB medium supplemented with 50 μM acetosyringone. The choice of explants as starting material and the addition of a suitable concentration of hygromycin to the selection medium were other important factors for the successes in producing a high efficiency of transformed rice plants. The 6-8 weeks-old scutellum-derived embryogenic calli were shown to be starting material in these transformation experiments. Selection medium based on NB medium supplemented with 40 mg/l hygromycin is an optimized medium for select transformed rice cells from a mixed population of transformed and untransformed cells, and selection should be applied immediately after the cocultivation for obtaining the efficient transformation. A percentage of transformation between 15 and 18.37% was obtained from calli grown on this medium.

The present study also indicated that selection medium based on NB medium was more suitable than selection medium based on R_2 medium. *Agrobacterium tumefaciens* strain EHA105, AGL1 and LBA4404 were able to transform calli of KDML105, but

EHA105 was definitely more effective than the others. The GUS gene and GFP gene present as the reporter can be expressed in calli, leaves and roots of KDML105. They can be used to assess the efficiency of transformation. Moreover, unlike the assay for GUS gene expression, which is enzymatic and cytotoxic, GFP can be detected in living cell. This suggested that GFP gene could also be useful in developing and optimizing transformation protocol.

These regeneration and transformation procedures could be possible to apply to other recalcitrant cultivars of rice and with modification to other monocot species. Confirmation of transformation by Southern blot analysis for the presence of the foreign genes and by transmission of foreign genes expression in the progeny in a Mendelian manner should be further done.

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Appendix

Table 1 Formulation of basal NB medium.

Compound		Amount per liter (mg/l)
N6 macro elements	KNO_3	2830
	$(NH_4)_2SO_4$	463
	KH_2PO_4	400
	$CaCl_2.2H_2O$	165
	$MgSO_4.7H_2O$	185
	$FeSO_4.7H_2O$	27.8
	Na_2EDTA	37.3
B5 micro elements	$MnSO_4.H_2O$	10
	H_3BO_3	3
	$ZnSO_4.7H_2O$	2
	KI	0.75
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$CoCl_2.6H_2O$	0.025
B5 vitamins	Myo-inositol	100
	Nicotinic acid	1
	Pyridoxine HCl	1
	Thiamine HCl	10
	L-proline	500
	L-glutamine	500
	Casein hydrolysate	300

Table 2 Formulation of basal R_2 medium.

Compound		Amount per liter (mg/l)
R_2 macro elements	KNO_3	4000
	$(NH_4)_2SO_4$	330
	$NaH_2PO_4.H_2O$	312
	$MgSO_4.7H_2O$	246
	$CaCl_2.2H_2O$	146
	$FeSO_4.7H_2O$	12.5
	Na_2EDTA	1.7
R_2 micro elements	$MnSO_4.H_2O$	1.6
	H_3BO_3	2.83
	$ZnSO_4.7H_2O$	2.2
	$Na_2MoO_4.2H_2O$	0.125
	$CuSO_4.5H_2O$	0.195
LS vitamins	Thiamine HCl	1

Table 3 Formulation of AB medium.

Compound		Amount per liter (mg/l)
AB buffer	K_2HPO_4	1500
	NaH_2PO_4	200
AB salt	NH_4Cl	1000
	$MgSO_4.7H_2O$	300
	KCl	150
	$CaCl_2.2H_2O$	150
	$FeSO_4.7H_2O$	2.5
	Glucose	5 g/l
	Agar	15 g/l

Table 4 Formulation of AAM liquid medium.

Compound		Amount per liter (mg/l)
AA macro elements	$Na_2HPO_4.2H_2O$	169.6
	$MgSO_4.7H_2O$	500
	KCl	2950
	$CaCl_2.2H_2O$	150
	$FeSO_4.7H_2O$	28
AA micro elements	$MnSO_4.4H_2O$	10
	H_3BO_3	3
	$ZnSO_4.7H_2O$	2
	KI	0.75
	$Na_2MoO_4.2H_2O$	0.25
	$CuSO_4.5H_2O$	0.0387
	$CoCl_2.6H_2O$	0.025
AA amino acids	Glycine	7.5
	Arginine	174
	Glutamine	876
MS vitamins	Myo-inositol	100
	Nicotinic acid	0.5
	Pyridoxine HCl	0.5
	Thiamine HCl	0.5
	Casamino acids	500
	Sucrose	68.5 g/l
	Glucose	35 g/l
	Acetosyringone	100 µM

Table 5 Formulation of X-gluc solution.

Composition	Amount
Phosphate buffer	50 mM
X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid	1 mg/ml
Triton X-100	0.5%
Methanol	20%

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