Establishment of in *Vitro* Regeneration Systems and Gene Transformation into *Indica* Rice (*Oryza sativa* L.) Landrace *Hom Kra Dang Ngah*

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A Thesis Submitted in Fulfillment of the Requirements for the Degree of

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Thesis Title Establishment of in *Vitro* Regeneration Systems and Gene Transformation into *indica* Rice (*Oryza sativa* L.) Landrace *Hom Kra Dang Ngah*

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Major Program Plant Science

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ABSTRACT

Indica rice, landrace *Hom Kra Dang Nagh*, is a kind of favorable rice in Thailand and no regeneration and genetic transformation systems has been reported earlier. In order to improve its quality by the genetic transformation, the present study developed a tissue culture and plantlet regeneration systems, established *Agrobacterium*-mediated transformation systems and molecular analysis to confirm introduction of foreign gene into putative rice transformants. Mature embryos from seeds were used to induce callus and establish regeneration system. The optimum culture medium was obtained by manipulating different concentrations of various plant growth regulators (PGRs). Experimental results showed that addition of 2 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) in combination with 1.0 mg/L α-naphthaleneacetic acid (NAA), 1.0 mg/L 6-benzyladenine (6-BA) and 0.5 mg/L kinetin (Kn) to callus induction medium which was basal MS medium gave the most suitable for embryogenic callus induction at 56.3%. The calli derived from seeds were proliferated on different culture media, such as MS, N6, Agricultural Research Development Agency（ARDA) supplemented with different concentrations of NAA, Kn and 6-BA. In addition, different types of carbohydrates as carbon source and concentrations of phytagel were also investigated. The results showed that ARDA medium supplemented with 0.5 mg/L NAA in combination with 1.0 mg/L 6-BA, 2.0 mg/L Kn, 82 mM sorbitol and 1 g/L casein hydrolysate, solidified with 0.3% phytagel gave the highest responsive for plantlet regeneration at 75%. Finally, the

transformation experiment was carried out by optimizing three important parameters: age of calli, infection time and optical density (OD) of *Agrobacterium* suspension. The results showed that the six-week-old embryogenic calli (EC) infected with bacterial suspension at OD_{600} of 0.6 for 20 minute gave the highest frequency of *gus* gene expression at 83.5%. After selecting transformed embryogenic callus on culture medium containing 0.5mM glyphosate, 78.6% of the callus could survive and have a normal growth under this condition. Polymerase chain reaction (PCR) revealed the integration of target genes into rice genome.

ชื่อวิทยานิพนธ์ การพัฒนาระบบการชักนำพืชต้นใหม่ในหลอดทดลอง และการถ่ายยีนเข้าสู่ข้าวชนิด Indica (*Oryza sativa* L.) พันธุ์พื้นเมืองหอมกระดังงา

ผู้เขียน Mrs. Yinxia Zhang

สาขาวิชา พืชศาสตร์

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บทคัดย่อ

ข้าวหอมกระดังงาเป็นข้าวชนิดหนึ่งที่นิยมรับประทานในประเทศไทย อย่างไรก็ตามยังไม่มีรายงานการศึกษาการชักนำการพัฒนาเป็นพืชต้นใหม่ และการปลูกถ่ ายยีนในข้าวพันธุ์ดังกล่าวมาก่อน เพื่อปรับปรุงพันธุ์ข้าวชนิดนี้ให้มีคุณภาพลักษณะที่ต้องการด้วยวิธีการถ่ายยีน ในการศึกษานี้จึงได้พัฒนาระบบการเพาะเลี้ยงเนื้อเยื่อ และการชักนำการสร้างพืชต้นใหม่ที่มีประสิทธิภาพ พัฒนาระบบการถ่ายยีนที่มีประสิทธิภาพผ่านอะโกรแบคทีเ รีย และตรวจสอบประสิทธิภาพการถ่ายยีนด้วยการใช้เครื่องหมายโมเลกุลเพื่อยืนยันการคัดเลือกแคลลัส หรือต้นที่ได้รับการถ่ายยีน ในการศึกษาใช้เมล็ดข้าวที่สุกแก่ของข้าวหอมกระดังงามาชักนำแคลลัส และส่งเสริมการพัฒนาเป็นพืชต้นใหม่ ผลที่ได้จากการศึกษานี้ พบว่าอาหารที่เห มาะสมต่อการเพาะเลี้ยงเติมสารควบคุมการเจริญเติบโตชนิด และความเข้มข้นที่แตกต่างกัน อาหารสูตร Murashige และ Skoog (MS) เติม 2,4-dichlorophenoxyacetic acid (2,4-D) เข้มข้น 2 มิลลิกรัมต่อลิตร α-napthaleneacetic acid (NAA) เข้มข้น 1 มิลลิกรัมต่อลิตร 6-benzyladenine (BA) เข้มข้น 1 มิลลิกรัมต่อลิตร และ Kinetin (Kn) เข้มข้น 0.5 มิลลิกรัมต่อลิตร ให้การชักนำเอ็มบริโอเจนิคแคลลัสได้ดีที่สุด 56.3 เปอร์เซ็นต์ แคลลัสเพิ่มปริมาณได้ดีบนอาหารสูตร คือ MS N6 และ ARDA เติมสารความคุมการเจริญเติบโต NAA Kn และ BA ความเข้มข้นต่างๆ นอกจากนี้ยังพบว่า แหล่งคาร์บอน และ ความเข้มข้นของวุ้นไฟต้าเจลมีผลต่อการเพิ่มปริมาณแคลลัส การเพาะเลี้ยงแคลลัสบนอาหารสูตร Agricultural Research Development Agency (ARDA) เติม BA เข้มข้น 1 มิลลิกรัมต่อลิตร Kn เข้มข้น 2 มิลลิกรัมต่อลิตร ซอร์บิทอล เข้มข้น 82 มิลลิโมลาร์ เคซีนไฮโดรไลเสท (CH) เข้มข้น 1 มิลลิกรัมต่อลิตร และไฟต้าเจล เข้มข้น 0.3 เปอร์เซ็นต์ ให้การพัฒนาเป็นพืชต้นใหม่สูงสุด 75 เปอร์เซ็นต์ สำหรับการถ่ายยีนในเบื้องต้นพบว่า อายุของแคลลัส ระยะเวลาในการจุ่มแช่ และความเข้มข้นของเชื้ออะโก รแบคทีเรีย มีผลต่อความสำเร็จ แคลลัสอายุ 6 สัปดาห์ จุ่มแช่ในเชื้ออะโกรแบคทีเรียมเข้มข้น OD_{600} เท่ากับ 0.6 เป็นเวลา 20 นาที ให้การ

แสดงออกของยีน *gus* สูงสุด 83.5 เปอร์เซ็นต์ เมื่อคัดเลือกแคลลัสบนอาหารที่เติมสารไกลโฟเสทเข้มข้น 0.5 มิลลิโมลาร์ ให้อัตราการรอดชีวิตสูงสุด 78.6 เปอร์เซ็นต์ และพบการปรากฏของยีนดังกล่าวหลังจากการปลูกถ่ายยีนเมื่อตรวจสอบโดยเทคนิคโพลีเมอเรสเชนรีแอคชัน (PCR)

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Yinxia Zhang

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List of Abbreviations and Symboles

ARDA	=	Agricultural research development agency
AS	=	Acetosyringone
BA	=	6-benzyladenine
bp	=	Base pair
cef	=	Cefotaxime sodiam
CH	=	Casein Hydrolysate
CIM	=	Callus induction medium
CTAB	=	Hexadecyltrimethylammonium bromide
2,4-D	=	2,4-Dichlorophenoxyacetic acid
DMRT	=	Duncan's multiple range test
DNA	=	Deoxyribonucleic acid
EPSPs	=	5-Enolpyruvylshikimate 3-phosphate synthase
EC	=	Embryogenic calli
dNTP	=	Deoxynucleotide triphosphate
EDTA	=	Ethylenediaminetetraacetic acid
gus	=	β-glucuronidase gene
GUS	=	β-glucuronidase gene
HCl	=	Hydrochloric acid
IAA	=	Indole-3-acetic acid
IBA	=	Indole-3-butyric acid
Km	=	Kanamycin
Kn	=	Kinetin
L	=	Litre
M	=	Molar
mg	=	milligram
M1	=	DNA ladder1000 bps

List of abbreviations and symbols (Continued)

MS	=	Murashige and Skoog (medium)
NAA	=	α- Naphthaleneacetic acid
N_6	=	Chu medium
$NaCl_2$	=	Sodium chloride
Na_2EDTA	=	Sodium ethylenediaminetetraacetate
OD	=	Optical density
PCR	=	Polymarese chain reaction
RCBD	=	Randomized complete block design
SAS	=	Statistical analysis software
SCM	=	Subculture medium
SD	=	Standard deviation
SE	=	Somatic embryo
SIM	=	Shoot induction medium
TDZ	=	Thidiazuron
TE	=	Tris EDTA
TAE	=	Tris-acetic acid-disodium ethylenediaminetetraacetic acid
TBE	=	Tris-boric acid- disodium ethylenediaminetetraacetic acid
Tris	=	Tris (hydroxymethyl) aminomethane
µ l	=	Microlitre
µ mol	=	Micromolar

List of Papers and Proceedings

Zhang, Y.X. and Te-chato, S. 2012. Callus induction and plantlet regeneration from mature embryos of *indica* rice (*Oryza sativa* L.) cultivar *Kra Dang Ngah*. Journal of Agricultural Technology 8: 2423-2433.

Zhang, Y.X. and Te-chato, S. 2013. Improved plantlet regeneration systems in *indica* rice (*Oryza sativa* L.) landrace *Hom Kra Dang Ngah.* Journal of Agricultural Technology. (Accepted)

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Zhang, Y.X. and Te-chato, S. 2013. The Effect of Plant Growth Regulator on plantlet regeneration in *indica* rice landrace *Hom Kra Dang Nagh*. The 1st From Plant Science to AEC. Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Thailand. 13-14 August 2013 (Oral presentation).

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ขอมอบใบประกาศเกียรติคุณให้แก่

Yinxia Zhang and Sompong Te-chato

ร่วมนำเสนอผลงานภาคบรรยาย

เรื่อง Effect of Plant Growth Regulators on Plantlet Regeneration in indica Rice Landrace Hom Kra Dang Ngah

ในการประชุมวิชาการพืชศาสตร์ ครั้งที่ ๑

"พืชศาสตร์สู่ประชาคมอาเซียน"

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คณบดี

SUMMARY OF CONTENTS

CHAPTER Ⅰ

GENERAL INTRODUCTION

General Introduction

Background

Plant transformation technique becomes important technology in current research of molecular biology and breeding. It makes flow of genetic material possible among animal, plants and microbes due to its capacity to break reproduction barriers. It also provides a desirable tool for a series of researches on plant physiology, biochemistry, development, genetics and so on. A reliable basis for the possibility of transgenic plants will produce expected products not only synthesized in animals (including human) and microbes before fully exploiting plant cell-totipotency but also overwhelming advantage of plants as natural biochemical factory. With its characteristics of high efficiency and rapidity in delivering one or more target genes at a time, transgenics plant turns out to be powerful means in gene function, identification and purposeful improvement of certain genetic traits.

Rice, one of the most important staple food crops in the world, is consumed by more than one third of the world population. Researches on rice biotechnology have been actively pursued to produce transgenic rice plants with improved yield and quality, increased resistance to biotic and abiotic stresses, and value added grains such as golden rice (Lee *et al.*, 2002). Tissue culture via somatic embryogenesis will be a key step in gene transfer and plant regeneration in rice biotechnology. The genetic transformation of rice can be accomplished through *Agrobacterium* co-cultivation of embryogenic calli (Hiei *et al.*, 1994). In general, embryogenic calli is more suitable for gene delivery and regeneration of transgenic rice plants. Therefore, proliferation of the embryogenic calli with the high regeneration capacity is a prerequisite for the successful production of transgenic rice plants.

In spite of the great progress in rice improvement using biotechnology, transformation system in *indica* rice remains difficulty (Zhang *et al.*, 2005). It appears

that *indica* rice is more specific than the *japonica* rice to various tissue culture conditions and generally less responsive to callus induction (Mikami and Kinoshita, 1988). There is no published data regarding the improvement of *indica* rice varieties using genetic transformation. This background and transformation system for the *indica* rice genotypes will be a key step towards the improvement of agronomical and nutritional quality of rice. To address this problem, the present study will be established. Regeneration system and a robust transformation for the introduction of agronomically-important genes into favorite *indica* rice, landrace *Hom Kra Dang Ngah* variety, are described. The *indica* rice, landrace *Hom Kra Dang Ngah*, has shorten harvesting period at 120-150 days, height at 125 cm, one hundred seeds weight 3g and has red color of brown rice as shown in Figure1.1 and Figure 1.2. This variety is widely distributed in *Ra-Ngae* or *Tak Bai* district, Narathiwat province, southern part of Thailand.

Figure 1.1 The characteristics of *indica* rice landrace *Hom Kra Dang Ngah.*

Figure 1.2 *Hom Kra Dang Ngah* seeds (left), brown rice (middle) and white rice (right)

Literature Review

Rice (*Oryza sativa* L.) is a major staple food and one of the most important crops in the worldwide. More than half of the world's population depends on rice for its major daily source of energy and protein. With the increase in population and reduction of arable land, together with the biotic and abiotic stress rice production is under great pressure. Breeding new rice varieties using genetic engineering techniques is one of the ways to increase rice yield and improve the total rice production. In recent year, the genetic transformation technology as a key technology of genetic engineering of rice has made a great progress. At the same time, the rapid, efficient, stable and large-scale genetic transformation system in most *japonica* rice was established. On this basis, a lot of important foreign genes, such as insect and disease resistance, stress tolerance, quality improvement, nutrition gene were transferred into this rice (Chen *et al.*, 2009; Zhu, 2012). *Indica* and *japonica* are two subspecies groups of cultivated rice in the world. Approximately 80% of rice production bases on *indica* (Wanichananan *et al.*, 2010). However, the transformation of *indica* rice is still difficult due to low induction rate of embryogenic callus and plantlet regeneration after transformation. Many *indica* rices are particularly genotype-dependent. Although a few successful genetic transformation was reported, it is still lack of commonly used genetic transformation technologies for *indica* rice and there are many problems to challenge in the practical application.

The history of *Agrobacterium*-mediated genetic transformation in *indica* rice research

Genetic transformation mediated by *Agrobacterium* in *indica* rice started in1992, Chan *et al.* who firstly induced the calli from the immature root. The gene was transformed into calli of rice which was proved by both enzyme digestion and Southern blotting detection. But plantlet regeneration was not obtained. Hiei *et al.*

(1994) constructed some unique vectors called ‘super-binary’ vector and added the acetosyringone in infection liquid medium which have additional *vir* genes in the binary plasmid itself. This modification led to achievement of high transformation efficiency in *japonica* rice (Hiei *et al.*, 1994). This success initiated the study of genetic transformation system of *indica* rice. So far, genetic transformation of some *indica* rice, such as *elita indica*, *Pusa Basmatic*, *R2* and some china *indica* cultivar were obtained (Zhang *et al.*, 1997; Azhakanandam *et al.*, 2000; Kumria *et al.*, 2001; Kumria *et al.*, 2002; Hoque *et al.*, 2005). After that, the focus of the study moved to establish the genetic transformation of the genotype-independent and recalcitrant cultivar from the genotype-dependent weak and easily transformatiom species (Kumar *et al*., 2005; Lin *et al*., 2005; Hiei and Komari, 2006; Hiei *et al*., 2008).

In order to breakthrough the restriction of genotype transformation in *indica* rice, some researchers tried a non-tissue culture by *Agrobacterium*-mediated genetic transformation. Lin *et al.* (2009) used the mature embryos of soaked seeds, pierced by a needle and then soaked in the *Agrobacterium* inoculum under vacuum infiltration. The inoculated seeds were germinated and grown to maturation (T_0) under non-sterile conditions without the use of tissue culture. However, the rate of transformation is low, and the embryogenic primordium of seeds has been formed. In addition, it is difficult to determine whether the T-DNA integrated into the genome of meristematic tissue or cells. So that generation of transgenic plants requires a lot of molecular analysis work (Supartana *et al.*, 2005). Therefore, the technology is still difficult to apply.

So far, there has not been a suitable common platform of genetic transformation method for all kinds of *indica* rice. Accordingly, there are still many problems to be solved in establishing a high efficient and stable genetic transformation system of *indica* rice. Thus, it is necessary to deeply study the factors affecting the *indica* rice transformation frequency.

Procedures for transformation of rice

Systems of *Agrobacterium*-mediated transformation have been well established for many dicotyledonous plants. However, monocotyledonous plants, in particular cereal plants, were originally outside the host range of *A. tumefaciens*. Thus, the initial development of system for genetic transformation of monocotyledons was slow. Attention was focused on other methods, such as delivery of DNA to protoplasts and microprojectile bombardment of cells and tissues.

Early success in the production of transgenic rice involved electroporation (Shimamoto *et al.*, 1989; Toriyama *et al.*, 1988; Veluthambi *et al*., 1989) or the polyethylene glycol-mediated (PEG-mediated) transfer of genes (Datta *et al.*,1990; Hayashimoto *et al.* 1990). Procedures for protoplast-mediated transformation of rice have certain problems. It still remains difficult to regenerate plants from protoplasts of elite *japonica* and the majority of *indica* varieties, although some successful results were reported (Ayres *et al.*, 1994). Plant regenerated from protoplasts are often sterile and phenotypically abnormal (Li *et al.*, 1990). Some of these problems are probably related to the use of cells that have been cultured for long periods. To avoid these problems, scutellar tissue of immature embryos was used for isolation of protoplast (Ghosh *et al.*, 1994). Other problems include the integration of multiple copies of genes into the genome, the fragmentation and rearrangement of genes (Wu *et al.*, 1995) and occasional non-Mendelian inheritance of transgenes (Peng *et al.*, 1995).

Microprojectile bombardment, which is also called the biolistic method or the particle gun method, has been attempted in many laboratories. Christou *et al.*(1992) reported that immature embryos are good targets for biolistic transformation of important cultivars of *japonica*, *javanica*, and *indica* rice. With further improvements in technique, the bombardment of rice embryos and cultured cells has become a routine method for transformation of *japonica* rice (Li *et al.*, 1993). Successful transformation of *indica* rice varieties using cells in suspension culture was described in recent reports (Zhang *et al.*, 1996).

Another method for the direct transfer of genes to intact tissues, developed for rice, is the electroporation of mature zygotic embryos. Xu *et al*. (1994) reported the production of fertile transgenic *indica* rice by this method. Compared to protoplast-mediated methods, this method using intact cells and tissues are less dependent on specific cultivars (Ayres *et al.*, 1994). Various problems, including insertion of multiple copies of foreign genes (Finnegan *et al.*, 1994) and the non-Mendelian inheritance of transgenes (Spencer *et al.*, 1992), have been discussed in other crop plants.

Factors affecting *Agrobacterium*-mediated genetic transformation in *indica* Rice

In recent years, the genetic transformation as a key technology of genetic engineering of rice has made a great progress. At the same time, the rapid, efficient, stable and large-scale genetic transformation system in most *japonica* rice was established. On this basis, a lot of important foreign gene, such as insect, disease resistance, stress tolerance, quality improvement, and nutritional gene were transferred into *japonica* rice (Chen *et al.*, 2009; Zhu, 2012). However, the transformation of *indica* rice is still difficulty due to the low induction rate of embryogenic calli and plantlet regeneration. Many *indica* rices are particular genotype-dependent (Kumar *et al.*, 2005; Lin *et al.*, 2005; Hiei and komari, 2006; Hiei and komari, 2008). Although a few successful genetic transformation in *indica* rice was reported (Chan *et al.*, 1992; Xiao *et al.*, 2008; Lin *et al.*, 2009; Wang *et al.*, 2010), generally, it is still lack of commonly used genetic transformation technologies for *indica* rice and there are many problems which challenge in the practical application.

The basic prerequisite for the potential use in genetic transformation in *indica* rice improvement is the establishment of the efficient plant regeneration system. Successful regeneration system mainly depends on genotype, explant type, medium composition, plant growth regulator and culture environment (Khanna and

Raina, 1999).

Influencing factors of high efficiency rice genetic transformation system mediated by *Agrobacterium*, include *Agrobacterium tumefaciens* strains, vectors, rice genotype, transformed receptor, co-culture conditions, selectable marker gene and regeneration procedure etc. (Hiei *et al.*,1997).

Genotype

The differences of genotype is the most significant factor in many factors that affect the successful of genetic transformation of rice (Gao and Hong, 1999; Wang *et al.*, 2007). In general, glutinous rice has the highest culture ability, followed by *japonica* hybrid rice, *indica* hybrid rice, respectively. Even with the same type, the tissue culture ability is different from variety to variety (Chen and Qing, 2007). Glaszmann *et al.* (1987) assessed the isozyme polymorphisms to divide rice varieties into six varietal groups of genotype I to genotype VI, class I is pure *indica* and class VI is pure *japonica* rice, in between is *indica*, partial *indica*, and partial *japonica*. Pure *indica* varieties are recalcitrant to tissue culture and transformation, such as Nan Jin 11, Suewon 258, IR series (Hiei *et al.*, 1997). Partial *indica* varieties with genetic background of *japonica* rice are relatively easy, such as Basmati 370, Basmati 385, Kasalath (Rashid *et al.*, 1996; Xiao *et al.*, 2008; Wang *et al.*, 2010). Especially, Kasalath cultivar of *indica* rice has been used as a model cultivar for transformation.

Type and physiological status of the explants

The type of explant is also an important factor affect the successful in genetic transformation. The mature embryo, immature embryo, spikelet, coleoptile, leaves, anthers and meristem tip of seedlings and the organ can be used for callus induction. But the callus state and differentiation ability are distinct from the different

explant obtained (Ning *et al.*, 2007). Two types of explants, mature embryo and immature embryo are commonly used in *Agrobacterium*-mediated transformation in *indica* rice. Immature embryo is a favorable system of transformation and regeneration by *Agrobacterium*-mediated gene transformation. Because it has strong ability of division and easily form a large number of embryogenic calli (Hoque *et al.*, 2005; Vijayachandra *et al.*, 1995). The frequency of transformation through the use of immature embryo expalnt was nearly 2 times higher than mature embryo source (Huang *et al.*, 2000). The preparation and manipulation of immature embryos is troublesome because only a limited number of immature embryos can be obtained at the right developmental stage (8-12days) after pollination (Hiei and Komari, 2008). So, it does not meet the need of genetic transformation experiment whole year. Mature embryo is not restricted by the growing season, manipulation is easy and can provide conservation material for genetic transformation. But the frequency of transformation is low (Wang *et al.*, 2002; Ge *et al.*, 2006). In case that highly efficient differentiation of mature seed-derived calli was developed. Applicable *Agrobacterium*-mediated transformation using *indica* rice calli would be of significant benefit to the field. Therefore, recent years a lot of study used mature embryo as receptor material.

A few other explants besides immature embryos or the callus derived from them or mature seeds have been used for successful *Agrobacterium*-mediated transformation. Rice seedling sections and inflorescences were transformed efficiently when the explants were preincubated in necrotic reduction medium (Enrıquez-Obregon *et al.*, 1998; 1999). Yet, if the meristematic tissues were not pretreated with necrotic reduction agents, the transformation efficiency was low (May *et al.*, 1995; Park *et al.*, 1996; Kisaka and Kameya, 1998). The source of the explants had a significant effect on transformation frequency. Zhao *et al.* (2000) found that immature embryos from field-grown stock plants could be transformed more efficiently than immature embryos from greenhouse-grown stock plants of sorghum. It also observed that immature embryos obtained from healthy and strong wheat plants from growth

chambers yielded higher transformation frequencies than stressed stock plants grown under similar conditions. It had also been noticed a significant effect of the embryo response in tissue culture and a subsequent decrease in transformation frequency if stock plants in the growth chamber were sprayed with a fungicide.

The selection of Agrobacterium tumefaciens strains

At present, the main *Agrobacterium tumefaciens* strains which were used for rice transformation include LBA4404, EHA101 and their derivative strains EHA105, AGL1 (Cheng *et al.*, 2004). Raineri *et al.* (1990) transformed embryogenic callus of two rice cultivars using 8 different *Agrobacterium* strains. Variety Fujisaka 5 inoculated with LBA4404 strain conferring kanamycin resistance and β-D-glucuronidase (*gus*) produced callus that grew on selective levels of kanamycin and this tissue fluoresced upon incubation with GUS substrate. Strain LBA4404 was reported to be suitable transformation for cv. Fujisaka 5 (Raineri *et al.*, 1990). Yi *et al.* (2001) and Lin *et al.* (2009) discovered that EHA105 was more sensitivity to transform receptor than LBA4404 and AGL1. Generally, the research of several *indica* rice transformation used LBA4404, EHA105 and AGL1.

Hiei *et al.* (1994) tested the efficiency of various combinations of two strains and two vectors. The strains were the ‘ordinary’ strain LBA4404 (Hoekema *et al.*, 1993) and the ‘super-virulent’ strain EHA101. The vectors were pIG121Hm, a derivative of the ‘normal’ binary vector pBIN19 (Bevan, 1984), and pTOK233, a derivative of a ‘superpl3790si binary’ vector pTOK162 (Komari., 1996). In transformation experiments, LBA4404 (pTOK233) was slightly more effective than both LBA4404 (pIG121Hm) and EHA101 (pIG121Hm) with the *Tsukinohikari* cultivar, and it was definitely the most effective with the *Koshihikari* cultivar. For unknown reasons, EHA101 (pTOK233) was not very effective in transformation of rice. In recent results, LBA4404 (pTOK233) was definitely efficient in transformation of *javanica* rice (Dong *et al.*, 1996). These data suggests that an ‘ordinary’

vector/strain combination resembles, improved, combinations in the transformation of cultivars that are easy to grow in tissue culture. By contrast, the choice of vectors and strains is important for the transformation of difficult cultivars. However, only limited numbers of strains have been tested to date and the use of various other strains might further improve the efficiency of transformation. Generally, the vector backbone has significant affected transformation process, as dual binary vector (Komari *et al.*, 1996) and additional *vir* genes enhanced transformation efficiency (Vain *et al.*, 2004). For the effective elimination of the *Agrobacterium* after transformation, Wang *et al.* (1997) inserted a castor bean CAT1 intron into the selectable marker gene for hygromycin resistance, thereby, abolishing the expression of the gene in *Agrobacterium* and rendering it susceptible to hygromycin.

Culture medium composition

The composition of the co-cultivation medium is an important factor that affects the efficiency of transformation, and media that can support the active division of cells are preferable. A number of culture media formulations have been established and published. A widely used basal medium for culturing mature seed of rice is MS (Murashige and Skoog, 1962). At present, the basal media, included MS, N6, B5, CC and NB etc. are also generally used. The optimal culture conditions of different rice varieties differ greatly. According to different varieties adjusting of various components of culture medium by increasing concentration of organic ingredients, such as vitamins and inositol can significantly improve the texture of *indica* rice callus (Liu *et al.*, 2005; Wang *et al.*, 2008). Firstly, maximum constituent of culture medium is required for callus formation, growth and development of *indica* rice. Liu *et al.* (2005) improved proportion of nitrate-nitrogen and ammonium nitrogen in MS maximum constituents and optimized medium L3, DL3 which suitable for *indica* rice mature embryo subculture and differentiation.

Secondly, various and concentration of minimum elements are the

main reason which caused the differences of callus induction and growth. Ge *et al.* (2006) optimized the medium S for callus subculture through increasing the $MnSO_4 \cdot 4H_2O$ and other trace elements, base on MS maximum constituent, which improved plantlet regeneration from embryogenic callus.

Finally, gelling agents also play role in plantlet regeneration and transformation. Culture media solidified with a gelling agent are better for co-cultivation than liquid media. When co-cultivation is carried out in liquid medium for several days with rice calli, the transient expression of GUS after co-cultivation is extremely low. Optimization of the medium may be necessary, depending on the genotype of rice or the type of material, and it can be achieved by monitoring the transient expression of marker genes. Several laboratories with different genotypes and explants adopted a similar medium recipe. MS medium or a modified MS-based medium was shown to be suitable for inoculation and co-culture in several report of rice transformation (Dong *et al.*, 1996; Enriquez-Obregon *et al.*, 1999; Mohanty *et al.*, 1999; Lucca *et al.*, 2001). Ishida *et al.* (1996) reported transformation of maize immature embryo using LS-based (Linsmaier and Skoog, 1965) medium, and N6-based medium failed to generate transformed plants. Zhao *et al.* (2001) showed that N6-based medium was also suitable for inoculation and co-culture of immature maize embryos, resulting in transgenic plants. Similarly, the addition of $CaCl_2$ in the medium was increased transformation efficiency in barley (Kumlehn *et al.*, 2006).

Co-culture conditions

Agrobacterium tumefaciens-mediated transformation included steps such as attachment, *vir* gene stimulation, T-DNA processing, transportation and integration, which completed during co-cultivation period (Yang *et al.*, 2011). So, the key of successful transformation is to master the technique and time of co-cultivation. The efficiency of co-cultivation was affected by the bacterial cell density, infection methods, infection time, co-cultivation temperature and time etc.. Some authors

reported that calli immersed in a culture of the pre-induced bacteria under shaking condition was the best way for transformation (Yi *et al.*, 2001). Infection time is one of the key factor affecting successful transformation experiments mediated by *Agrobacterium*. Research has shown that infection for 20 min gave the better result for transformation (Wang and Tian, 2009). The co- cultivation temperature also play important role on the successful transformation because the temperature requires to induce *vir* (viru lence region) gene activity normally at 20 to 30℃. So the temperature of activation *vir*D and *vir*G must below 28℃. Hiei *et al.* (1994) reported that the suitable temperature to co-cultivation was 22℃.

A significant factor enhancing transformation of rice species is desiccation of explants prior to, or post *Agrobacterium* infection. Arencibia *et al.* (1998) reported that air drying sugarcane suspension cells prior to inoculation under laminar flow conditions for 15–60 min slightly improved T-DNA delivery and subsequently increased transformation efficiency, but the actual desiccation was not stringency defined in this report. Similarly, air-drying calluses derived from rice suspension cultures for 10–15 min increased the transformation efficiency at 10-folds higher than that of control without air-drying (Urushibara *et al.*, 2001). It is unclear to the investigators what mechanisms affected by air-drying, but it is possible that plasmolysis or wounding may be related. The effect of air-drying on other explants of rice, such as embryogenic calli and precultured immature embryos was not evaluated. Using the same air-drying conditions gave different response in different species. It was shown that air-drying precultured immature embryos and embryogenic calli in wheat prior to inoculation did not have the same effect as in sugarcane and rice (Cheng, *et al*., 2004). However, Cheng *et al.* (2003) reported that desiccation of precultured immature embryos, suspension culture cells, embryogenic calli of wheat, and embryogenic calli of maize greatly enhanced T-DNA delivery and plant tissue recovery after co-culture, leading to stable transformation frequency and plant tissue recovery after co-culture. This treatment was not only effective in monocot species, but also improved T-DNA delivery in recalcitrant dicot species such as soybean

suspension cells based on the preliminary study of Cheng and Fry (2000). Although the molecular mechanism of desiccation during co-culture remains unclear, it is known that desiccation significantly suppresses the growth of *Agrobacterium*, similar to the effect seen with silver nitrate. In addition, maize embryogenic calli from the desiccation treatment with 20 mM silver nitrate recovered better than explants co-cultured under non-desiccation conditions (with H_2O). Furthermore, osmotic treatments and abscisic acid (ABA) treatment before and during inoculation, and during co-culture, did not have the same effect on T-DNA delivery as the desiccation treatment.

The effect of temperature during co-culture on T-DNA delivery was first reported in dicot species. *Agrobacterium* co-culture at temperature of 22℃ found to be optimal for T-DNA delivery in *Phaseolus acutifolium* callus and tobacco leaves (Dillen *et al.*, 1997). However, in another report, co-culture at 25℃ led to the highest number of transformed plants of tobacco, even though 19℃ was optimal for T-DNA delivery (Salas *et al.*, 2001). These results indicate that the optimal temperature for T-DNA delivery may not be optimal for stable transformation with a given species and explant. The optimal temperature for stable transformation should be evaluated with each specific explant and *Agrobacterium* strain involved (Salas *et al.*, 2001). In monocots, the co-culture temperature for most of the crops ranged from 24 to 25℃, and in some cases, 28℃ was used (Rashid et al., 1996; Arencibia et al., 1998; Enrıquez-Obregon et al., 1998). The effect of lower temperature (23℃) on T-DNA delivery and stable transformation was also evaluated. Kondo et al. (2000) tested the effect of four temperatures, namely 18℃, 20℃, 22℃, and 24℃ on T-DNA delivery with garlic calli. The highest transient GUS expression was observed at 22℃, in which 64% of the total calli showed GUS activity. The ratio of GUS-stained calli to total calli decreased by 85% at 20℃ and by 69% at 24℃.

Chemicals such as acetosyringone for *vir* induction are recommended in most of the mococot transformation protocols (Hiei *et al.*, 1994; Ishida *et al.*, 1996; Tingay *et al.*, 1997; Zhao *et al.*, 2000). When acetosyringone was omitted, the level of

transient GUS expression was low and stable transformed plants could not be regenerated in rice or onion (Rashid *et al.*, 1996; Hiei *et al.*, 1997; Zheng *et al.*, 2001). However, some explants of monocot species could be efficiently transformed without the aid of external vir induction chemicals after special treatment. For example, meristematic sections of sugarcane pre-treated with an antinecrotic mixture (Enrıquez-Obregon *et al.*, 1999), and pre-cultured immature embryos and embryogenic calli of wheat co-cultured under desiccation conditions (Cheng *et al.*, 2003), could be efficiently transformed.

Agrobacterium density. Hiei *et al.* (1997) reported that transformation of rice was possible when the *Agrobacterium* density was between 1.0×10^{6} and 1.0×10^{10} colony-forming units cfu/ml, and the optimal concentration was approximately 1.0×10^{10} cfu/ ml (Hiei *et al.*, 1994). The same density of *Agrobacterium* was successfully used later in maize (Ishida *et al.*, 1996) and adopted by many other laboratories for various genotypes and explants in rice. Transient GUS activity increased with higher *Agrobacterium* density, but the callus initiation frequency was reduced and peak transformation frequency was achieved with *Agrobacterium* at 0.5×10^{10} cfu/ ml. Similar results were reported with sorghum immature embryos (Zhao *et al.*, 2000). Experiments with various explants of wheat showed that higher *Agrobacterium* density could increase transient GUS expression, but it was not correlated with higher stable transformation frequency (Cheng *et al.*, 1997).

Construct genes containing the reporter gene: green fluorescent protein (*gfp*) or red fluorescent protein (*rfp*) can be detected by the use of fluorescent microscopy or UV transillumination. It can be optimized infection way, infection time and co-culture conditions in *indica* rice (Yi *et al.*, 2008; Shi, 2010). In order to optimise this technology, Toki (2006) and Saika and Toki (2010) suggested that the incubation temperature should be 25℃ for 3 days. The green fluorescent protein (*gfp*) in the callus can be observed to express strongly.

Plant growth regulators

Plant growth regulators play a key role in rice tissue culture, in which a high auxin and cytokinin ratio usually used for initiation of the embryogenic callus, while a low ratio is used for the regeneration of plantlets. It is suggested that plant growth hormones function by mediating the signal transduction cascade that leads to reprogramming of the expression of embryogenic genes (Dudits *et al.*, 1995). Auxins, especially 2,4-D, are essential for induction and proliferation of the callus, but they also cause slow regeneration, which could lead to the loss of embryogenic competence. Cytokinin may also increase the growth rate of pre-embryogenic masses (Kommamine *et al.*, 1992). Synthetic auxin, 2,4-D, is important to initiate and sustain embryogenic callus growth in rice and has been used as the only growth regulator in callus induction medium (Seraj *et al.*, 1997; Khanna *et al.*, 1998; Lee *et al.*, 2002; Ozawa *et al.*, 2003; Lin and Zhang, 2005). It is also reported that combination of 2,4-D with kinetin was more effective in producing an embryogenic callus while 2,4-D alone only produced a non-embryogenic calli (Fan *et al.*, 2002; Wang *et al.*, 2004). Some reports suggested that the addition of 6-BA could enhance the quality of the initiated callus (Reiffers and Freire, 1990; Tian, 1994; Trejo-Tapia *et al.*, 2002) and further differentiation of calli (Tang *et al.*, 2000). Auxin in term of picloram and dicamba in combination with 2,4-D was superior in anther culture of *indica* rice (Silva, 2010). So, the type and the concentration of plant growth hormones are the main function to improve the ability of tissue culture.

Antibiotics

Antibiotics such as cefotaxime, carbenecillin and timentin have been used regularly in *Agrobacterium*-mediated transformation of crops following co-culture to suppress or eliminate *Agrobacterium* (Cheng *et al.*, 1996; Bottinger *et al.*, 2001; Sunikumar and Rathore, 2001). Although cefotaxime worked well in

Agrobacterium-mediated transformation of rice and maize initially, it was later found that cefotaxime at a concentration of 250 mg/L had a detrimental effect to maize Hi II callus (Ishida *et al.*, 1996), Callus formation was greatly reduced when cefotaxime at concentration ranging from 50 to 250 mg/L was added in the callus induction medium, and consequently transformation frequency was reduced to 3-folds in comparision with carbenicillin (100 mg/L). Carbenicillin at 100mg/L was used for all the subsequent experiments (Zhan *et al.*, 2001). Carbenicillin has been the antibiotic of choice in reports of *Agrobacterium*-mediated transformation of wheat and maize (Cheng *et al.*, 1997, 2003; Zhang *et al.*, 2003). On the other hand, 100 mg/L kanamycin was economical and improved the transformation efficiency in white spruce by enrichment of transformed tissue in budforming callus (Le *et al.*, 2001) and increased the proportion of positively transformed shoots during subculture on kanamycin containing medium in peanut and pigeon pea (Sharma and Anjaiah, 2000; Thu *et al.*, 2003).

Selectable marker

The most widely used selectable markers for transformation of crops are genes encoding hygromycin phosphotransferase (*hpt*), phosphinothricin acetyltransferase (*pat or bar*), and neomycin phosphotransferase (*nptII*). Use of these marker genes under the control of constitutive promoters such as the 35S promoter from cauliflower mosaic virus, the ubiquitin promoter from maize, works as efficiently for selection of *Agrobacterium*-transformed cells as for biolistics-mediated transformation. For asparagus and banana, the *npt II* gene under the control of the nopaline synthase promoter has been used successfully to select stable transformants with kanamycin (May *et al.*, 1995; Limanton-Grevet and Jullien, 2001). The positive selectable marker phosphomannose isomerase was firstly used for *Agrobacterium*-mediated transformation of sugar beet and was recently used to enhance transformation in sorghum (Joersbo *et al.*, 1998; Lucca *et al.*, 2001; Gao *et al.*, 2005).

To improve selectable marker genes for crops, Wang *et al.* (1997) inserted introns into the coding region of *hpt* as the strategy used in enhancing transgene expression in monocot species (Simpson and Filipowics, 1996). The intoduction of introns into the *hpt* not only improved transformation frequency in rice, but also reduced copy numbers of the marker gene. Furthermore, inserting the introns into the marker gene also enabled better control of *Agrobacterium* growth during the transformation process (Wang *et al.*, 1997). This modified selectable marker enhanced stable transformation with elite rice and barley cultivars as well (Upadhyaya *et al.*, 2000; Wang *et al.*, 2001). Glyphosate insensitive plant containing 3-enolpyruvylshikimate-5-phosphate synthases (EPSPS) genes derived from bacterial that degrades glyphosate some bacteria contain glyphosate oxidoreductase (GOX) gene which have the same mechanism to EPSPS and have been used in some laboratories to generate transgenic plants in wheat and maize with biolistics-mediated transformation approaches (Armstrong *et al.*, 1995; Zhou *et al.*, 1995; Russell and Fromm, 1997; Howe *et al.*, 2002). Among those genes, CP4, has been successfully used in *Agrobacterium* transformation of wheat (Cheng *et al.*, 2003; Hu *et al.*, 2003).Transformation frequency was comparable to biolistics-mediated transformation in wheat (Hu *et al.*, 2003) when a desiccation-based protocol was used.

Objectives

The objectives of the study were to establish tissue culture system in *indica* rice landrace *Hom Kra Dang Nagh* and used tissue culture as a tool for the transformation by *Agrobacterium* strain EHA 105 harbouring plasmid pCAMBIA 1301 with 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) as desirable and β-glucuronidase (GUS) as reporter gene.

1. Establish the regeneration system in *indica* rice *Hom Kra Dang Ngah* variety.
2. Establish the *Agrobacterium*-mediated transformation system and obtained the transgenic plantlets, then apply PCR to analyze transgenic plants.

CHAPTER Ⅱ

Development of Tissue Culture and Plantlet Regeneration Systems

Experiment 1

Callus Induction and Plantlets Regeneration from Culturing Mature Zygotic Embryos of Landrace *Hom Kra Dang Ngah* rice

EXPERIMENT 1

Introduction

Plant tissue culture is wildly regarded as one of the attractive areas with the potential to regenerate fertile plants using different types of explants. In the last three decades, various tissue culture techniques are being used for the genetic improvement of rice plant throughout the world. Routinely, tissue culture system including callus induction and plantlet regeneration is a fundamental requirement for successful genetic transformation (Li *et al*., 2007; Seraj *et al*., 1997). The callus initiation from cultured parts or explant of a species, proliferation of callus and subsequently plantlet regeneration are first step in tissue culture among cereals (Snezana *et al*., 2005). However, the potential for callus induction and its regeneration have been reported to be a species specific (El-Bakry and Ahmed, 2002; Barry-Etienne *et al*., 2002). It is highly rely on genotypes, explant types, carbohydrate sources, plant growth regulators, basal salts of culture medium and culture conditions (Rueb *et al*., 1994). Many authors reported on the optimization of tissue culture system of rice cultivars, especially the use of plant growth regulators (Ge *et al*., 2006; Lee *et al*., 2002; Rashid *et al*., 2001; Zaidi *et al*., 2006; Zhu *et al*., 1996).

Plant growth regulator plays a key role in rice tissue culture, in which a high auxin and cytokinin ratio usually used for initiation of the embryogenic callus, while a low ratio used for the regeneration of plantlets. It is suggested that plant growth regulator function by mediating the signal transduction cascade that leads to reprogramming of the expression of embryogenic genes (Dudits *et al.*, 1995). Auxins, especially 2,4-D, are essential for induction and proliferation of the callus, but they causes a low frequency of plantlet regeneration, which might lead to the loss of embryogenic competence. Cytokinin may also increase the growth rate of pre-embryogenic masses (Komamine *et al.*, 1992). Synthetic auxin, 2,4-D, is important to

initiate and sustain embryogenic callus growth in rice and has been used as the only growth regulator in callus induction medium (Lee *et al.*, 2002; Ozawa *et al.*, 2003; Lin and Zhang, 2005). It is also reported that combination of 2,4-D with kinetin was more effective in producing an embryogenic callus while 2,4-D alone produced only a non-embryogenic calli (Wang *et al.*, 2004). Some authors suggested that the addition of 6-BA could enhance the quality of the initiated callus (Tian *et al*., 1994) and further differentiation of calli (Tang *et al.*, 2000). Auxin in term of picloram and dicamba in combination with 2,4-D was superior in anther culture of *indica* rice (Silva, 2010). So, the type and the concentration of plant growth regulatros are the main factor improving the ability of plant tissue culture.

Plant regeneration from embryogenic callus has achieved initially in *japonica* rice varieties (Nishi *et al*., 1973). Successful regeneration of fertile plants has been limited in *indica* rice varieties (Kyozuka *et al*., 1988; Raman *et al*., 1994). It is also a reason that the transformation of *indica* rice is still difficulty due to the low induction rate of embryogenic calli and plantlet regeneration. Many *indica* rices are particular genotype-dependent (Kumar *et al*., 2005; Lin and Zhang, 2005; Hiei and Komari, 2006; Hiei and Komari, 2008). Although a few success of genetic transformation in *indica* rice has been reported (Chan. *et al*., 1992; Xiao *et al*., 2008; Lin *et al*., 2009; Wang *et al*., 2010), it has strong recalcitrant nature and genetype-independent, even with the same types. Tissue culture ability is different from varieties to varieties (Chen and Qing, 2007). So far, there is no report on the tissue culture of *Hom Kra Dang Ngah* cultivar. Hence, the purposes of this chapter are 1) to design a number of cultures to assess the comparative effect of different plant growth regulators on the efficiency of callus induction and plantlet regeneration from scutellum derived from mature zygotic embryos of recalcitrant *indica* rice variety *Hom Kra Dang Ngah* and 2) to establish plantlet regeneration systems from mature zygotic embryos of *indica* rice cultivar for further gene transformation experiment.

Materials and Methods

Plant material and its preparation

Mature seeds of *Oryza sativa* L. cultivar *Hom Kra Dang Ngah* were used as explant source. Mature and healthy seeds were selected and dehusked manually. The surface disinfestation was carried out by immersing in 70% ethanol for 1 min, followed by 20% Clorox (1.25% NaClO) in the presence of a few drops of wetting agent "Tween 20" for 5 minutes. Finally, the explants were rinsed with sterile distilled water for four to five times in laminar air flow bench.

Callus induction

The surface sterilized seeds were cultured on solidified MS medium, containing 3% sucrose, 0.75% agar-agar powder and different concentration of 2,4-D ranging from 1 to 4 mg/L. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121^{o}C, 1.07 kg/cm^{2} for 15 minutes. All cultures were placed in the culture room at 25-27 ^{o}C, under 16 hour photoperiod of 2.5 µmol/m^{2}/sec. After being cultured for one month the frequency of callus induction was recorded and statistically compared.

Enhancing callus formation

In order to enhance the frequency of callus induction, the surface sterilized seeds were cultured on MS medium supplemented with 2,4-D at the optimum concentration (from callus induction experiment) in combination with various kinds and concentrations of other plant growth regulators, which was 0.5

mg/L or 1 mg/L 6-BA together with 1 mg/L NAA or 1 mg/L IAA with Kn 0.5 mg/L. The treatments were as follow;

(1) 1 mg/L 2,4-D and 1.5 mg/L TDZ
(2) 2 mg/L 2,4-D, 1.0 mg/L NAA and 1.0 mg/L6-BA
(3) 2 mg/L 2,4-D, 1.0 mg/L NAA, 1.0 mg/L6-BA and 0.5 mg/L Kn
(4) 2 mg/L 2,4-D, 1.0 mg/L IAA, 0.5 mg/L6-BA and 0.5 mg/L Kn
(5) 4 mg/L 2,4-D, 1.0 mg/L NAA, 1.0 mg/L 6-BA and 0.5 mg/L Kn
(6) 4 mg/L 2,4-D, 1.0 mg/L IAA, 0.5 mg/L 6-BA and 0.5 mg/L Kn

All culture media were supplemented with 1 g/L casein hydrolysate (CH), 3% sucrose and solidified with 0.75% agar. The cultured seed were incubated at the same conditions as previously mentioned. After one month of culture, the percentage of seed forming callus and the size of callus were recorded and statistically compared.

Callus proliferation

The calli produced on the above medium were transferred to different proliferation medium. Those media were MS supplemented with various kinds and concentrations of plant growth regulators as follow;

(1) 1 mg/L 2,4-D
(2) 2 mg/L 2,4-D
(3) 3 mg/L 2,4-D
(4) 4 mg/L 2,4-D
(5) 1 mg/L 2,4-D and 1.5 mg/L TDZ
(6) 1 mg/L 2,4-D and 0.5 mg/L NAA, 0.5 mg/L 6-BA

(7) 1 mg/L 2,4-D and 0.5 mg/L NAA, 0.5 mg/L 6-BA, 0.25 mg/L Kn

(8) 1 mg/L 2,4-D and 0.5 mg/L IAA, 0.25 mg/L 6-BA, 0.25 mg/L Kn

(9) 2 mg/L 2,4-D and 0.5 mg/L NAA, 0.5 mg/L 6-BA ,0.25 mg/L Kn

(10) 2 mg/L 2,4-D and 0.5 mg/L IAA, 0.25 mg/L 6-BA, 0.25 mg/L Kn

All culture media were supplemented with 1g/L CH, 3% sucrose and solidified with 0.75% agar. After being cultured for 4 weeks the percentage of browning response and frequency of embryogenic callus formation were recorded and statistically compared. Embryogenic and non-embryogenesis callus were determined and distinguished under the stereo microscope.

Plantlet regeneration

In order to optimize plantlet regeneration through somatic embryogenesis, the calli were transferred from proliferation medium to the regeneration medium. The medium was MS supplemented with difference kinds and concentrations of various plant growth regulators at the optimum concentration (from proliferation experiment). CH at 1 g/L was also added to this medium. Within 4 weeks, growth and development of the calli were recorded and statistically compared among those kinds and concentrations of plant growth regulators.

Statistical analysis

The experiment was arranged as a randomized complete block design (RCBD) with 3 replicates per treatment. Analysis of variance (ANOVA) was applied to determine which kind and concentration of plant growth regulators were the best for callus induction, proliferation and plantlet regeneration. Data were analyzed using the SAS program Version 6.11 (SAS Institute, Cary, NC). A probability level of 5%

(=0.05) was chosen for all statistical inferences. Mean among treatments was separated by least significant difference (LDS).

Results and Discussion

Callus induction

The scutellum region of the seeds swelled at 7 days. However, a few callus formation took place after being cultured for 4 weeks. Increase in concentration of 2,4-D to 3 mg/L promoted the increment of callus formation from 20 to 33%. However, higher concentration than this inhibited callus induction frequency. Without the addition of 2,4-D in the medium callus formation was not obtained (Table 2.1). Similar result was also reported by Zuraida *et al*. (2010). An optimum concentration of 2,4-D for callus induction varied depending on the explant source and genotype of rice (Raina, 1987). Our result in the present study revealed that 2,4-D at concentration of 1-4 mg/L can produce callus from mature seeds of rice cultivar *Hom Kra Dong Ngah*, while the percentage of callus induction is still low. Ge *et al*. (2006) reported that 2,4-D alone in culture medium induced a low frequency of callus induction in rice. In the present study, it concluded that 2,4-D alone was not the best treatment to induce embryogenic callus in *Hom Kra Dong Ngah* rice. The characters of four types of the callus were classified in different concentrations of 2,4-D containing medium. The first type was yellowish-brown compact obtained from 1.0 mg/L 2,4-D containing medium. The second type was yellow compact obtained from 2.0 mg/L 2,4-D containing medium. The third type was yellowish-white granular obtained from 3.0 mg/L 2,4-D containing medium. The last type was yellow friable one obtained from 4.0 mg/L 2,4-D containing medium. All characters of calli were shown in Figure 2.1.

Table 2.1 The frequency of callus induction from mature seed of rice *Hom Kra Dang Ngah* cultured on MS medium supplemented with different concentrations of 2.4-D for 4 weeks.

2.4-D (mg/L)	Callus induction frequency (%)	Characters of callus
0	0b	No callus formation
1.0	20.0a	Yellowish brown compact
2.0	25.0a	Yellow compact
3.0	33.9a	Yellowish white granular
4.0	31.2a	Yellow friable
F-test	*	
C.V. (%)	61.3%	

*Significant difference at $p < 0.05$.

Means of callus induction frequency with the same letter are not significantly different using LSD.

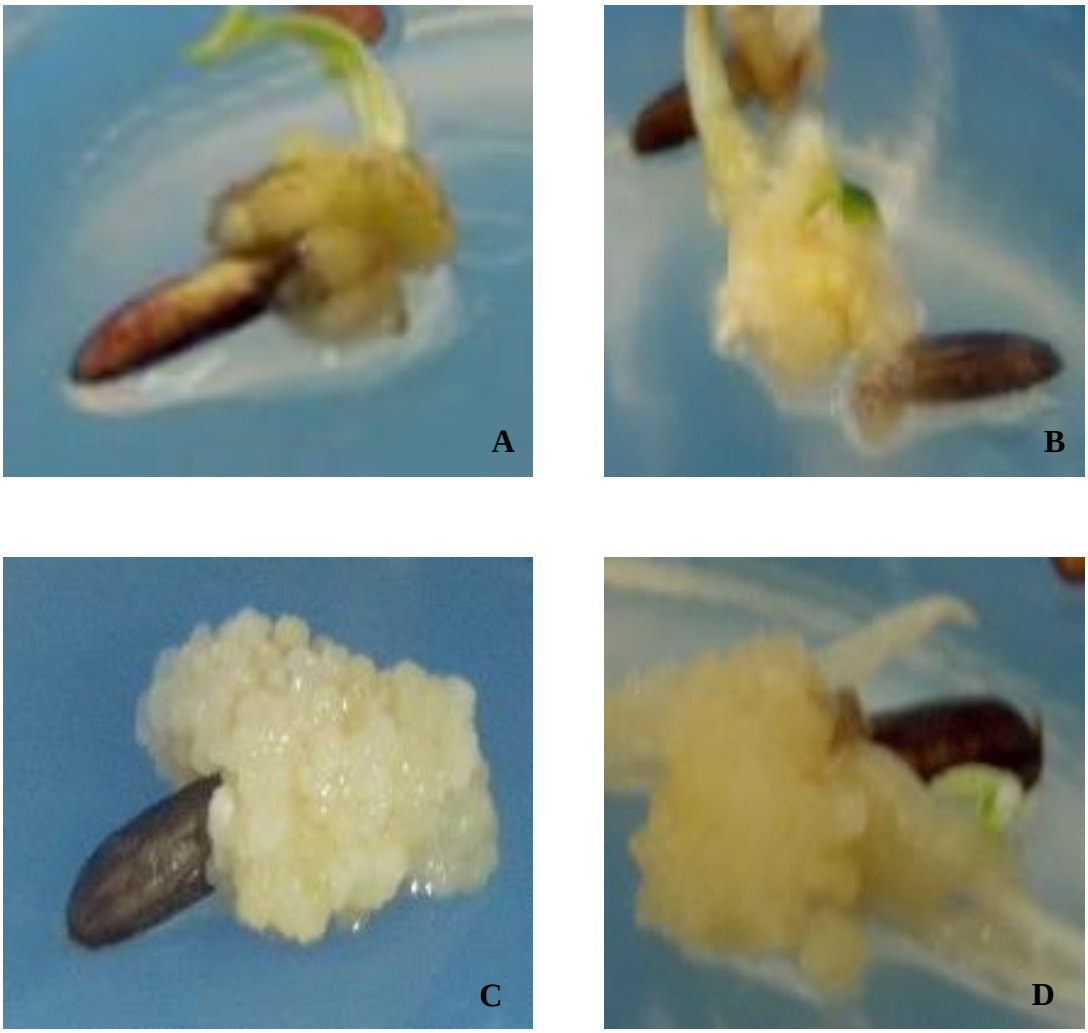


Figure 2.1 Callus induction of *Hom Kra Dang Ngah* rice on MS medium supplemented with different concentrations of 2,4-D for 4 weeks.

A: 1 mg/L 2,4-D

B: 2 mg/L 2,4-D

C: 3 mg/L 2,4-D

D: 4 mg/L 2,4-D

Enhancing callus formation

When the seeds were inoculated on the basal medium containing different auxin and cytokinin, the mature seeds swelled within 3-4 days and after being cultured for 7 days callus formation occurred. These calli proliferated with the passage of time and an excellent callus growth was obtained after 4 weeks of culture. The results were shown in Table 2.2. The frequency of callus induction was significantly enhanced to 56.3% and the quality of callus improved when the seeds were cultured on the MS medium supplemented with 2 mg/L of 2,4-D, 1.0 mg/L of NAA, 1.0 mg/L of 6-BA and 0.5 mg/L of Kn. The analysis of variance showed significant different effect of plant growth regulators on callus induction rate. This suggested that different concentrations of plant growth regulators containing MS medium are effective in callus induction of *indica* rice like those reported by Chowdhry *et al*. (1993) and Shazia *et al*. (2005).

Most of these calli were friable embryogenic, hard and yellowish-white in colour (Figure 2.2). The callus induced on MS medium containing 2.0 mg/L of 2,4-D, together with different auxin and cytokinin was creamy white, compact with grow green spots, usually which also were classified as embryogenic type. The green spots were further developed into shoots. Very few callus was induced on MS medium containing 1.0 mg/L 2,4-D and 1.5 mg/L of TDZ. This callus was compact, hard, white in color and had lower growth rate than that on another culture media. Some of which were embryogenesis. The callus initiated on MS medium containing 4.0mg/L of 2,4-D and another different concentrations of auxin and cytokinin was somewhat compact with yellow to white color. The size of those calli was small and some were brown in color (Table 2.3). Only a small percentage of this callus turned into embryogenic callus in nature (Figure 2.3).

Table 2.2 The frequency of callus induction from mature seeds rice *Hom Kra Dang Ngah* cultured on MS medium supplemented with different plant growth regulators for 4weeks.

Treatment	2,4-D	NAA	6-BA	IAA	Kn	TDZ	Callus induction
	mg/L						frequency (%)
1	1	0	0	0	0	1.5	23.3c
2	2	1	1	0	0	0	28.9b
3	2	1	1	0	0.5	0	56.3a
4	2	0	0.5	1	0.5	0	35.3b
5	4	1	1	0	0.5	0	31.6b
6	4	0	0.5	1	0.5	0	38.9b
F-test							*
C.V.(%)							33.2%

*Significant difference at $p < 0.05$.

Means of callus induction frequency with the same letter are not significantly different using LSD.

Table 2.3 The growth and morphological characters of callus induced on MS medium supplemented with different combinations of auxin and cytokinin for 4 weeks.

Treatment	Callus growth	Morphology of callus
1	+	white, compact
2	+	Yellowish-white, compact, no browning, seedling
3	++++	Whitish, green, friable and somewhat compact, copious, no browning, seedling
4	+++	Yellowish-white, friable and somewhat compact, seedling
5	++	Whitish, green, compact, a little browning, no seedling
6	++	Whitish, green, compact, a little browning, no seedling

Sign for callus growth: + = Small size; ++ = Medium size; +++ = Large size; ++++ = Very large size.

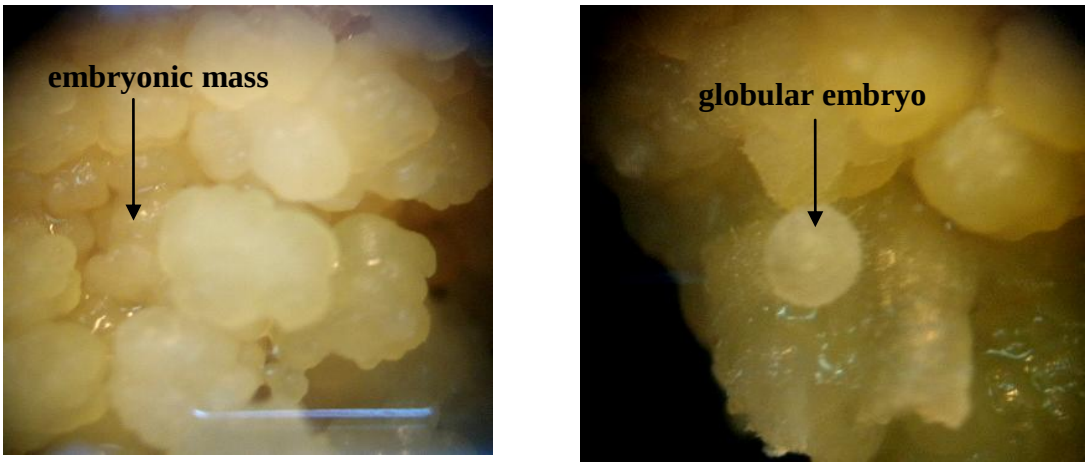


Figure 2.2 Morphological characters of embryogenic callus under the observation of stereo microscopy. The callus were induced on MS medium containing 2 mg/L 2,4-D , 1.0 mg/L NAA, 1.0 mg/L6-BA and 0.5 mg/L Kn for 4 weeks.

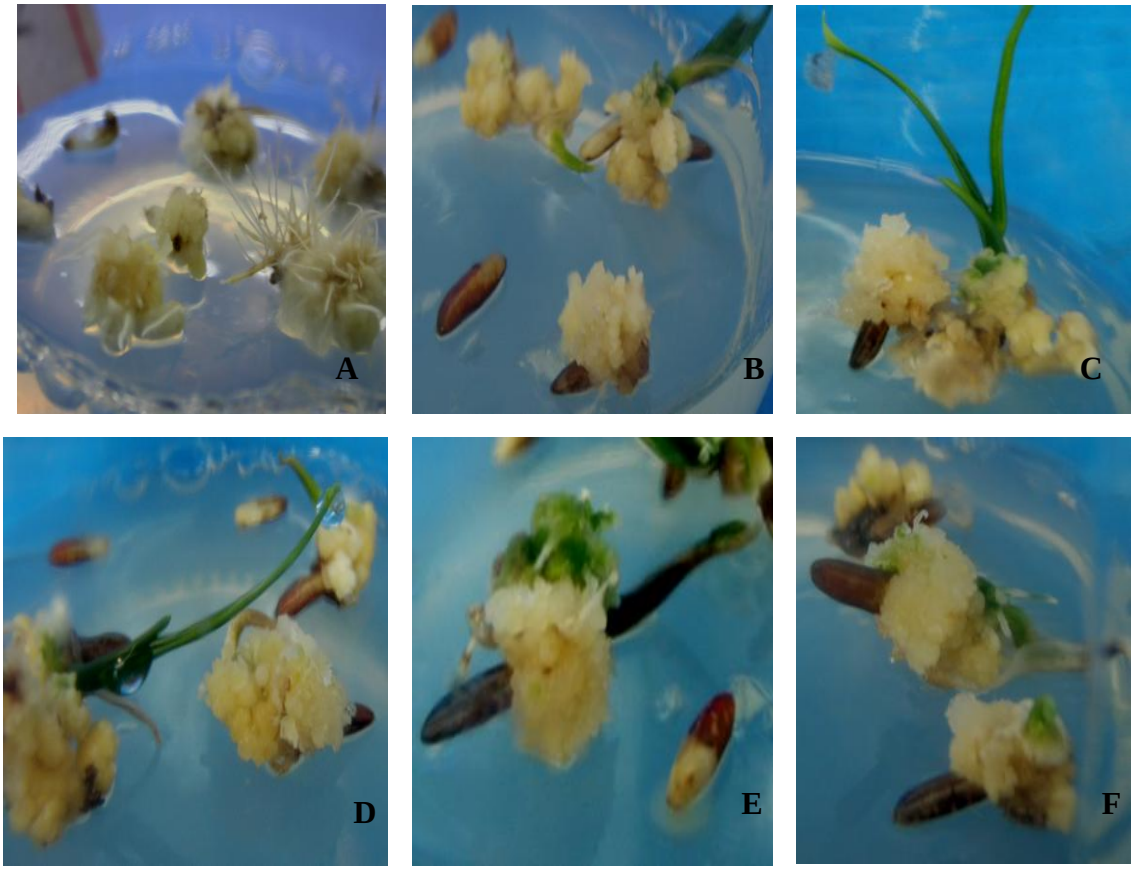


Figure 2.3 Morphological characters of calli on MS medium supplemented with different types and concentrations of plant growth regulators after being cultured for 4 weeks.

A: 1 mg/L 2,4-D and 1.5 mg/L TDZ

B: 2 mg/L 2,4-D and 1.0 mg/L NAA, 1.0 mg/L 6-BA

C: 2 mg/L 2,4-D and 1.0 mg/L NAA, 1.0 mg/L 6-BA ,0.5 mg/L Kn

D: 2 mg/L 2,4-D and 1.0 mg/L IAA, 0.5 mg/L 6-BA, 0.5 mg/L Kn

E: 4 mg/L 2,4-D and 1.0 mg/L NAA, 1.0 mg/L 6-BA, 0.5 mg/L Kn

F: 4 mg/L 2,4-D and 1.0 mg/L IAA, 0.5 mg/L 6-BA, 0.5 mg/L Kn

In this experiment, combination of two auxins, 2,4-D and NAA and two cytokinins, BA and Kn gave the best result in embryogenic callus formation. Addition of 6-BA, Kn and NAA to the callus induction media in the presence of 2,4-D was more effective for the production of embryogenic calli than using 2,4-D alone. The results were in agreement with Ge *et al*. (2006). Moreover, addition of Kn and NAA to the callus induction media can improve the quality of calli (Figure 2.3C). The calli obtained from those plant growth regulators containing medium were friable, hard, some compact, more granular structure and green spots, while the calli on the medium containing 2,4-D alone were mucilaginous, smooth and hardly to regenerate into plantlets (Figure 2.1). So the culture medium containing 2 mg/L of 2,4-D, 1.0 mg/L of NAA, 1.0 mg/L of 6-BA and 0.5 mg/L of Kn was the most effective for callus induction.

Callus proliferation and regeneration

Calli obtained from the above trial were sub-cultured onto the fresh media supplemented with the same kinds and concentrations of plant growth regulators or half concentration of plant growth regulators in order to induce further development of embryogenic calli. During 3 weeks of the sub-culture period, some treatments showed more callus browning. The calli which were cultured on medium containing 2,4-D alone at the same concentration with previous study produced higher percentage of browning ranging from 13.6 to 100% (Table 2.4). Browning forming calli decreased when 2,4-D in culture medium increased from 1 to 4 mg/L. However, plantlet regeneration was more difficult. Similar results were reported in Malaysian *indica* rice and *jaumala indica* rice. Maintenance of those calli on high concentration of 2,4-D containing culture medium for a longer period played inhibitory effect on plantlet regeneration (Aparna and rashied, 2004; Zuraida *et al*., 2010). Therefore, using 2,4-D alone was not the suitable treatment to induce embryogenic callus in

indica rice variety *Hom Kra Dang Ngah* and also due to a higher browning response after sub-culture.

When the calli were sub-cultured on MS medium supplemented with 1 mg/L or 2 mg/L 2,4-D in combination with 1.5 mg/L TDZ or 0.5 mg/L NAA, 0.5 mg/L 6-BA, 0.25 mg/L Kn or 0.5 mg/L IAA, 0.25 mg/L 6-BA, 0.25 mg/L Kn. The percentage of browning produced after sub-culture was 0-27.2%, lower than the medium containing 2,4-D alone. Moreover, the combination of 1.0 mg/L 2,4-D with 0.5 mg/L NAA or IAA, 0.25-0.5 mg/L 6-BA and 0.25 mg/L Kn resulted in the most effective for embryogenic callus induction without browning. The callus from these treatments was yellow in color and produced green spots (Figure 2.4). The present study indicated that these calli were easy to regenerate. Furthermore, the calli grown on 1.0 mg/L 2,4-D, 0.5 mg/L IAA, 0.25 mg/L 6-BA and 0.25 mg/L Kn produced shoot buds within 3 weeks of culture (Figure 2.4A).

In this present study, the somatic embryos derived from previous experiments were transferred to MS medium with 1.5 mg/L TDZ and 1.0 mg/L 2,4-D in order to induce shoot induction. TDZ showed a remarkable regenerative ability in tissue culture of *indica* rice variety *Hom Kra Dang Nga* (Figure 2.4 D). It was not only shortened the time of regeneration but also enhanced number of shoot formation. Similar results were obtained from Tian *et al*. (1994) and Jutta *et al*. (2007) who reported that TDZ was more efficient on improving regeneration from mature seeds of rice than the combination of 2,4-D with NAA. However, TDZ did not promote callus induction in *jaumala* rice (Aparna, 2004). However, this present study, TDZ in combination with 2,4-D was not the effective treatment for callus induction and proliferation but the most suitable for shoot induction.

In summary, the results from this study showed that concentrations and combinations of plant growth regulators had greatly influence on the proliferation of callus. Combination of 1.0 mg/L 2,4-D with 0.5 mg/L IAA, 0.25 mg/L 6-BA and 0.25 mg/L Kn was the most suitable for callus proliferation. Similar findings were reported in MR 219 rice (Syaiful *et al*., 2009; Zuraida *et al*., 2010). But different point

of view also reported. Such as the opinion that the incorporation of NAA and Kn in the callus induction medium supplemented with 2 mg/L 2,4-D did not significantly improve the callus induction frequency (Shahsavari *et al*., 2010). It was reported that the *indica* rice has recalcitrant character in nature and genotypes-dependent (Rashid *et al*., 1996). In addition, 1.5mg/L TDZ was the most suitable for shoot induction.

Table 2.4 The browning percentage of callus after sub-culture on MS medium supplemented with various plant growth regulators for 3 weeks.

Treatment	2,4-D	NAA	6-BA	IAA	Kn	TDZ	Callus
	mg/L						browning (%)
1	1	0	0	0	0	1.5	100
2	1	0	0	0	0	0	26.7
3	1	0.5	0.5	0	0	0	3.6
4	1	0.5	0.5	0	0.25	0	0
5	1	0	0.25	0.5	0.25	0	0
6	2	0	0	0	0	0	20.3
7	2	0.5	0.5	0	0.25	0	16.0
8	2	0	0.25	0.5	0.25	0	27.2
9	3	0	0	0	0	0	13.6
10	4	0	0	0	0	0	0

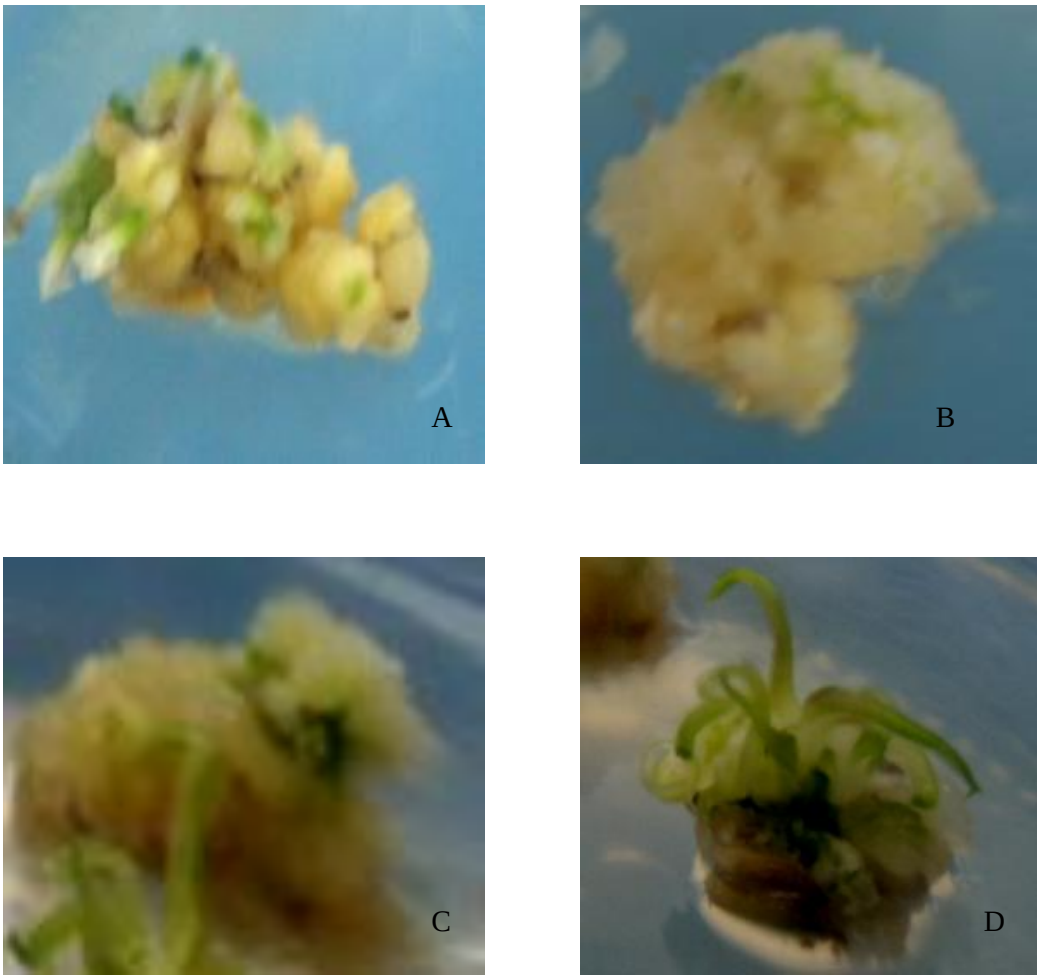


Figure 2.4 Somatic embryo initiation, turned into green and started to shoot induction after 3 weeks of sub-culture on MS medium supplement with different plant growth regulators.

A: 1.0 mg/L of 2,4-D, 0.5 mg/L of IAA, 0.25 mg/L of 6-BA, 0.25 mg/L of Kn

B: 1.0 mg/L of 2,4-D, 0.5 mg/L of NAA, 0.5 mg/L of 6-BA, 0.25 mg/L of Kn

C: 1.0 mg/L of 2,4-D, 1.5 mg/L of TDZ

D: Shoot elongation on medium containing 1.0 mg/L 2,4-D and 1.5 mg/L TDZ and more shoot induction after subsequence sub-culture on plant growth regulators-free-basal MS medium

Conclusion

The present study confirmed the production of embryogenic calli, somatic embryos as well as shoot induction of *indica* rice cultivar *Hom Kra Dang Ngah*. The somatic embryogenesis system was preliminarily established and can be used for genetic engineering purposes to produce high yield and quality of new *indica* rice variety through genetic transformation technology.

CHAPTER Ⅱ

Development of Tissue Culture and Plantlet Regeneration Systems

Experiment 2

Improved Plantlet Regeneration Systems in *Indica* Rice (*Oryza sativa* L.) Landrace *Hom Kra Dang Ngah*

EXPERIMENT 2

Introduction

Rice (*Oryza sativa* L.) is a major staple food and one of the most important crops in the worldwide. More than half of the world's population depends on rice for its major daily source of energy and protein. With the increase in population and reduction of arable land, together with the biotic and abiotic stress rice production is under great pressure. Breeding new rice varieties using genetic engineering techniques is one of the ways to increase rice yield and improve the total rice production. In recent year, genetic transformation technology as a key technology for improvement desirable traits of rice has made a great progress. However, the transformation of *indica* rice is still difficult due to a low induction rate of embryogenic callus and plantlet regeneration after transformation. Many *indica* rices are particularly genotype-dependent.

The regeneration of calli is the most difficult problem in genetic transformation processes. The physiological activity of calli will be decreased with the selection and differentiation. A large number of browning calli appeared, which lead to cell die and difficult to differentiation. So, the decrement of browning calli is directly promoted the frequency of *in vitro* differentiation. In order to solve this problem, addition of ascorbic acid in culture medium was recommended. In addition, the presence of mannitol in subculture medium followed by regular transfer calli to fresh medium of the same component could amend browning of the callus (Reddy, 1986). Ye *et al*. (2001) reported that macro-elements of N_6 in combination with MS micro-elements are helpful to callus differentiation. Reducing quantity of sucrose in differentiation medium, pulsing sorbitol and addition of zeatin to culture medium enhance the percentage of callus differentiation in *indica* rice. L-proline, as an organic nitrogen and osmotic regulatory, can have a positive impact on calli and regeneration ability (Shahsavari, 2010).

Although there have been some successes in plant regeneration from rice tissue culture, the protocol developed is not applicable to all the cultivars. Therefore, the present study was carried out to develop a high frequency regeneration system in *indica* rice, landrace *Hom Kra Dang Ngha* in order to use as a tool for further efficient gene transformation techniuqes.

Materials and Methods

Plant material and explant preparation

Mature seeds of *Oryza sativa* L. landrace *Hom Kra Dang Ngah* were used as explant source. Mature and healthy seeds were selected, dehusked, and surface sterilized by the protocol described by Zhang and Te-chato (2012). Sterile seeds were sown on MS medium with different plant growth regulators (PGRs) for callus induction.

Callus induction and proliferation

Sterile mature seeds were cultured on solidified MS medium for callus induction. The medium were supplemented with 2 mg/L 2,4-D, 1 mg/L NAA, 1 mg/L 6-BA and 0.5 mg/L Kn containing 3% sucrose and 1 g/L CH. The medium was solidified with 0.75% agar-agar powder. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121^{o}C, 1.07 kg/cm^{2} for 15 min. All cultures were placed in the culture room at 27±2^{o}C, under 16 hour photoperiod. After 4 weeks of culture, the frequency of callus induction was recorded and statistically compared.

The calli initiated on above medium were transferred to proliferation medium which was MS supplemented with 3% sucrose, 1 mg/L 2,4-D and 0.5 mg/L NAA, 0.5 mg/L 6-BA, 0.25 mg/L Kn and 1 g/L CH. The medium was solidified with 0.75% agar-agar powder. After being cultured for 3 weeks the yellowish-white

compact or granular embryogenic calli were selected and transferred to shoot induction medium (SIM).

Plantlet regeneration

Effect of culture media

To understand the effects of culture media on plantlet regeneration, three basal culture media were used in the present experiment. Those were MS, N_6 (Chu *et al*., 1975), and ARDA (Te-chato and Yenchon, unpublished data) medium. All culture media were supplemented with 3% sucrose, 0.5 mg/L NAA, 1.0 mg/L BA, 2.0 mg/L Kn and 1 g/L of CH and adjusted to pH 5.7 by 1N KOH and solidified with 0.75% agar-agar powder. The cultures were placed in the culture room under the same conditions as mentioned in materials and methods section. After 3-4 weeks of culture, the percentage of green spots forming calli and time required for green spot formation were recorded and compared statistically.

Effect of phytagel

The best result of culture medium obtained from the above experiment was selected and used for further studying the effect of phytagel on plantlet regeneration. The medium was supplemented with 3% sucrose, 0.5 mg/L NAA, 1.0 mg /L BA and 2.0 mg/L Kn. The culture medium was adjusted to pH 5.7 by 1 N KOH and solidified with different concentrations of phytagel (0.17, 0.25, 0.3 and 0.35%) in comparison with 0.75% agar-agar powder (as control treatment). Embryogenic calli obtained from CIM were transferred to various concentrations of phytagel solidified medium. After 4 weeks of culture the frequency of green spots

forming callus, percentage of plantlet regeneration and a number plantlets per callus were recorded and compared statistically.

Effect of different ratios of cytokinin (BA+Kn) to auxin (NAA)

Different ratios of auxin and cytokinin based on their concentrations were used to find out an appropriate ratio for induction of plantlet regeneration. The experiment was consisted of 4 treatments as follow;

(1) 1.0 mg/L BA+2.0 mg/L Kin+1.0 mg/L NAA; ration of BA+Kn/NAA=3:1
(2) 1.5 mg/L BA+2.0 mg/L Kin+1.0 mg/L NAA; ration of BA+Kn/NAA=3.5:1
(3) 1.0 mg/L BA+2.0 mg/L Kin+0.5 mg/L NAA; ration *of* BA+Kn/NAA=6:1
(4) 1.5 mg/L BA+2.0 mg/L Kin+0.5 mg/L NAA; ration of BA+Kn/NAA=7:1

Embryogenic calli obtained from CIM were transferred to different ratios of cytokinin (BA+Kn) to auxin (NAA) containing medium. After 4 weeks of culture the frequency of green spots forming calli, percentage of plantlet regeneration and a number of plantlets per callus were recorded and compared statistically. At the same time, root formation was recorded.

Effect of some osmotic regulatory substances and organic nitrogens

Two osmotic regulatory substances, sorbitol and L-proline and two organic nitrogens, glutamine and CH were supplemented to the best resulting SIM. L-proline at concentrations 0, 500 mg/L, glutamine at 0, 300, 500 mg/L, casein

hydrolysate at 1 g/L and 82 mM sorbitol were added to the SIM, together with 3% sucrose and solidified with optimal concentration of phytagel from previous experiment. For PGRs, 0.5 mg/L NAA together with 1mg/L BA and 2.0 mg/L Kn were employed. After being cultured for 4 weeks the frequency of shoot regeneration and a number of regenerated shoot were recorded and statistically compared.

Root induction and acclimatization

The regenerated plantlets at 3-4cm in height were excised and transferred to culture tubes (25x125mm) containing 10ml of basal MS medium without PGRs or supplemented with 0.1 mg/l IAA for root initiation. After vigorous roots appear complete plantlets were transferred to clay soil containing in 12 inch plastic pots covered with plastic bottle and acclimatized in the greenhouse at 28-30^{o}C supplied by natural light conditions until the plants were healthy enough and ready to transfer to field conditions.

Statistical Analysis

The experiment was designed as a randomized complete block design (RCBD) with 3 replicates per treatment. Analysis of variance (ANOVA) was applied to indicate which variety or combination was optimal and gave the best results for plantlet regeneration. Data were analyzed using the SAS program Version 6.11 (SAS Institute, Cary, NC). A probability level of 5% (p=0.05) is chosen for all statistical inferences. Means among treatment were separated by least significant difference (LSD).

Results and Discussion

Callus induction and proliferation

In this study, calli produced from suitable media (obtained from previous study) were obtained after 4 weeks of inoculation. After being transferred to subculture medium for proliferation for 4 weeks, two types of callus were obtained, embryogenic and non-embryogenic ones (Figure 2.5). Calli that were creamy white, some compact, friable and globular defined as embryogenic calli, which further developed into plantlets (Figure 2.5A). By contrast, the non-embryogenic calli were completely yellow or bright brown in color with soft or compact in texture (Figure 2.5B) couldn't develop into shoots.

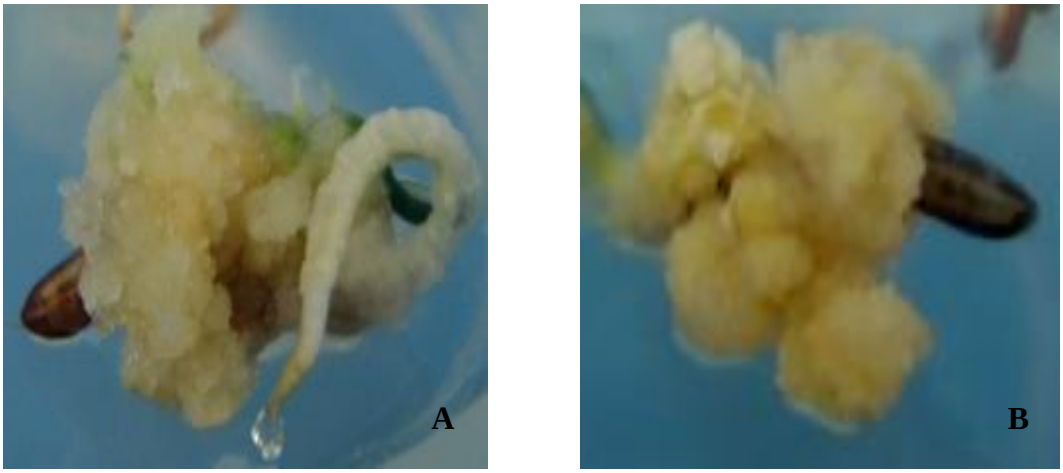


Figure 2.5 Morphological characters of embryogenic callus and non- embryogenic callus on MS medium after being cultured for 4 weeks.

A: Embryogenic callus: creamy white, some what compact, friable and globular

B: Non-embryogenic callus: completely yellow or bright brown in color with soft and compact texture

The result of this study was the same with Wang *et al*. (1997) who reported plantlet regeneration in rice through somatic embryogenesis. They observed that rice produced two types of calli viz., embryogenic and non-embryogenic which morphological characteristic like the characteristics described above. According to the characteristics of calli obtained from the present study, former is produced on the surface region, grew faster and are rise to shoots through somatic embryogenesis. So, it is easier to regenerate into plantlet.

Plantlet regeneration

Effect of culture media on plantlet regeneration

The embryogenic calli transferred to different plantlet regeneration culture media for improving rice plantlet regeneration system showed different response in regeneration ability on the three different types of culture media. The earliest time for green spot formation in callus was 20 days of culture on ARDA medium followed by N6 medium. Among those three culture media ARDA gave the best results in both time and frequency for green spot formation. After 20 days of culture on plantlet regeneration media, the calli developed green spots and a maximum of 61.3% green spot produced calli were obtained on ARDA medium after 30 days of culture. Those green spots developed into shoots. In N_6 medium, the calli at 8.6% developed green spots after the same time of culture (30 days of culture). However, this phenomenon was not observed on MS medium (Figure 2.6 and Figure 2.7). The results indicated that the regeneration frequency (0.0-61.3%) was significantly different among the three tested culture media (Figure 2.7). With respect to medium types, the highest regeneration frequency at 61.3% took place on ARDA medium while the lowest regeneration frequency at 0% was obtained from MS medium. For this reason, ARDA medium was applied for culturing callus in the next experiments.

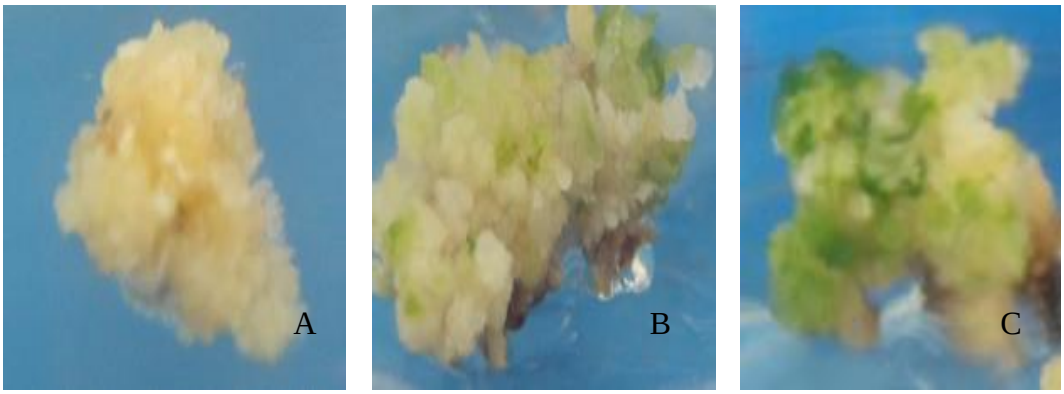


Figure 2.6 Development of green spots in embryoenic calli cultured on different culture media after being cultured for 30 days.

A: MS medium

B: N_6 medium

C: ARDA medium

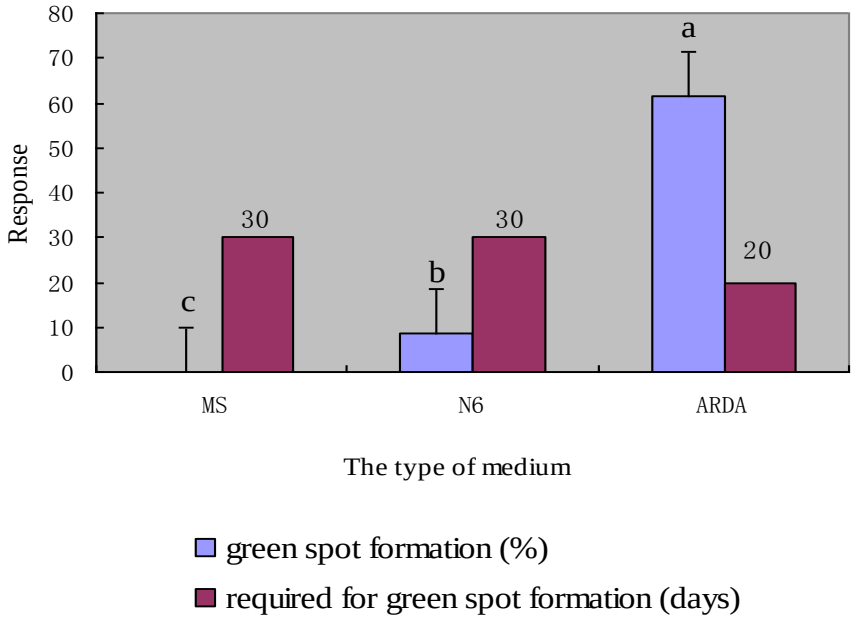


Figure 2.7 Effect of culture media on green spot formation frequencies and time required for green spot development. (bars: standard error; histogram with the different letter is significant difference at $p<0.05$).

In the present study, the regeneration systems of landrace *Hom Kra Dang Ngah* were optimized. The findings revealed that the composition of culture media had significant effect on plantlet regeneration. Among the three culture media, the ARDA provided a maximum percentage of plantlet regeneration. Many authors have reported the use of different culture media, such as MS, N_6, B_5 and NB etc. in tissue culture of rice species (Liu *et al.*, 2005; Wang *et al.*, 2007; Ge *et al.*, 2006). The composition and strength of mineral salts in the media is the main reason which caused the differences of callus induction and plantlet regeneration. Ge *et al.* (2006) optimized the medium S for callus subculture through increasing the $MnSO_4 \cdot 4H_2O$ and other trace elements, base on MS maximum constituent, which improved the regeneration of embryogenic callus. In this present study, the concentration of $MnSO_4 \cdot 4H_2O$ and other trace elements in ARDA medium are higher than that in MS and N_6. Therefore, it might be one of the result that improve plantlet regeneration in landrace *Hom Kra Dang Ngah* rice like the report of Ge *et al.* (2006). It is well known that *indica* rice has strong genotype unique, so, it is essential to optimize culture media separately for each genotype before carrying out any transformation experiments (Zaidi *et al.*, 2006; Afolabi *et al.*, 2008).

Effect of phytagel on plantlet regeneration

Gelling agent also play important role in callus induction subsequent to plantlet regeneration. Both types and strength of them have significant important in those responses. The embryogenic calli exhibited a variable response with respect to agar-agar powder and phytagel concentration in the plantlet regeneration medium. Firstly, the ANOVA results indicated that the frequency of green spots forming calli and percentage of plantlet regeneration on phytagel solidified medium were significant difference from those obtained on agar-agar powder solidified one (Table 2.5). Therefore, phytagel was more suitable for plantlet regeneration than agar powder. Secondly, higher strength of the phytagel (more than 0.25%) was more beneficial for

the differentiation of calli and plantlet regeneration than the low concentration (0.17%). Some calli cultured on the low concentration phytagel turned into green spots but were not able to develop into shoots. So the percentage of plantlet regeneration was as low as 8.3%. However, most of the green spots developed in a low concentration of phytagel (2.5%) containing medium formed a large number of multiple shoots after transferring to culture on medium containing higher concentration of phytagel (>0.25%). Meanwhile, most of the green spots developed in high concentration of phytagel (>0.25%) gave a large number of multiple shoots after transferring to culture medium containing higher concentration of phytagel (0.3-0.35%) (Table 2.5). Moreover, It was observed that phytagel at concentration of 0.3% promoted the highest frequency of green spot forming calli at 100% and produced the highest number of regenerated shoots per callus at 7 shoots with a frequency of 61.0%. Phytagel at 3.5% also gave frequency of green spot forming calli at 100% (same as that of 3%) but frequency of shoot formation was slightly higher (63.3%). Even though frequency of shoot formation was slightly higher but a number of shoots produced was slightly lower at 6 shoots per callus. The two concentrations of phytagel, 0.3% and 0.35% exhibited non significant difference on plantlet regeneration frequency, however, significant differences were found with the other two concentrations (0.17% and 0.25%) (Table 2.5). So, the concentration of phytael at 0.3% was chosen and used in the following experiments.

Table 2.5 Effect of types and concentrations of gelling agents containing SIM on plantlet regeneration capacity after being cultured for 4 weeks.

Gelling agent (%)	Calli forming green spots(%)	Plantlet regeneration frequency (%)	No. of shoots/ callus
Agar(0.75%)(control)	32.1b	8.3c	3
Phytagel(0.17%)	0.0c	0.0d	0
Phytagel(0.25%)	87.5a	21.5b	5
Phytagel(0.30%)	100a	61.0a	7
Phytagel(0.35%)	100a	63.6a	6
F-test	*	*	
C.V. (%)	71.1	96.1	

*Significant difference at $p < 0.05$.

Means of those parameters with the same letter within column are not significantly different using LSD.

Agar is thought to contain agropectins with its sulphate side groups and some other organic impurities that might have inhibitory effects on the explant growth and callus proliferation (Bhojani and Razdan, 1996). Phytagel is said to be free of such impurities as have been found in agar-agar powder. In the present study, the different results between phytagel and agar-agar powder were investigated and the obtaining results were similar to those reported by Shaukat *et al*. (2004). It was clearly observed that phytagel at concentration of 0.3 to 0.35 could significantly improve plantlet regeneration from green spots forming calli.

The increase in concentration of phytagel resulted in the increment of plantlet regeneration frequency. Available of water under low concentration of gelling agent and high humidity of *in vitro* culture conditions were reported to decrease the plantlet regeneration frequency from calli (Bhojani and Razdan, 1996). So, in this study, 0.3% phytagel was the most suitable for plantlet regeneration in *indica* rice

landrace *Hom Kra Dang Ngah* in comparison with the normal concentration of 0.17-0.25%. The contribution of these gelling agents might adjust the humidity, available of water and mineral salts uptake by the callus under *in vitro* culture conditions like those reported by Zaidi *et al.* (2006).

Effect of different ratio of cytokinin (BA+Kn) to auxin (NAA)

In this experiment the results showed that different ratios of (BA+Kn) to NAA gave the various frequencies of differentiation and plantlet regeneration. The ratio of (BA+Kn) to NAA at 6:1 gave the highest frequency of green spots forming calli and plantlets regeneration at 75.5% and 33.3%, respectively. Plantlets obtained on that ratio of PGRs containing medium produced roots at the same time (Figure 2.8D). Lower ratio of those PGRs at 3:1 or 3.5:1 gave the lower percentage of green spots forming calli and plantlets regeneration (Table 2.6). In addition, the plantlets obtained on these PGRs containing medium didn't produce roots (Figure 2.8C). The results from this study suggested that subsequent plantlet regeneration from green spots forming calli depends upon the ratio of (BA+Kn) to NAA. In this case, the ratio of (BA+Kn) to NAA at 6:1 was the most suitable for induction of green spots forming calli, plantlet regeneration and root formation.

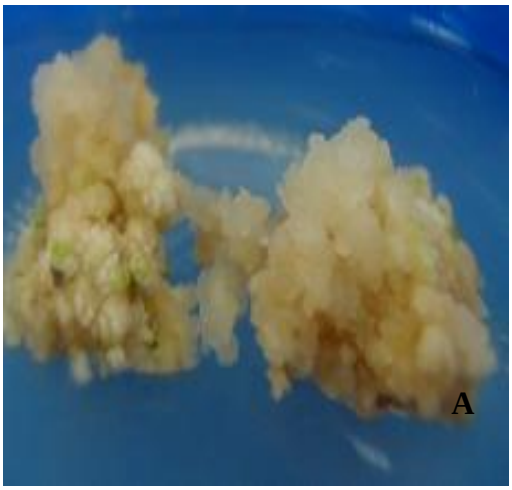
Table 2.6 Effects of different ratio of (BA+Kn) to NAA containing ARDA medium on the response of calli after being cultured for 4 weeks.

NAA ———	BA mg/L	Kn ———	Ratio of (BA+Kn):NAA	Green spots forming calli (%)	Plantlet Regeneration (%)	Root formation
0.5	1.0	2.0	6:1	75.5a	33.3a	Yes
0.5	1.5	2.0	7:1	69.3a	32.1a	Yes
1.0	1.0	2.0	3:1	45.8c	20.3b	No
1.0	1.5	2.0	3.5:1	52.5 b	25.4b	No
F-test				*	*	
C.V.(%)				22.9	21.9	

*Significant difference at $p < 0.05$.

Means of callus response frequency with the same letter within column are not significantly different using LSD.

In most tissue culture experiments, a high auxin to cytokinin ratio is used for initiation of embryogenic callus whereas a low ratio of those PGRs is used for plantlet regeneration (Ge *et al*., 2006). Usually, cytokinins have negative regulators of root growth and development (Werner *et al*., 2003). Auxins are also thought to exert control over the cell cycle by regulating key genes (Blilou *et al*., 2002; Del Pozo *et al*., 2002). The cell cycle is therefore under the influence of both PGRs. Hence, auxin to cytokinin ratio is important for the control of many developmental processes, including organ regeneration from differentiated tissue. The type of interaction may be synergistic, antagonistic or additive (Coenen and Lomax, 1997) depending upon the tissue, its developmental stage and culture conditions (Jaillais and Chory, 2010). Together, these mechanisms coherently coordinate developmental decisions (Busch and Benfey, 2010) culminating into callogenesis, organogenesis or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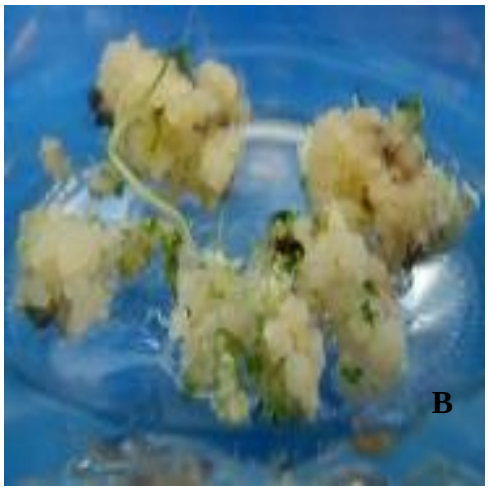
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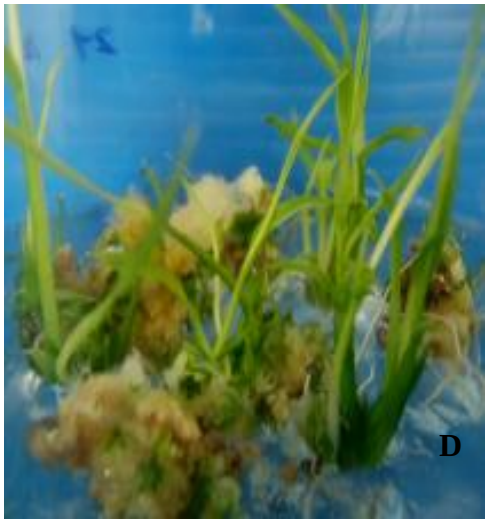
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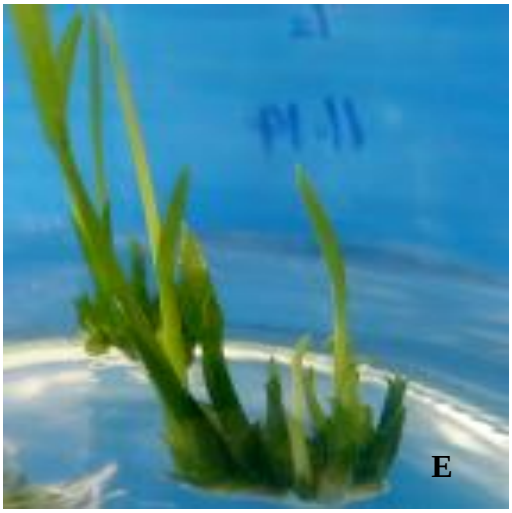
E


F

Figure 2.8 The different stages of plantlet regeneration from somatic embryos or green spots developed in embryogenic calli after being cultured on SIM medium with 0.5 mg/L NAA, 1 mg/L BA and 2.0 mg/L Kn.

A. Proliferation of callus on CIM after 3 weeks of subculture

B. Green spot formation on SIM after 3 weeks of the first round of plantlet regeneration

C. Shoots together with roots produced on SIM supplemented with 1.5 mg/L 6-BA + 0.5 mg/L NAA + 2.0 mg/L Kn after 3 weeks of culture

D. Shoots developed from green spots on SIM after 3 weeks of second round regeneration

E & F. Shoot or cluster of shoots at 3-4 cm in height just after transfer to PGR-free MS medium for root initiation

G & H. Root formation from shoot or cluster of shoots after 3 weeks of culture on PGR-free MS medium

embryogenesis. The present study has also demonstrated that the auxin to cytokinin ratio is decisive to *in vitro* response of plant tissues.

The higher ratio of cytokinin to auxin enhanced the plantlet regeneration. In addition, the ratio of (BA+Kn) to NAA ranging from 3:1 to 7:1 promoted plantlet regeneration in landrace *Hom Kra Ngah* rice. However, the ratio at 6:1 showed the highest efficiency. Lu *et al*. (2006) also reported that the ration of (BA+Kn) to NAA at 7.7:1 was suitable for regeneration in *indica* rice cultivar boB. In case of *indica* rice cultivar Super Basmati, a low ration of Kn to NAA at 3:1 was reported to be the most suitable for plantlet regeneration (Faiz *et al*., 2012). The different responses of the cultivar might be due to strong genotype-independent and recalcitrant in *indica* rice. So, it is necessary to regulate the ratio of cytokinin to auxin according to various genotypes in order to promote regeneration frequency.

Effect of some osmotic regulatory substances and organic nitrogens

Organic nitrogen in term of CH alone at concentration of 1 g/L never promoted plantlet regeneration from green spots forming calli. Decrease in concentration of CH to 300 mg/L in combination with L-proline and glutamine at the same concentration of 500 mg/L slightly improved plantlet regeneration (27.3%). Replacement of the two organic nitrogens, CH in combination with sorbitol at concentration of 82 mM gave significant results. Sorbitol provided the highest plantlet regeneration percentage at 75% (Table 2.7).

Table 2.7 Effect of osmoregulatory substances and organic nitrogen on plantlet regeneration response after being cultured on SIM for 4 weeks.

Treatments	Cultured calli	Green spots forming calli (%)	Plantlet regeneration (%)
CH (1g/L)	28	20	0c
CH (300mg/L)+L-proline (500mg/L)+glutamine (500mg/L)	28	22	27.3b
CH (1g/L)+sorbitol (82mM)	28	26	75a
F-test			*
C.V. (%)			90.9

*Significant difference at $p < 0.05$.

Means of plantlet regeneration frequency with the same letter within column are not significantly different using LSD.

The response of landrace *Hom Kra Dang Ngah* to tissue culture was affected by the type and concentrations of nitrogen sources and some osmoregulatory substance. Our results showed that sorbitol was more effective than L-proline, glutamine and CH with respect to regeneration response. It was reported that CH stimulated callus induction and regeneration frequencies and it provides a source of amino acids. These findings are in conformity with those reportedbby Zaidi *et al.* (2006) and Afolabi *et al.* (2008), in which the positive effect of CH had been shown.

Proline is sometime act as an osmotic regulatory and can have a positive impact on regeneration ability. Our findings showed only a slight effect of L-proline and glutamine on *in vitro* regeneration response of landrace *Hom Kra Dang Ngah* rice. However, 500-600 mg/L L-proline has been recommended for rice tissue culture experiments (Ge *et al.*, 2006; Afolabi *et al.*, 2008). The impact of

incorporating appropriate sorbitol on the plantlet regeneration was also examined. Adding 82mM sorbitol to medium combined with 1 g/L CH created a positive impact on regeneration frequency. This finding is similar to the previous research of Shahsavari *et al*. (2010). In fact, they reported that addition of appropriate amounts of sorbitol in the culture media increased regeneration rate drastically. It seems using sorbitol in tissue culture acts as primary carbon source to enhance regeneration frequency of embryogenic calli (Geng *et al.,* 2008).

Root induction and acclimatization

The shoots at height of 3-4 cm excised and transferred to rooting medium responded in different way. This evidence depended upon the former culture media. Shoots on ARDA medium with 0.5 mg/L NAA together with 1.5 mg/L BA with 2.0 mg/L Kn produced complete roots on PGR-free MS medium after 3 weeks of culture (Figure 2.8G). On the other hand, shoots on ARDA medium supplemented with 1 mg/L NAA together with 1 mg/L BA with 2.0 mg/L Kn produced roots on MS medium with 0.1 mg/L IAA (Figure 2.8H). The frequency of root induction reached at 100% in both PGR-free and IAA containing MS medium. All *in vitro* regenerated plantlets had well-development root system upon transferring complete plantlets to pot containing clay soil and covering with 12 inch plastics under waterlog condition for one week, a 100% of survival rate of plantlets was obtained (Figure 2.9).

Figure 2.9 Survival of plants and morphological characteristics of plant after being transferred to soil for one month.

Conclusion

Many factors affecting plantlet regeneration have been reported in tissue culture of rice species, such as medium composition, plant growth regulators, nitrogen sources, carbon source and so on. In addition, the callus induction medium and proliferation medium together with PGR are important role for plantlet regeneration.

In this study, successful optimizations on the regeneration systems of *indica* rice landrace *Hom Kra Dang Ngah* were performed on ARDA medium supplemented with (BA+KN) and NAA at ratio of 6:1. The culture medium was solidified with phytagel at concentration of 0.3%. Addition of 82 mM sorbitol in combination with 1 g/L CH to SIM gave the highest frequency of plantlet regeneration at 75%. After 7 days of acclimatization, 100% of survival rate was obtained. The regenerated plantlets were morphological healthy and normal. The high regeneration frequency will lead to improve it by genetic transformation technology.

CHAPTER Ⅲ

Establishment of *Agrobacterium*-mediated Transformation Systems of Local *Indica* Rice *Hom Kra Dang Ngah* Variety

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Establishment of *Agrobacterium*-mediated Transformation Systems of Local *Indica* Rice *Hom Kra Dang Ngah* Variety.

Introduction

Rice transformation technique has become important technology in current research of molecular biology and breeding and has been reported by several laboratories. Rice improvement has been carried out by sexual hybridization (Repellin *et al*., 2001), electroporation and *Agrobacterium*-mediated gene transformation (Marchand *et al*., 2007), particle gun bombardment (Baisakh *et al*., 2001), protoplast and *Agrobacterium*-mediated gene transformation (Martino *et al*., 2007). Rice genetic transformation has taken rapid strides since the first transgenic rice plant was produced few years ago (Raineri *et al*., 1990; Baba *et al*., 1986). During the last two decade, a significant breakthrough occurred. Genetic transformation of *indica* rice, such as elita *indica*, Pusa BasmaticI, R_2 and so on had been reported (Azhakanandam *et al*., 2000; Kumria *et al*., 2001; Kumria *et al*., 2002; Hoque *et al*., 2005). So far, the focus of the study moved to establish the genetic transformation of the genotype-independent and recalcitrant cultivar (Hiei and komari, 2008).

Agrobacterium tumefaciens-mediated are routine methods and considered preferable due to simple procedure and low cost, high transformation efficiency, the capacity to transfer relatively large segments of DNA with defined ends, the low copy number of transgenes inserted into the host genome and the expected Mendelian heredity of transgenic plant (Hiei *et al*., 1997, 1994; Shibata and Liu, 2000). Therefore, it is very important to establish efficient and widely used protocol for varieties of rice and extend it to other recalcitrant species. It is also a complement to traditional breeding. However, there are many factors affecting the efficiency of transformation, such as the type and age of explants, strains of

Agrobacterium, expression vector, selectable marker genes and selecting agents, as well as various conditions of tissue culture (Hiei *et al*., 1997; Cheng *et al*., 2004). Among those factors, the genotype of the explants is considered as a crucial one that can hardly be overcome or complemented through optimizing other external factors (Tie *et al*., 2012).

According to the reports in *indica* rice, it is known that their species is recalcitrant genotype and low efficiency of transformation (Wanichananan *et al*., 2010). Nowadays, there is no published data regarding the improvement of *indica* rice varieties using genetic transformation. It is still lack of commonly used genetic transformation technologies for *indica* rice. So the objectives of present study were to examine some key factors affecting gene transformation and established the *Agrobacterium*-mediated transformation systems of local *indica* rice cultivar *Hom Kra Dang Ngah*.

Materials and methods

Plant material

Indica rice (*Oryza sativa* L.) landrace *Hom Kra Dang Ngah* was used for callus induction and transformation. Mature and healthy seeds were selected, manually dehusked, and surface sterilized followed the protocol as described by Zhang and Te-chato (2012).

Callus induction and plantlet regeneration

Sterile seeds were placed on callus induction medium (CIM) which was MS medium supplemented with 3% sucrose, 2 mg/L 2,4-D, 1 mg/L NAA, 1 mg/L 6-BA, and 0.5 mg/L Kn and 1 g/L CH. The medium was solidified with 0.75%

agar-agar powder. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121^{o}C, 1.07 kg/cm^{2} for 15min. All cultures were placed in the culture room at 27±2^{o}C, under 16 hour photoperiod. After 1 month of culture, the embryogenic calli were transferred to subculture medium (SCM) which was MS medium containing 1 mg/L 2,4-D, 0.5 mg/L NAA, 0.5 mg/L 6-BA and 0.25 mg/L Kn. The other supplements in the subculture medium were the same as in CIM and cultured for further 1 month. Embryogenic calli were then transferred to regeneration medium (RM) which was reported by Zhang and Te-chato (2013). The medium was ARDA supplemented with 0.5 mg/L NAA, 1 mg/L 6-BA and 2 mg/L Kn and modified by adding 82 mM sorbitol and 1 g/L casein hydrolysate. Finally, the culture medium was solidified with 0.3% phytagel.

Testing the sensitivity of the callus to glyphosate

Embryogenic calli were inoculated on SCM with four different concentrations of glyphosate (0, 0.15, 0.3 and 0.5 mM) and cultured at 27±2^{o}C, under 16 hour photoperiod for 2 weeks. The sensitivity was determined according to the growth status of the calli.

Strain of Agrobacterium and its preparation

Agrobacterium strain EHA 105 harbouring the plasmid pCAMBIA1304 with β-glucuronidase (*gus*) reporter gene and glyphosate resistant gene (*epsps*) as shown in Figure 3.1 was used for transformation experiment.

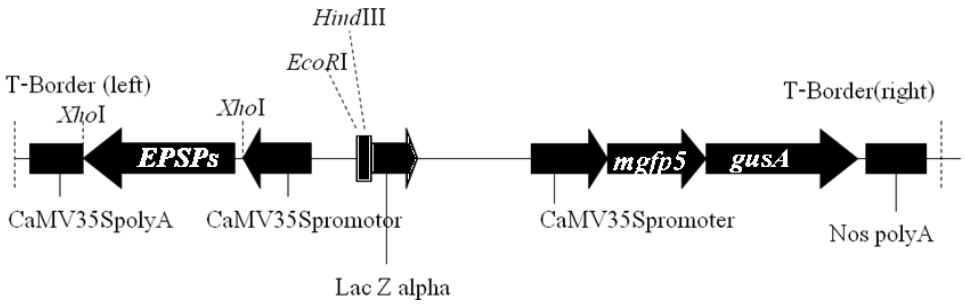

Figure 3.1 The T-DNA regions of the cointegrate vector pCAMBIA1304 with β-glucuronidase (*gus*) and glyphosate resistant gene (*epsps*) used for rice transformation. (source: Kasetsart Univerity Kamphaeng Saen, Nakhorn pathom, 2012).

One loopful of bacterial stock was streaked onto LB solidified medium with 50 mg/L kanamycin containing in 20 × 900 mm glass plate for correct transformed colony selection. The cultures were incubated at 28℃ in the dark for 2 days. From this culture, one single colony of bacterium was picked out and incubated into 20 ml of LB liquidified medium containing 50 mg/L kanamycin and 200 μM acetosyringone in 125ml Erlenmeyer flask. The cultures were maintained on a rotary shaker at 75-100 rpm at 28℃ for 16-18 h. After that the optical density (OD_{600}) of bacterial cell suspension was adjusted to 0.3, 0.6 and 0.8 with liquidified CI medium containing 200 μM acetosyringone as *Agrobacterium* inoculum and directly used for infection.

Infection and co-cultivation period

The 6 and 8-week-old embryogenic calli obtained on SCM were cut into approximate size of 0.5 cm pieces and inoculated by submerging in 20 ml of the *Agrobacterium* solution for 15, 20 and 30 minutes. Then the calli were blotted dry on sterile filter paper to remove remnant *Agrobacterium* and then transferred to co-

culture medium (CIM medium containing 200 μM acetosyringone). The co-cultivation was maintained in the dark at 26℃ for 3 days, followed by thoroughly washing in sterile distilled water at 3-5 times, then washing with liquid CIM medium containing 500 mg/L cefotaxime. The washed calli were blotted dry on sterile filter paper to remove excess moisture and transferred to *Agrobacterium* elimination medium (SCM medium containing 300 mg/L cefotaxime) to get rid of the excess of *Agrobacterium* attached to the calli for 2 weeks. After that the inoculated calli were transferred to seletction medium, which was SCM medium supplemented with maximum concentration of glyphosate obtained from testing its sensitivity. The cultures were placed in culture room at 28^{o}C under 16 h photoperiod and subcultured at 2 weeks of interval for 4 weeks. After 4 weeks of selection, the small transgenic glyphosate resistant embryogenic calli were recorded.

GUS histochemical assay

After 3 days of co-cultivation, the rice embryogenic calli were washed with sterile distilled water and immersed in GUS assay buffer containing 50 mM phosphate buffer (pH 6.8), Triton X-100 and 1mM histochemical substrate 5-bromo, 4-chloro-3-indolyl-β-D-glucuronide (X-Gluc-Duchefa, The Netherlands) according to method as described by Jefferson (1987). The reaction mixture was incubated overnight at 37 ^{o}C. After X-gluc treatment the calli were washed thrice in 70% methanol. The presence of blue color as the expression of GUS (β-glucuronidase) was recorded. The frequency of transient transformation was expressed as the ratio between the number of callus showing blue spot (GUS expression) by the total number of callus kept for staining. Proper control for GUS histochemical assay was done with the calli having no *Agrobacterium* infection.

Polymerase chain reaction (PCR) analysis

The genomic DNA of putative transformed and non-transformed calli was isolated according to the method described by Te-chato (2000). The samples of DNA were subjected to PCR amplification using *gus* and *epsps* primers along with positive (plasmid DNA) and negative control (non-transformed DNA) to confirm the presence or absence of transgenes in the primary transformed calli. The primer sequences were as followed: the *gus* gene fragment primer sequence F-primer 5′-CTGCGACGCTCACACCGATAC-3′ and reverse primer sequence R-primer 5′-TCACCGAAGTTCATGCCAGTCCAG-3′. The *epsps* gene primers forward: 5′-CCATTCCGCTCGAGATGGCACAAATTAACAACATGGC-3′ and reverse: 5′-ATCCACCGCTCGAGCGGTCATCAGGCAGCCTTCGTAT-3′. PCR was performed for *gus* and *epsps* in a 20 μL reaction volume containing 2μL 10×PCR buffer, 4 μL 1mM dNTP, 0.5 μL each of 10 mM primer, 1.0 U of *Taq* polymerase and 20 ng of DNA template. Amplification was carried out in a programmable thermal cycler under following conditions, predenature at 94 ^{o}C for 5 min, followed by 30 cycles of denature at 94 ^{o}C for 40 s, annealing at 60 ^{o}C for 30 s, extension at 72 ^{o}C for 1min, with a final extension at 72 ^{o}C for 5 min. Amplification products were analyzed by electrophoresis at 100 V, 80 A for 45 min on 1.5 % agarose gel dissolved in TBE buffer followed by staining with ethidium bromide and detection under UV illumination.

Statistical analysis

The experiment was arranged as a randomized complete block design (RCBD) with 3 replicates per treatment. Analysis of variance (ANOVA) was applied to indicate which treatment was the best for transformation. Data were analyzed using

the SAS program Version 6.11 (SAS Institute, Cary, NC). A probability level of 5% (p=0.05) was chosen for all statistical inferences.

Results and Discussion

The foreign gene transformation mediated by the *Agrobacterium tumefaciens* is the result of *Agrobacterium tumefaciens* strains and plant cell interaction. Several factors were found to have an impact on the efficiency of *Agrobacterium tumefaciens*-mediated transformation, including Ti plasmid, plant cell response capability, co-cultivation with *Agrobacterium* and the regeneration ability of the transformants (Gao and Hang, 1999). *Agrobacterium tumefaciens*-mediated transformation included steps such as, attachment, *vir* gene induction, T-DNA processing, transporting and integration, which completed during co-cultivation period (Liu and Zhang, 2005). So, the key of successful transformation is to master the technique and time of co-cultivation.

Embryogenic calli induction and plantlet regeneration

The embryogenic calli are the most amenable source material for genetic transformation of *indica* rice. In this study, two types of callus which were embryogenic and non-embryogenic callus were obtained on CIM after 4 weeks of inoculation (Figure 3.2A). Embryogenic calli were creamy white, some compact, friable and globular (Figure 3.2B) which were further developed into plantlets (Figure 3.2C).

The embryogenic calli were transferred to SCM for proliferation. After 3 weeks of subculture, fast growing embryogenic calli and green spots were obtained (Figure 3.2 B). Upon transferring the calli to RM for plantlet regeneration, a high frequency of plantlet regeneration at 75% was obtained (Figure 3.2 C).

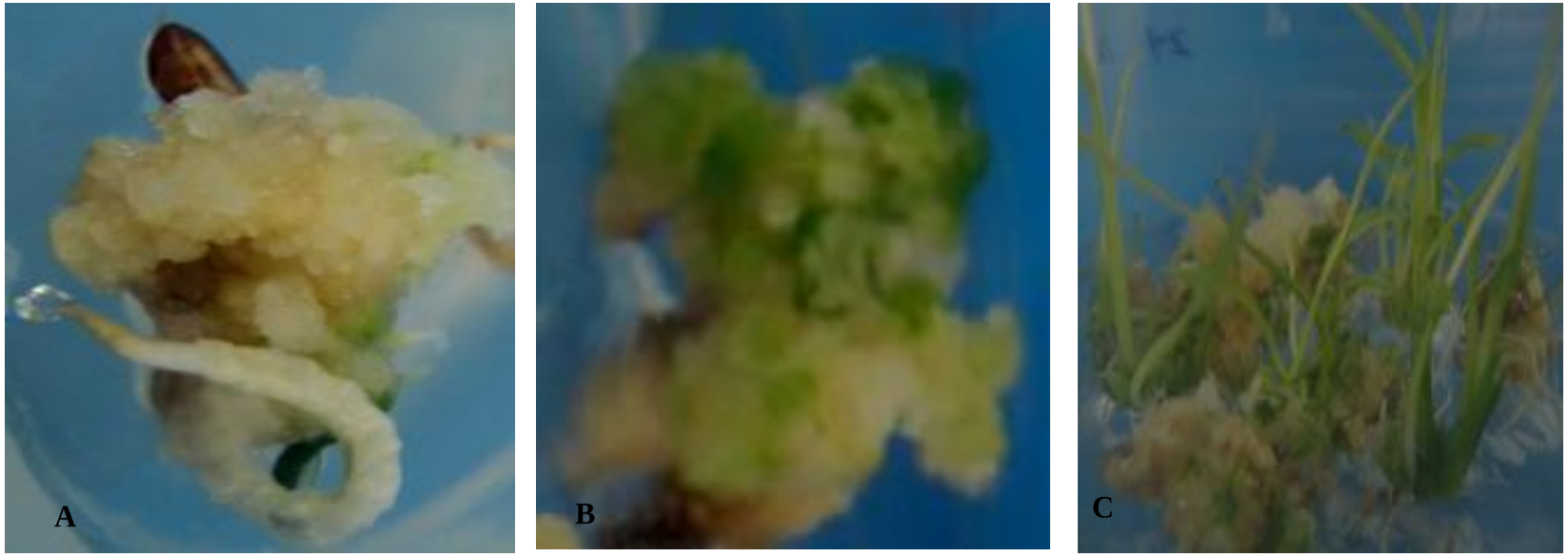

Figure 3.2 Embryogenic callus induction, proliferation and regeneration of *indica* rice cv.*Hom Kra Dang Ngah*.

A. Callus with embryogenic structures derived from mature seed after 1 month on CIM

B. Proliferation of callus on SCM after 3 weeks of subculture

C. Plantlet regeneration from embryogenic callus after 2 months on RM

Embrogenic calli and regeneration system are the prerequisite for genetic transformation mediated by *Agrobacterium* in *indica* rice. Plant regeneration from embryogenic callus has achieved initially in *japonica* rice varieties (Nishi *et al*., 1973). Successful regeneration of fertile plants has been reported to limit in *indica* rice varieties (Rueb *et al*., 1994). It is also a reason that the transformation of *indica* rice is still difficulty, due to the low induction rate of embryogenic calli and plantlet regeneration. Many *indica* rices are particular genotype-dependent (Hiei and komari, 2006; Hiei and komari, 2008). Although a few successful genetic transformation in *indica* rice has been reported (Lin *et al*., 2009; Wang *et al*., 2010) it has strong recalcitrant nature and genotype-dependent. In present study, the high efficiency of regeneration system was established using mature embryo as explant and cultured on MS medium supplemented with 2 mg/L 2,4-D, 1 mg/L NAA, 1 mg/L 6-BA and 0.5 mg/L Kn containing 3% sucrose and 1 g/L CH. After 1 month of culture, the embryogneic calli were transferred to proliferation medium which was ARDA medium supplemented with 0.5 mg/L NAA, 1 mg/L 6-BA and 2 mg/L Kn and cultured for further 1 month. Finally, the calli were transferred to plantlet regeneration medium which was MS solidified with phytagel at concentration of 0.3%, 82 mM sorbitol in combination with 1 g/L CH. The highest frequency of plantlet regeneration at 75% was obtained. This culture system in callus-based *indica* rice was used for transformation system.

The sensitivity of calli to glyphosate

The present study aims to optimize glyphosate concentration for selection procedure. After culturing the calli on SCM containing different concentrations of glyphosate for 2 weeks, all calli survived without the supplemented with glyphosate (Figure 3.3A). Glyphosate at concentration higher than 0.3 mM caused browning and necrosis of the calli. Glyphosate at concentration of 0.5 mM caused partial dead of the calli after 2 weeks of culture (Figure 3.3D). Among the

different concentrations of glyphosate, 0.5 mM was found to be a suitable concentration for selection of gene transfer in *indica* rice cultivar *Hom Kra Dang Ngah* (Figure 3.3D). So, 0.5mM was concluded to be the minimum concentration, which effectively prevented calli to grow. This experiment provided the basis for the choice of glyphosate concentration in selection media for transformation experiments.

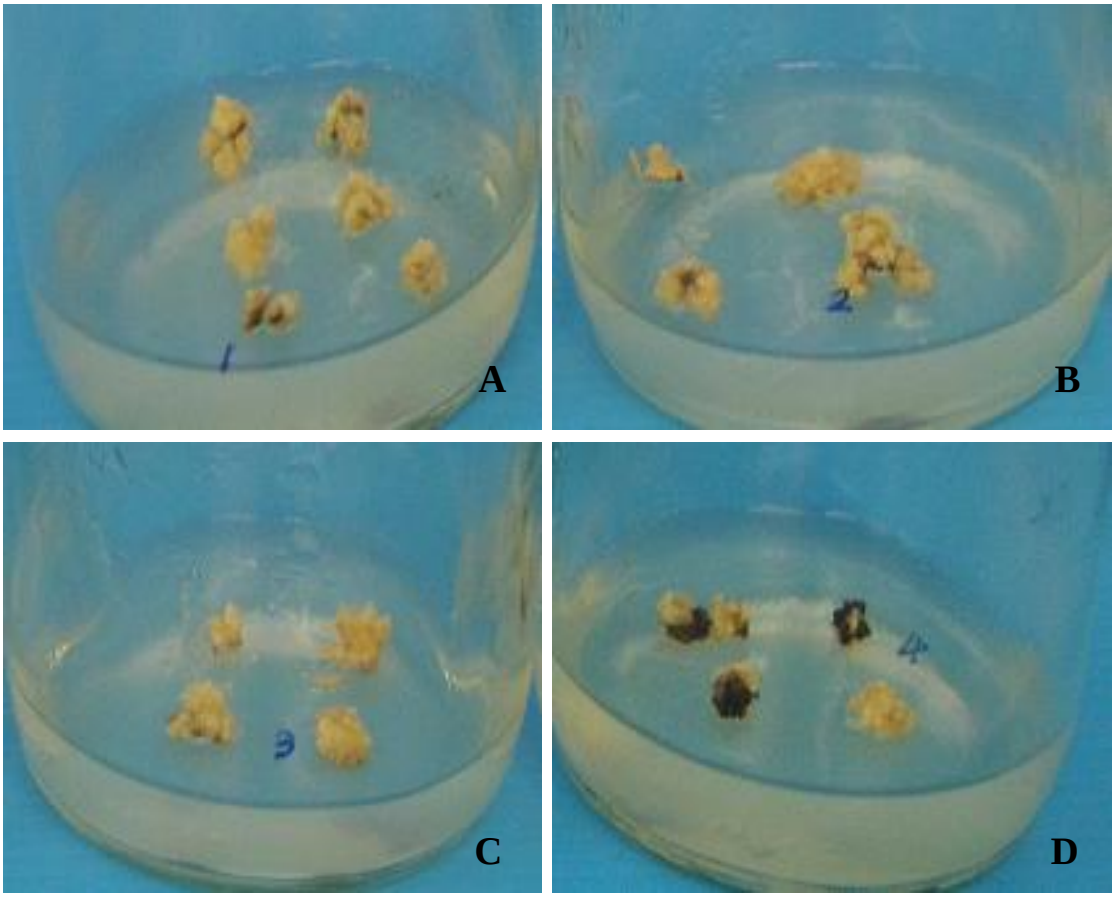

Figure 3.3 Growth status of embryogenic calli of *indica* rice cv. *Hom Kra Dang Ngah* on culture medium containing various concentrations of glyphosate after 2 weeks of culture.

A: without glyphosate

B: 0.15mM glyphosate

C: 0.3mM glyphosate

D: 0.5mM glyphosate

Effects of callus age on gene transformation

The age of explant plays important role in transformation. In order to investigate the effect of callus age on T-DNA delivery and transformation efficiency, 6 and 8-week-old calli were separately immersed in *Agrobacterium* suspension at OD_{600} of 0.8 for 20 minutes. Through assaying *gus* gene histochemical activity and observing the frequency of *gus* gene expression, the results indicated that 6-week-old calli showed more strong sensitivity to transient *gus* gene expression than that of 8-week-old calli. Frequency of *gus* gene expression obtained from 6-week-old calli was 70.5% whereas 8-week-old calli gave only 51.3% (Table 3.1). According to the blue zone and the color of spots, non-transgenic calli didn't show the appearance of blue color of *gus* activity (Figure 3.4A). By contrast, the 8-week-old transgenic calli showed small zone and a little blue color (Figure 3.4B). In the case of 6-week-old transgenic calli, they showed blue zone at the entire surface of transformed calli with dark blue color (Figure 3.4C). In addition, transformed 8-week-old calli were difficult to eliminate *Agrobacterium* leading to finally contamination. So, 6-week-old calli were better and used for following experiment.

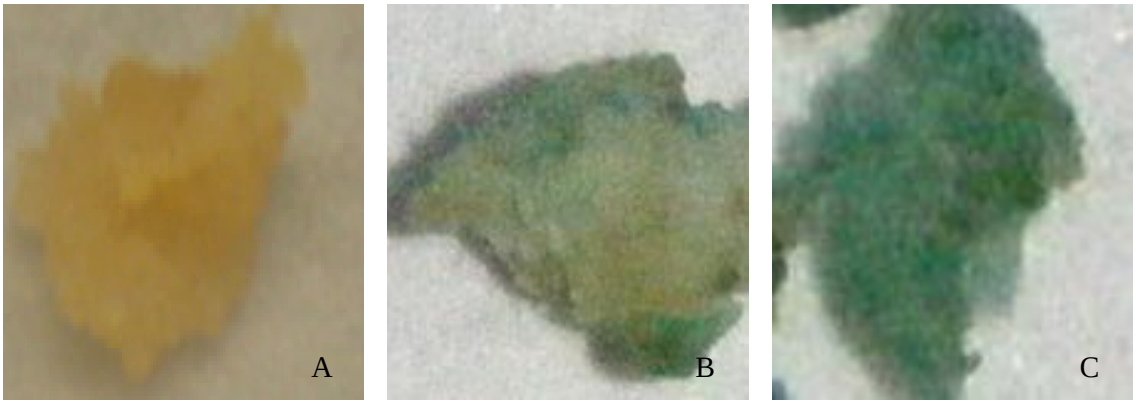

Figure 3.4 Histochemical assay of *gus* expression in transformed calli of rice cultivar *Hom Kra Dang Nga* at different ages with *Agrobacterium* suspension at OD_{600} of 0.8 for 20 minutes.

A. Non transformed calli (negative control)

B. Transformed 8-week-old transgenic calli with small and a little blue color

C. Transformed 6-week-old transgenic calli with blue zone at the entire surface of infected calli

Table 3.1 Ages of calli, density of *Agrobacterium* and infection time affecting gene transformation in term of GUS expression.

Callus age	OD_{600}	Infection time (min)	GUS expression （%）
6-week-old	0.3	10	5.3
		15	33.5
		20	56.7
	0.6	10	40
		15	68.6
		20	83.5
	0.8	20	70.5
8-week-old	0.3	10	0
		15	14.6
		20	20.5
	0.6	10	32
		15	36
		20	46.3
	0.8	20	51.3

Callus cultures are excellent sources of cells for the production of transgenic rice (Hiei *et al*., 1997; Rashid *et al*., 1996). The use of actively growing, embryogenic calli is one of the most important factors in efficient transformation. Short-term and long-term culture significantly affected the efficiency of transformation. Liu *et al.* (2005) studied the effect of callus age on transient *gus* expression. The results have shown that the 42-day-old calli performed better and exhibited maximum number of blue zone in the variety *Pusa Basmati 1.* Similar result was also found in this present study. Six-week-old calli gave better results in genetic transformation than 8-week-old calli. This might be due to the different between physiological status of those two callus ages. The 6-week-old calli has strong capability of division due to the cells are in mitogenetic status. So it benefits the *Agrobacterium* to attach. In addition, mitogenetic cells promote T-DNA to integrate into genomic DNA of rice. Therefore, 6 weeks of sub-cultured calli were easier to transformation than those of short-term and long-term cultured calli.

Density of Agrobacterium and infection period

The bacterial density and infection time are also important factors in transformation experiments mediated by *Agrobacterium*. In the present study, 6-week-old calli were used for genetic transformation. During the transformation process, different concentrations of *Agrobacterium* (OD_{600}=0.3, 0.6, 0.8) and the duration of inoculation (10, 15, 20 min) gave different results. After 3 days of co-culture, histochemical activity of *gus* gene and the frequency of calli indicated that *gus* gene response increased with the increasing time of infection (Figure 3.5). Lower bacterial density (OD_{600}=0.3) and shorter time of infection (10 min) decreased the frequency of transformation (Table 3.1). The highest frequency of gene transformation according to detecting of blue spot was obtained at 83.5% from 20 minute infection time and the OD_{600} of bacterial density at 0.6 (Figure 3.5F).

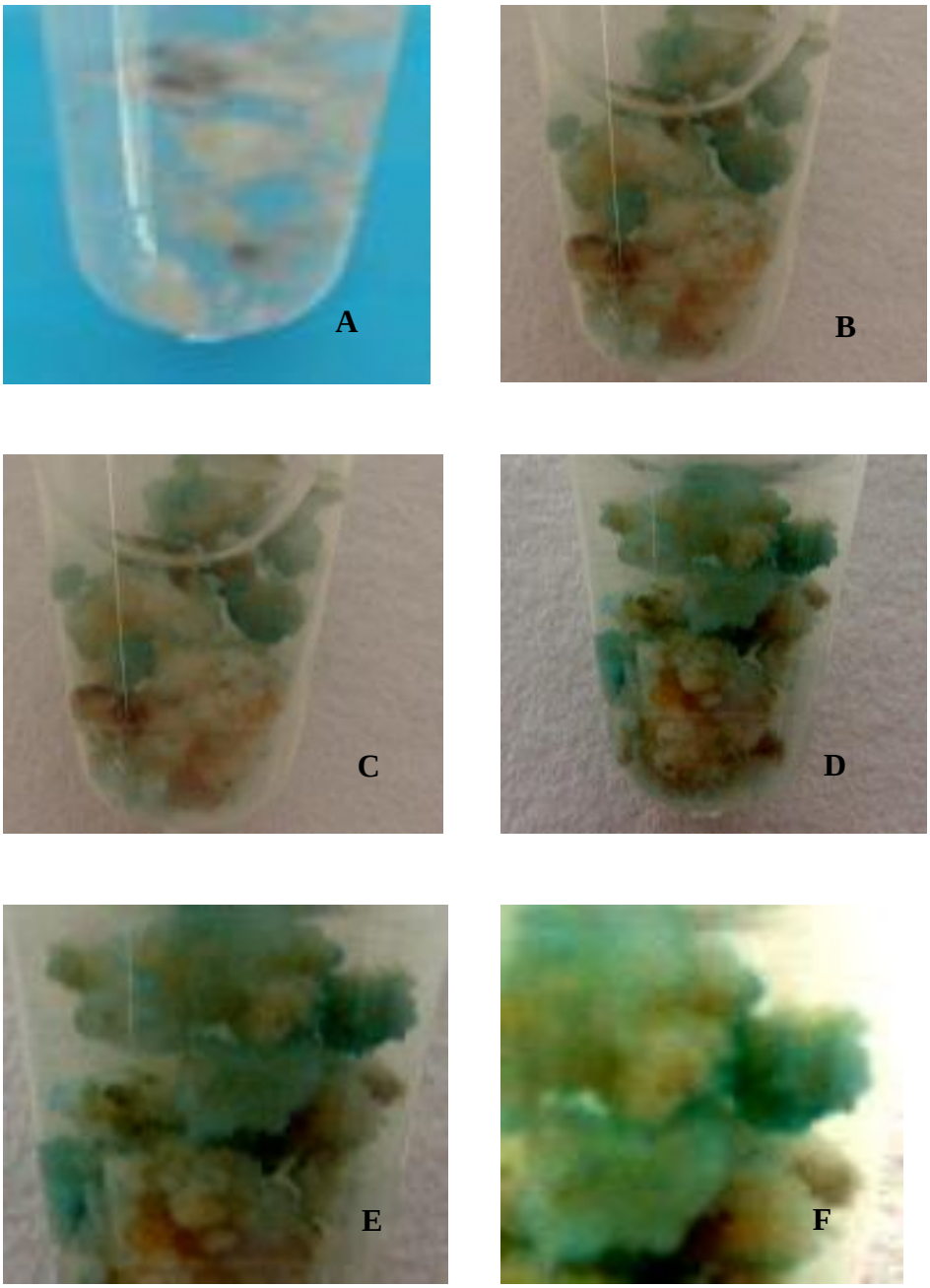

Figure 3.5 Histochemical assay of *gus* expression in transformed calli of rice, *Hom Kra Dang Nga* at different densities of *Agrobacterium* for different times of infection.

A: The callus excised from non-transformants (control)

B: Calli inoculated in OD_{600} =0.3 and infected time for 10min

C: Calli inoculated in OD_{600} =0.3 and infected time for 15min

D: Calli inoculated in OD_{600} =0.6 and infected time for 10min

E: Calli inoculated in OD_{600} =0.6 and infected time for 15min

F: Calli inoculated in OD_{600} =0.6 and infected time for 20min

The efficiency of co-cultivation was affected by the bacterial cell density, infection methods, infection time, co-cultivation temperature and so on. Bacterial density plays an important role in the transformation. According to many reports, the OD_{600} of bacterial density at range from 0.3-1 was suitable for transformation (Aananthi *et al*., 2010； Baskaran and Dasgupta, 2012). In the present study, the OD_{600} of bacterial density at 0.6 showed the highest transformation efficiency at 83.5% as the result of assaying *GUS* activity.

Infection time is also one of the key factors affecting successful in transformation experiments mediated by *Agrobacterium*. Research has shown that infection 10-20 min gave the best result in transformation (Wang and Tian, 2009). Long time of infection leads to contamination, while short time of infection can't promote *Agrobacterium* attachment to calli completely and T-NDA processing. In the present study, calli infected for 10, 15 and 20 minutes gave the increment in the expression of *GUS* response. Infect time at 20 minutes showed the highest response of *GUS* activity.

Polymerase chain reaction (PCR) analysis

The result of gene transformation at molecular level by PCR analysis revealed that the calli with the *gus* gene and *epsps* resistance were transformed. The genomic DNA was isolated from the putative transgenic calli and non-transgenic calli as control. The DNA samples were amplified with *GUS* primers and *EPSPs* primers separately for PCR analysis. Nine of ten putative transgenic calli were found to contain the transgene with the expected band size at 441bp, while non-transformed

callus could not amplify the band size of 441 bp (Figure 3.6). These results preliminarily confirmed that *Agrobacterium tumefaciens* could transfer the T-DNA into the *indica* rice and the transgene integrated into the genomic DNA. In terms of *epsps* gene, however, only one from ten DNA sample showed amplification of this sequences as expected size of 1600bp though these transformants showed amplification of *gus* gene of the expected size (Figure 3.7).

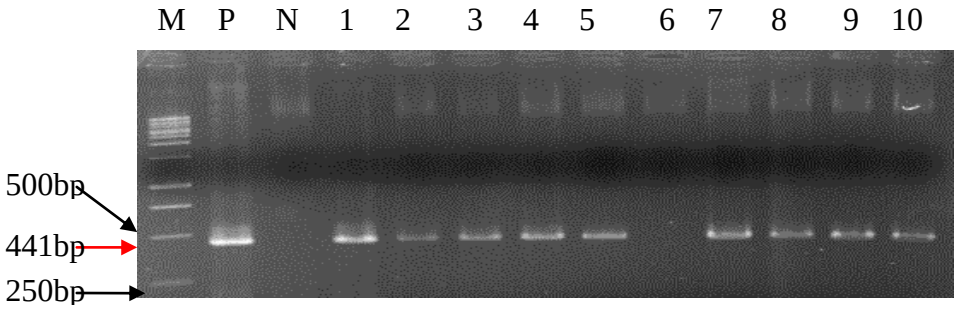

Figure 3.6 PCR analysis of genomic DNA of putative transgenic calli to detect the presence of *gus* gene. M: 1kb size marker (Promega). P: positive control. N: negative control. lane 1-10 were transformants. The red arrow indicates the expected PCR product of the *gus* gene.

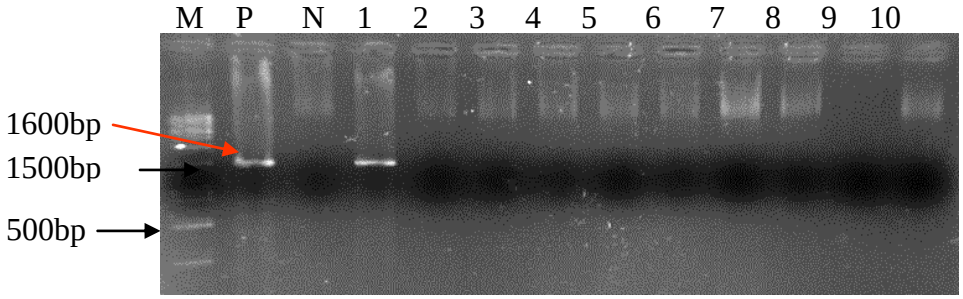

Figure 3.7 PCR analysis of genomic DNA of putative transgenic calli to detect the presence of *epsps* gene. M: 1kb size marker (Promega). P: positive control. N: negative control. lane 1-10 were transformants. The red arrow indicates the expected PCR product of the *epsps* gene.

Conclusion

In the present study, the high-efficiency regeneration system and succeeded in establishing an efficient gene transfer system in *indica* rice cultivar *Hom Kra Dang Ngah* by *Agrobacterium*-mediated method were reported. The results of our study suggested that 6-week-old embryogenic calli derived from mature seeds infected in optical density of *Agrobacterium* at 0.6 for 20 minutes were the most suitable for transformation. The stable *gus* gene and *epsps* gene integration were further confirmed by PCR, which showed stable transformation of the gene at T_0 level. In summary, this report described the use of *Agrobacterium tumefaciens* strain EHA 105 (pCAMBIA1304-*EPSPS*) to transfer screenable and report genes into *indica* rice cultivar *Hom Kra Dang Ngah* and the tissue culture standardization and optimization of transformation conditions in *Hom Kra Dang Ngah* might help in the transformation of other related genotypes for their genetic improvement.

CHAPTER IV

Concluding Remarks

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Concluding Remarks

Hom Kra Dang Ngah has been favorite cultivar of southern people due to it contains high nutritional element. The present research aimed to improve its quality through genetic transformation technique. The followed results were obtained through the present study.

Firstly, the plantlet regeneration systems were established, which included the callus induction on MS medium supplemented with 2 mg/L of 2,4-D in combination with 1.0 mg/L of NAA, 1.0 mg/L of 6-BA and 0.5 mg/L of Kn and the frequency of embryogenic callus induction was at 56.3%. The calli derived from seeds proliferated well on MS medium supplemented with 1 mg/L of 2,4-D in combination with 0.5 mg/L of NAA, 0.5 mg/L of 6-BA and 0.25 mg/L of Kn. In terms of plantlets regeneration, ARDA medium supplemented with 0.5 mg/L NAA in combination with 1.0 mg/L 6-BA, 2.0 mg/L Kn, 82 mM sorbitol and 1 g/L CH, solidified with 0.3% phytagel gave the highest frequency of plantlet regeneration at 75%.

Secondly, the transformation system was well established through *Agrobacterium tumefaciens*-mediated methods and obtained the transgenic plantlets by optimizing important parameters which were 6-week-old embryogenic calli, 20 minutes of infection with bacterial suspension at OD_{600} of 0.6 and selection medium containing 0.5 mM glyphosate. By those conditions the highest frequency of *gus* gene expression at 83.5% was obtained.

Finally, the target genes were confirmed to integrate into rice genome by polymerase chain reaction (PCR).

The results suggested that the improved medium for the tissue culture standardization and the optimized conditions for transformation system were effective in achieving high efficient transformation of novel varieties of *indica* rice. Since the

indica rice genotypes descried here were widely grown in southern of Thailand. It seems likely that the regeneration protocols developed here might help in transformation of similar genotypes for crop improvement strategies in future.

Reproducible transformation of *indica* rice varieties with desired gene is very important for the development of cultivars with valuable agronomic traits in the world. Further work and optimization of the transformation techniques will be needed to achieve the goal. Although we have not yet been successful to get transgenic plants from the transformed calli, the good response in transient *GUS* assay indicates that this might be possible in the future.

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Appendix A

Preparation of solution buffers and reagents

1. DNA isolation by the modified Te-chato method

1.1 TE buffer

- 500 µl of 20 mM Tris-HCl (pH 8.0)
- 200 µl of 0.1M EDTA (pH 8.0)

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave.

1.2 10% SDS

- 5 g of SDS

Adjust the volume to 50 ml with distilled water and sterilize using an autoclave.

1.3 5M Ammonium acetate

- 38.54 g of ammonium acetate

Adjust the volume to 50 ml with distilled water and sterilize using millipore filter

1.4 0.5 M EDTA

- 37.224 g EDTA

Add about 700 ml H_2O and add 16-18 g of NaOH pellets for adjust pH to 8.0 by with a few more pellets, EDTA won't dissolve until the pH is near 8.0. Bring total volume to 1 L with ddH_2O.

1.5 1.0 M Tris-HCl (pH 8.0)

- 121.1 g of Tris-HCl

Dissolve in about 700 ml of H_2O. Bring pH down to 8.0 by adding concentrated HCl.

1.6 TE buffer

- 500 µl of 1.0 M Tris-HCl (pH 7.5)
- 200 µl of 0.25M Na2EDTA (pH 7.0)

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave.

1.7 5X TAE buffer

- 121.1g of Tris Base
- 28.5 ml of Acetic acid
- 50.0 ml of 0.5M Na_2EDTA (pH 8.0)

Adjust the volume to 500 ml with distilled water and sterilize using an autoclave. To make 1X TAE buffer before using.

1.8 5X TBE buffer

- 216 g of Tris Base
- 110 g of Boric acid
- 80 ml of 0.5M Na2EDTA (pH 8.0)

Adjust the volume to 4 L with distilled water and sterilize using an autoclave. To make 0.5X TBE buffer before using.

2. Histochemical detection of GUS gene

X-Gluc solution：

0.2 M NaHPO4，pH 7.0	100 mls
0.2 M $Na_2HPO4 \cdot 7H_2O$	62 mls (53.614 g)
0.2 M $NaH_2PO4 \cdot H_2O$	38 mls(27.598 g)
DI H_2O	94 mls
0.1 M $K_3[Fe(CN)_6] \cdot 3H_2O$	1 ml (6.586 g)
0.1 M $K_4[Fe(CN)_6] \cdot 3H_2O$	1 ml (8.448 g)
0.5 M Na_2EDTA	4 mls (93.06 g)
200 mg X-Gluc	
sodium phosphate buffer	50 ml
Triton-x	20 uls

Kept in refrigerator at 4 ^oC

Appendix B

Composition of Nutrition of Culture Media

Table 1 Composition of nutrition of Murashige and Skoog (MS) medium

Component	Volume(mg/l)
Major elements	
NH_4NO_3	1,650.000
KNO_3	1,900.000
KH_2PO_4	170.000
$CaCl_2.2H_2O$	440.000
$MgSO_4.7H_2O$	370.000
KI	0.830
H_3BO_3	6.200
$MnSO_4.H_2O$	16.900
$ZnSO_4.7H_2O$	10.600
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.250
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.800
Na_2EDTA	37.300
Myo-inositol	100.000
Nicotinic acid	0.500
Pyridoxine HCl	0.500
Thaiamine HCl	0.100
Glycine	2.000
Sucrose(g)	30.000
Agar(g)	7.500
pH	5.7

Appendix B (Continued)

Table 2 Composition of nutrition of ARDA medium

Component	Volume(mg/l)
NH_4NO_3	1025
KNO3	950
$CaCl_2.2H_2O$	268
$Ca(NO_3)4H_2O$	278
$MgSO_4.7H_2O$	185
K_2SO4	495
KH_2PO4	170
H_3BO_3	6.2
$MnSO_4.H_2O$	16.9
$ZnSO_4.7H_2O$	9.6
KI	0.415
$CuSO_4.5H_2O$	3.138
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.013
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.3
Organic elements	
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thaiamine HCl	0.55
Glycine	2
Sucrose(g)	30
Agar(g)	7.50
pH	5.7

Appendix B (Continued)

Table 3 Composition of nutrition of N_6 medium

Component	Volume(mg/l)
$(NH_4)_2SO_4$	463.000
KNO_3	2830.000
KH_2PO_4	400.000
$CaCl_2.2H_2O$	166.000
$MgSO_4.7H_2O$	185.000
KI	0.800
H_3BO_3	1.600
$MnSO_4.4H_2O$	4.400
$ZnSO_4.7H_2O$	1.500
$FeSO_4.7H_2O$	27.800
Na_2EDTA	37.300
Organic elements	
Nicotinic acid	0.500
Pyridoxine HCl	0.500
Thaiamine HCl	0.100
Glycine	2.000
Sucrose(g)	30.000
Agar(g)	7.500
pH	5.7

Appendix B (Continued)

Table 4 Composition of nutrition of LB medium

Component	Volume(g/l)
Pepton	10
Yeast extract	5
NaCI	5
PH	7.0
Agar	7.0

PAPER 1

Callus Induction and Plantlet Regeneration from Mature Embryos of *Indica* Rice (*Oryza sativa* L.) Cultivar *Kra Dang Ngah*

PAPER 2

Improved Plantlet Regeneration Systems in *Indica* Rice (*Oryza sativa* L.) landrace *Hom Kra Dang Ngah*

PAPER 3

Establishment of *Agrobacterium*-mediated Transformation System of *Indica* Rice *Hom Kra Dang Ngah* Variety

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List of Publications and Proceedings

Zhang, Y.X. and Te-chato, S. 2012. Callus induction and plantlet regeneration from mature embryos of *indica* rice (*Oryza sativa* L.) cultivar *Kra Dang Ngah*. Journal of Agricultural Technology 8: 2423-2433.

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