GENETIC TRANSFORMATION OF PAPAYA (*Carica papaya*, L.) CULTIVAR KAPOHO BY PARTICLE BOMBARDMENT

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ABSTRACT

Papaya transformation systems were developed by Fitch et al. (1991) at the University of Hawaii, and transgenic 'Sunset' papayas with papaya ringspot virus (PRV) coat protein (cp) gene showed complete resistance to papaya ringspot virus (PRV) in the field tests (Manshardt et al, 1994). In our studies, we transformed 'Kapoho' papaya, the major crop on the Big Island, Hawaii, based on Fitch's (1991) papaya transformation systems, and obtained transgenic 'Kapoho' papaya plants.

The coat protein (cp) gene of PRV, along with a kanamycin selective marker gene (neomycin phosphotransferase, NPTII) and a β-glucuronidase (GUS) reporter gene, were constructed into the same plasmid vector by our collaborators at Cornell University and transformed into papaya tissue by particle bombardment. Transgenic 'Kapoho' papaya plants were obtained following somatic embryogenesis from hypocotyl callus on kanamycin selective medium and showed GUS positive expression.

Immature zygotic embryos were excised and bombarded with gold particles. Following different treatments of indole-3-butyric acid (IBA), chimeric hypocotyls were harvested on germination medium 20 days after bombardment. Somatic embryogenesis from sections of chimeric transgenic hypocotyls occurred on induction medium and the transgenic embryos were cultured on selective induction medium or maturation medium with different concentrations of kanamycin for

eight months. Then, the embryos were regenerated on germination medium without kanamycin.

GUS was assayed in all experimental steps, and different GUS positive results were observed at different developmental stages. ELISA assays of coat protein and NPTII in chimeric transgenic hypocotyls showed positive expression and a high efficiency of transformation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BA	=	6-Benzylaminopurine
CaMV	=	Caulifower Mosaic Virus
cp	=	Coat protein
2,4-D	=	2,4-Dichlorophenoxyacetic Acid
ELISA	=	Enzyme-Linked Immunosorbent Assay
GUS	=	β-glucuronidase
IAA	=	Indole-3-acetic Acid
IBA	=	Indole-3-butyric Acid
LB	=	Luria Bertani
MS	=	Murashige and Skoog Media (1962)
NAA	=	Naphthaleneacetic Acid
NPTII	=	Neomycin phosphotransferase II
PRV	=	Papaya Ringspot Virus
TMV	=	Tobacco Mosaic Virus
ToMV	=	Tomato Mosaic Virus
X-gluc	=	5-bromo-4-chloro-3-indolyl glucuronide

Chapter 1. Introduction

Papaya, received its nickname "fruit of kings" (PAC), is favored as a breakfast or dessert fruit and a vegetable staple in many countries. It is one of the most widely grown and economically important tree crops of the tropics and subtropics. In Hawaii, papaya is a principal commercial producing crop, and there were more than 18 million dollar value of utilized production from 2415 acres in 1992 (Statistics of Hawaiian Agriculture. 1994).

Currently, papaya production is severely limited in most parts of the world, including Hawaii, due to susceptibility to papaya ringspot virus (PRV) disease. Now papaya ringspot virus has reached a epidemic/devastating stage and acquired the entire papaya growing areas of the big island of Hawaii, threating the Hawaii papaya industry.

Papaya ringspot virus is a potyvirus, transmitted by aphids in nonpersistent manner (Yeh and Gonsalves. 1984). The symptoms of PRV on leaves showed reduced growth at the top of the plant, included new leaves mottled with yellow in mosaic patterns between the leaf veins, and new leaves also crinkled, bumpy or deformed rather than flat. On fruits, there were ring-shaped spots on the fruits, reduced fruit growth at the top of the fruit column, and reduced the size of fruits. On stems, symptoms included "water-soaked" spots and streaks on green stems and leaf petioles. The PRV infection papaya severely reduced fruit production and fruit quality (Nishina et al. 1989).

Conventional breedings have not shown good enough to select long-term PRV endeavor, and other control practices like quarantine, sanitation and elimination of infected trees have only obtained temporary solution to this problem.

Cross protection, deliberate use of a mild or attenuated virus strain to protect against economic loss by the severe strain of the same virus, might provide a useful and practical approach to PRV management, but it has many limitations. The major limitations include that the protected plants might produce symptoms due to infection by the mild strain of virus, the mild strain might escape to other crop as a pathogen, also the mild strain virus might reverse mutation to wild type characteristics, and reduced the crop yield.

Genetic engineering approaches including transfer viral coat protein gene, replicase gene, movement protein gene, or other viral gene into plant to disrupt the viral life cycle, showed high level of virus resistance for many crops (Gonsalves. 1993). Genetic engineering, use of in vitro techniques (recombinant DNA technology) for the deliberate manipulation of genes within or between species for the purpose of gene analysis and product improvement, offer new tools to transfer foreign genes into plant cells, combining in new ways to create improved crops.

Fitch et al, (1991) developed genetic transformation systems for papaya, and Manshardt et al (1994) reported transgenic 'sunset' papaya with papaya ringspot virus coat protein gene obtained completely resistance to papaya ringspot virus in the field testes. The mechanisms of coat protein-mediated resistance might be due to the transgene product 'coat protein' interfered with various stages of viral life cycle, such as

uncoating, translation, replication, cell-to-cell or long-distance movement, or vector-mediated transmission.

In this study, we introduced PRV cp genes, one isolated from the Hawaiian PRV mild strain HA 5-1 and one isolated from the Hawaiian PRV-Panaewa strain, into 'Kapoho' papayas by particle bombardment based on Fitch's 1991 transformation systems and combined with other tissue culture techniques. The objectives of this thesis work are to 1) effectively transform the foreign genes into immature zygotic embryos, 2) obtain chimeric transgenic hypocotyls from these immature zygotic embryos, 3) induced embryogenesis from callus of chimeric transgenic hypocotyl sections, 4) regenerate transgenic papaya embryoids.

Chapter 2. Literature Review

2.1 Papaya

Papaya (*Carica papaya* L.) is one of the most widely grown and economically important tree crops of the tropics and subtropics. It belongs to the dicotyledonous family Caricaceae, which consists of four genera: *Carica, Cylicomorpha, Jacaratia, and Jarilla* (Badillo, 1971). *Carica* is the largest genus with twenty-two described species, but *C. papaya* L. is the only species that has attained commercial importance.

Papaya is a short-lived perennial growing to 30 ft (9.14 m) high. Its hollow, herbaceous stem is usually unbranched. The deeply lobed, palmate leaves are borne on long, hollow petioles emerging from the stem apex. Flowers occur in leaf axils. Older leaves die and fall as the tree grows (Chia et al, 1989). Three types of plants are recognized based on flower type: female, hermaphrodite, and male. The commercial plantations are normally retained for three to four years, due to the tree too tall to harvest and the root disease problem to decrease production and fruit quality.

Papaya, received its nickname "fruit of kings" (PAC), is favored as a breakfast or dessert fruit and a vegetable staple in many countries. It is also processed for soft drinks, fruit jam, ice cream, canned slices and dry fruit. Papaya contain the protein-hydrolyzing enzyme 'papain', which is used in the brewing industry and in meat tenderizers; it is believed to aid digestion (Chia et al, 1989). Papaya is not only a good-testing fruit but also rich in vitamin A and C, mineral and fiber content.

Papayas are grown extensively in the lowland tropics of the gardens and door yards. World papaya annual production, 6.2 million tones (FAO 1994), ranks above strawberry and below grapefruit and pummelo (Manshardt 1992). In Hawaii, the papaya is cultivated on all of the major islands, ranked as the fourth most valuable agricultural commodity after sugarcane, pineapple, and macadamia nuts (Statistics of Hawaiian Agriculture. 1994). The principal commercial producing area is Kapoho in the Puna District along the eastern coast of the Island of Hawaii (Yee, 1970). There were more than 18 million dollar value of utilized production from 2415 acres in 1992 (Statistics of Hawaiian Agriculture. 1994). But there were only 13.8 million dollar value of utilized production from 2200 acres in 1994 (Statistics of Hawaiian Agriculture 1994), it was 8.6% less of utilized production and 215 harvested acres less than that of 1992 due to a virus disease called papaya ringspot virus (PRV) disease.

2.2 Papaya Ringspot Virus

Papaya ringspot virus is RNA virus, transmitted by green peach aphids in nonpersistent manner. Aphids can acquire virus by probing infected tissue for only a few seconds, retain the ability to transmit virus for several hours, and transmit virus to healthy plant with short probe lasting less than one minute (Yeh and Donsalves et al, 1984). Papaya ringspot virus belongs to potyvirus with two major strains, type P and type W. The strain of PRV-W is an economically important Cucurbit virus, does not infect papaya, only the P strain of PRV (PRV-P) causes extensive damage to papaya (Quemada et al, 1990).

Papaya ringspot virus is a devastating disease of papaya in the world. The symptoms of PRV on leaves showed reduced and poor growth at the top of the plant included new leaves mottled with yellow in mosaic patterns between the leaf veins and new leaves crinkled, bumpy or deformed with narrow rather than flat. On fruit, symptoms were ring-shaped spots on the fruits, reduced fruit growth at the top of the fruit column and reduce the size of fruits. On stems, symptoms included water-soaked spots and streaks on green stems and leaf petioles (Nishina et al, 1989). PRV infection papaya are serious flower abortion, and reduced fruit production and quality due to reduced sugar content. The occurrence of ringspots on the fruits severely distorts the fruit shape (zee, 1985).

Papaya ringspot virus (PRV) was first identified in the main papaya-growing area of Puna on the Big Island on May 5, 1992, continues to be of major concern for the industry (Isherwood, 1992). Currently, papaya ringspot virus has reached an epidemic/devastation stage and acquired the entire papaya growing areas of the state. The disease is serious enough in some areas and researchers of College of Tropical Agriculture and Human Resources (CTAHR) believe that the future survival of the papaya industry in Puna was at risk.

Conventional breedings have not showed good enough to select long-term PRV endeavor, and other control practices like quarantine, sanitation and elimination of infected trees have only obtained temporary solution to this problem.

2.3 Cross Protection

One of the few options available to growers is the use of cross-protection "deliberate use of a mild or attenuated virus strain to protect against economic loss by the severe strain of the same virus", and has some certain degree of success in Taiwan (Wang et al. 1987; Yeh et al. 1988) and in Hawaii (Ferreira et al. 1992). But the cross-protection has major limitations, including that the protection plants might produce symptoms due to infection by the mild strain of virus, the mild strain virus might escape to other crops as a pathogen, also the mild strain might reverse mutation to wild type characteristics, and the resistant characteristics could not express on next generation. However, cross protection is also limited by the requirement for a large-scale inoculation program for each new planting, a reduction in crop yield, and gradual losses of cross-protected plants due to superinfection by virulent strains (Gonsalves et al. 1989, Grumet, 1990 and Fitch et al, 1994).

2.4 Genetic Engineering

Genetic engineering approaches, use of in vitro techniques (recombinant DNA technology) for the deliberate manipulation of genes within or between species for the purpose of gene analysis and product improvement, offer new tools to transfer foreign genes into plant cells, combining in new ways to create improved crops. It opens up a new source of genetic variability that can be used in crop improvement, also those techniques can be mastered that they could be used in the design of plants that are higher yielding, more nutritious disease resistance or pesticide resistance (Zakri et al, 1987). Genetic engineering allows the incorporation of particular major genes into valuable crop cultivars with minimal disturbance to the cultivars genotype. Molecular genetic and

plant transformation technologies have made it possible to use novel approaches to increase disease resistance in plants and obtain the same trait to the next generation (Kung et al, 1993). A major advantage of using pathogen genes, rather than host genes, is that potentially useful pathogen genes can be more easily identified, isolated and cloned (Grumet, 1990). The objectives of genetic engineering including transfer viral coat protein gene or other viral gene into plant are to disrupt the viral life cycle, such as viral uncoating, replication, movement, or translation (Lindbo, 1993).

2.5 Coat Protein-Mediated Viral Protection

Since 1986, Powell-Abel et al produced transgenic tobacco plants expressing the coat protein (cp) gene of tobacco mosaic virus (TMV) and found that they were more resistant to the infection by Tobacco Mosaic Virus (TMV) than the nontransgenic controls. After that, there were more than fifty published reports of genetically engineered coat protein-mediated plant virus resistance in various systems and at least thirteen different groups of virus have been demonstrated the viral resistance (Grumet, 1995). These phenomenon, plants expressing the coat protein gene of a plant virus exhibit degrees of resistance or protection when challenge inoculated with that virus or closely related isolates, called coat protein-mediated protection and sparked researchers effort to develop transgenic plants that resist infection to a range of plant viruses (Gonsalves, 1993).

The resistance of transgenic plants that express genes encoding viral coat proteins to infection by the viruses from which the genes derived was termed coat protein-mediated resistance and has been

demonstrated for a variety of virus/host combination. The mechanism of coat protein-mediated resistance against virus requires accumulation of coat protein and does not seem to involve the induction of plant defense mechanisms. The resistance appears to be mainly based on the inhibition of virion disassembly in transgenic cells and disrupt the normal pathogenic cycle by causing the host to express a pathogen gene at wrong time, in the wrong amount, or in a counterfunctional form (Rebecca Grumet, 1995).

In its simplest form, coat protein-mediated protection could be due to the transgene product directly interfering with one or more viral processes. (1). Coat protein-mediated resistance is at least in part of inhibition of the TMV particle uncoating in inoculum (Wu and Beachy 1990). The expression of CMV coat protein gene was specific to infection by CMV virions, and interference with an early event in the infection process of uncoating virus particles, is probably involved in resistance of transgenic tobacco plants (Okuno et al, 1993). (2). The coat protein was required for full infectivity in rice plants, presumably by playing a role in long distance movement and possibly in cell-to-cell movement (Brugidon C et al, 1995). (3). In 1989, Beachy et al reported the helical virus-like aggregates of the coat protein are more effective inhibitors of viral replication and Silva, 1994, suggested that the defective C118 coat protein could be involved in resistance of this case, as virus replication was greatly reduced or completely eliminated in protoplast studies. The virus replication may occur in primary infected cells, but the initiation of the replication process in adjacent cells may be inhibited by the resident CMV-cp gene (Okuno et al 1993). Those multiple mechanisms may be ineffect and operate with different efficiencies in different transgenic

plants and different host-pathogen systems especially as the infected plant grew older since virus genomes are small (compared to other biotic pathogens), it is reasonable to expect the their proteins are often multifunctional. Therefore, expression of one virus gene product could theoretically alter multiple viral and/or host processes (Lindbo et al, 1993).

2.6 Characteristics of Coat Protein-mediated Protection

Lindbo et al (1993) described clearly that virus coat protein can be effective in conferring virus specific and broad spectrum resistance to viruses, although the results are some what confusing, the following generalizations of coat protein-mediated resistance can be made. (1). The resistance phenotype can be expressed as: a temporal delay in the development of symptoms; an attenuation of normal virus-induced symptoms; lower virus titer in infected transgenic tissue; the ability of infected plants to 'outgrow' infection; a percentage of plants which escape infection. (2). Resistance phenotypes usually can be overcome by high levels of virus inoculum. (3) Resistance is more effective in older plants than in younger plants. (4). Virus-specific coat protein-mediated resistance tends to be more effective than broad spectrum coat protein-mediated resistance. (5). Truncated or chimerical forms of coat protein can (in some cases) be used to increase the effectiveness and/or spectrum of coat protein-mediated resistance. (6). There does not appear to be a correlation between coat protein accumulation and resistance. (7). Many lines which accumulate coat protein showed susceptibilities identical to untransformed parental tissue.

2.7 Evaluation of Transgenic Plants under Field Conditions

The ultimate value of coat protein-mediated protection in controlling virus diseases will be determined under field conditions where the target crop is grown. The following field tests provided typically practical benefits towards controlling plant virus diseases.

Tomato Mosaic Virus

A first field test of tomato mosaic virus (ToMV) coat protein mediated protection was done in 1988 (Nelson RS). This field test used tomato transformed with the ToMV coat protein gene, and no more than 5% of the cp-expressing plants inoculated with ToMV exhibited viral systemic disease symptom by fruit harvest compared with 99% of the VF 36 plants. Under field conditions, transgenic tomato plants that express that coat protein (cp) gene of common (U_1) strain of tobacco mosaic virus (TMV) showed a high degree of resistance to the U_1 strain and to a more severe strain of ToMV, pV230; Transgenic tomato plants expressing the tomato mosaic virus (ToMV) showed a level of resistance to ToMV in the field. Those results demonstrated that although TMV and ToMV cp sequence are highly homologous, ToMV cp gene is more effective in control of ToMV in field-grown tomatoes that is the TMV cp gene (Sanders 1992). Currently Fuchs 1996, all transgenic plants remained symptomless through the crop cycle, and could not be detected by ELISA. Those transgenic developed to normal height, and showed a 17-fold increase in productivity along with a 44% increase in fruit weight compared with nontransformed control plants.

ZYMV

Arce-Ochoa et al, 1995 tested two transgenic squash hybrids XPH-1719 and XPH-1739 under field conditions and demonstrated an increased virus resistance over 'pavo' by producing a smaller percentage of symptomatic plants and a lower percentage of infected fruits. Even though the transgenic plants presumably were infected by aphids, their genetic resistance helped them to reduce viral replication (Arce-Ochoa et al, 1995). BIO/TECH designated different cp gene constructs for progeny and found the progeny from lines transformed with single or multiple cp gene constructs were tested for virus resistance under field conditions. Most transgenic lines remained nonsymptomatic throughout the growing season and produced marketable fruits while other lines showed a delay in the onset of symptoms and/or a reduction in symptom severity. A few lines failed to display any level of resistance depending on the cp gene used. The transgenic line designated ZW-20, which contained the cp genes from ZYMV and WMV2 in that most of the plants showed complete resistance.

Potato Virus

Malnoe et al, 1994, concluded that the transgenic Bt6 plants contained two copies of the cp gene acquired a complete resistance to primary infections and good resistance to second infection by PVY was confirmed in two successive field tests, and PVY coat protein-mediated resistance in transgenic Bt6 is not only effective in air part of the plant, but also on the spread of the virus through the tubes which is slowed down or inhibited.

Papaya Ringspot Virus

Transgenic papaya field test was established in Hawaii with Ro plants of transgenic papaya line 55-1 in September 1992, the resistance to PRV was equally effective whether cp + plants were inoculated manually or by natural aphid vectors (Manshardt et al 1992). This transgenic papaya was transformed with PRV cp gene of PRV strain HA 5-1 (Fitch et al, 1990) and expressed at low levels in Ro transgenic papaya plants, confers a high level of resistance to virulent Hawaii PRV under field conditions (Lius and Manshardt, 1994). Vegetative vigor of the resistant cp + plants were significant greater than that of PRV infected cp - control, also there is no deleterious pleotropic effects of the cp gene with respect to papaya morphology, fertility or fruit sugar content (Lius and Manshardt, 1994).

2.8 Papaya Transformation

There are many kinds of methods for plant transformation, such as direct DNA uptake, microinjection, virus vectors, pollen tube pathway, Agrobacterium, or particle bombardment, but the two well-known of them for papaya are Agrobacterium and particle bombardment (Fitch et al, 1991 and 1993).

The most developed system for higher plant transformation is derived from the tumor-inducing mechanism of the soil bacterium Agrobacterium tumefaciens (Klee et al. 1987). Agrobacterium tumefaciens is a gram-negative phytopathogen that causes the disease crown gall on a wide variety of dicotyledonous plants. The infection cycle of Agrobacterium is complex, involving a number of chemical signals and chemicals secreted by both pathogen and wounded plant cells of its hosts. The bacteria bind to the plant cells and transfer a portion of this

Ti plasmid, called the T-(transferred) DNA, to the plant cell. The T-DNA covalently integrates into plant nuclear DNA, where genes encoded by the T-DNA direct the synthesis of the phytohormones auxin and cytokinin, as well as novel low molecular weight metabolites called opines. Opines are secreted from the tumor cells and can be used as carbon and sometimes nitrogen sources by the inciting strain of Agrobacterium. Certain of these opines can also induce the conjugal transfer of the Ti plasmid between Agrobacterium cells (Kung et al, 1993). The transformed papaya callus was done with leaf disks via A. tumefacines (Pang 1988), but those calluses were not regenerated to plants. Chen (1992) and Cheng (1990) both obtained transgenic tissue, but they were both failed to regenerate plants.

The first transgenic papaya plants were regenerated from somatic embryos with PRV cp gene by particle bombardment (Fitch et al 1990). The transformation process is a direct gene transfer method, and as such has no direct effect on the chromosomal integration mechanism. The nature of the integration event arising from the process will depend upon the DNA being delivered and the biology of the organism being transformed (Sanford, 1990). The particle gun may overcome many of the limitations and have several fundamental advantages over other plant transformation. It can serve any organelle, cell, tissue or species as target, it is a rapid and very simple procedure, and it should facilitate the direct transformation of totipotent tissues such as pollen, embryos, meristems and morphogenic cell cultures (Sanford JC, 1990). The process can be defined as the introduction of substances into intact cells and tissues through the use of high-velocity microprojectiles. A microprojectile is any small coherent particle capable of being

accelerated, such that it penetrates cells and tissue. A microprojectile is made of high-density metals of tungsten or gold, 0.4 to 2.0 nm in diameter and carried the DNA on the surface of the particles, then the DNA is being 'shot' into cells via high-velocity microprojectile. The results showed higher transformation efficiency, and many crops had been demonstrated since Klein et al (1988) transferred successfully a chloramphenicol acetyltransferase (CAT) reporter gene in particle bombarded onion epidermal cells.

2.9 Papaya Tissue Culture

Callus induction

Callus induction is most often initiated with the growth regulator 2,4-D, and develops from the cut surfaces of the explants (Ammirato, 1983). Callus initiation, growth and phenotype were dependent on the explant source rather than the medium used (Mondal et al, 1994).

Papaya tissue cultures have been initiated for various reasons such as embryo rescue, before abortion of interspecific hybrids (Manshardt and Wenslaff, 1989). Litz et al (1993) obtained papaya callus in half-strength MS basal medium containing 0.4-5.0 mg/l NAA and 0.3-5.0 mg/l BA from cotyledon lamina of papaya seedlings and 1.2-5.0 mg/l NAA and 0.6-3.0 mg/l BA from midrib of papaya seedlings. Chen et al (1987) obtained callus from papaya seedling in half-strength MS basal medium containing 1.0 mg/l NAA and 0.5 mg/l kintin. Yie and Liaw (1977) obtained callus from stem tissue of seedlings in presence of full-strength MS supplemented with 1.0 mg/l NAA and 0.1 mg/l kintin. Somatic embryogenic calluses were produced efficiently on half-strength MS and

2,4-D induction medium after ten to fourth weeks of culture at 27C¡ in the dark by culturing hypocotyl sections from ten-day-old seedlings (Fitch, 1993). Callus cultures were obtained from petiole explants of papaya on MS medium containing 0.5-10.5 μM NAA in combination with 0.5-5 μM BA (Hossain et al, 1993). Cross sections of the petioles were induced to form callus with 62% efficiency on MS medium supplemented with 2.5 μM 2,4-D and 0.4 μM 6-BA (Yang et al, 1992).

Embryogenesis

Somatic embryogenesis is a developmental pathway in which embryos have been induced to form from a somatic cell or group of somatic cells. It can occur directly from cells of the explant tissue or indirectly induced and developed from a proliferated callus (Williams and Maheswaran, 1986).

Fitch et al 1990 developed a series of methods to induce somatic embryogenesis from immature zygotic embryos and hypocotyl callus for papaya. About 49 to 62% of the zygotic embryos produced embryogenic callus, and each zygotic embryo yielded hundreds of somatic embryos within five months of culture on media supplemented with 2,4-D (Fitch and Manshardt, 1990). Over 60% 'Kapoho' hypocotyl sections obtained somatic embryogenic callus after two months of culture on induction medium containing 4.5 mM 2,4-D and 7% sucrose (Fitch, 1993). Yang (1992) demonstrated that somatic calluses from petiole sections were cultured in MS medium with 5.0 mM 2,4-D produced numerous embryoids with 49% efficiency. Hard-green calli were transferred into MS medium containing 100 mg/L casein hydrolysate with specific BA-NAA

formulation, where they developed adventitious buds within two weeks of culture (Hossain et al, 1993).

Somatic embryo germination

Regeneration of plant from somatic tissue culture in vitro can follow one of two alternative pathways: somatic organogenesis or embryogenesis (Debergh et al, 1991).

DeBruijne et al (1974) obtained shoot regeneration and formation of somatic embryos in white s medium (1963) supplemented with BA and NAA. Yie and Liaw (1977) observed shoot regeneration and somatic embryogenesis in MS medium in presence of 1.0-2.0 mg/l kintin and 0.0-0.05 mg/l IAA. Litz et al (1983) observed shoot regeneration in MS medium with 0.05-1.0 mg/l BA with 0.0-0.2 mg/l NAA in cotyledon lamina callus of papaya. Different types of results indicate that media requirements for regeneration from papaya callus are also different in different genotype. Fitch (1990) published papaya regeneration from immature zygotic embryos and obtained transgenic papaya. This medium called 'germination' containing full strength MS salts (Murashige and Skoog, 1962),100 mg/L myo-inositol, 3% sucrose, 5 mg kinetin (Sigma Chemical Co.) and 1% Difco Bactoagar, the germination medium without kinetin was used for plant enlargement.

Root production

It was confirmed that IBA was the best auxin for root initiation of papaya, but IBA is not required or is inhibitory for root emergence and growth (Went 1939). However, shoots exposed for 4 weeks to auxin concentration that resulted in highest root initiation showed deleterious

levels of callus development and deterioration of shoot quality (Drew et al, 1993). High rooting percentages and high-quality adventitious root systems for papaya (*Carica papaya* L.) were obtained in vitro by appropriate auxin source and maximum rooting percentage (96%) was achieved by exposure of shoots to a medium containing 10 μM IBA for three days before transfer to a hormone-free medium (Drew et al, 1993). Yang et al (1992) demonstrated that individual shoots of 1.5 to 2.0 cm in length were excised and cultured on MS medium supplemented with varying levels of IBA, and obtained 80% plant establishment. 1.0-2.0-cm tall cuttings of micropropagated shoots were rooted on MS agar medium containing 5.0 μM IBA, the cuttings were transferred to vermiculite moistened with MS liquid medium when the roots were at least 0.5-cm long (Fitch 1993).

Establishment in soil

The plantlets with roots and 2 to 3 leaves were removed from tissue culture to vermiculite in plastic pots and covered with transparent plastic bags that were opened gradually over a two-week period to adapt the plantlets to ambient conditions (Yang et al, 1992). The other plant establishment procedures followed the method of Manshardt (1989): The individual shoots were severed at base, dipped in a talc powder mixed with 0.5% IBA, and planted in vermiculite supplemented with 1/4 MS salts. Roots formed within two weeks.

Fitch et al (1991) reviewed all media formulations reported in the literature for various types of papaya tissue cultures. In this review we only emphasized the induction medium of somatic embryogenesis form different kinds of explants of papaya (*Carica papaya* L.) (Table 2.1).

Table 2.1. Media used for somatic embryogenesis from different explants of papaya (*Carica papaya* L.)

Explant	Medium	Growth Reg.	Citation
H. S.	1/2 MS	2,4-D	Fitch, MMM. 1993
P. S.	1/2 MS	2,4-D and 6-BA	Yang, JS et al. 1992
R. S.	1/2 MS	NAA, Kinetin & GA_3	Chen, MH et al. 1987
I. Z. E.	1/2 MS	2,4-D	Fitch, MMM et al. 1990
S. S.	LS	2,4-D	Yamamoto et al.1989

H. S.= hypocotyl sections
P. S.= petiole sections
R. S.= root sections
I. Z. E.= immature zygotic embryos
S. S.= seedling sections
MS = Murashige and Skoog (1962)
LS = Linsmaier and Skoog (1965)

Chapter 3. Materials and methods

3.1 Plant materials

Immature fruits of the hermaphroditic papaya, *Carica papaya* L., cultivar Kapoho harvested about 120 to 135 days after pollination were peeled off green skin and soaked in 20% Clorox solution with two drops of Tween-20 per liter for 10 minutes. The fruits were air dried in a flow hood and seeds were dissected to expose the immature zygotic embryos, and these embryos were placed on 1% of water agar medium. The following day, one cotyledon was removed from each embryo to expose the apical meristem and radical, and the embryos were bombarded using a particle gun. The later experiments would be conducted immediately after bombardment. A single fruit provided all of the embryos of a certain age for each experiment.

3.2 Plasmids Chapter

PRV4: E. coli DH5a/pGA482GG/cp PRV4.

This binary vector pGA482GG plasmid contains the NPTII selective marker gene for resistance to kanamycin, the reporter β-glucuronidase (GUS) gene, and the coat protein (cp) gene isolated from the Hawaiian papaya ringspot virus of mild strain HA 5-1. This cp gene was engineered containing the first 16 amminoacids of the CMV-C coat protein fused to the Gln-Ser proteolytic cleavage site of PRV cp (aa 17-18), called PRV4 construct (Quemada et al, 1990).

P208: E. coli DH5a/pGA482GG/cp PRV HA-P-208:

This binary vector pGA482GG plasmid contains the selective NPTII marker gene for resistance to kanamycin, the reporter β-glucuronidase (GUS) gene, and the coat protein (cp) gene isolated from the Hawaiian papaya ringspot virus of the PRV-Panaewa strain. This cp gene was cloned at the Cln-Ser proteolytic cleavage site of PRV coat protein, called P208 construct (Quemada et al, 1990).

Those two cp genes were initially cloned into the plant expression vector pUC18cpexp (Slightom, 1991) at the Nco I site. The plant expression cassette in this vector contains the 35S cauliflower mosaic virus (CaMV) promoter sequences and the 35S CaMV terminator sequence. The cassette was excised and cloned into the HindIII site of the binary vector pGA482GG for plant transformation. The plasmid pGA482GG is resistance to gentmycin, so we use routinely Luris Bertani (LB: 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, added to 1000 ml H_2O and adjusted pH 7.4) medium containing gentamycin 60 mg/L to select for the presence of the plasmid pGA482GG in bacteria.

3.3 Transformation of E. coli

Plasmid DNA has to be transformed into bacterial cells to obtain the large number of replication, so the plasmid PRV4 and PRV208 were transformed into competent cells of E. coli (DH5a).

10 µl of each plasmid containing PRV4 or P208 was mixed with 150 µl DH5a competent cells in a sterile microfuge tube and incubated on ice for 40 minutes, then the cells were heat shocked at 37¡C for 5 minutes and the cells were transferred into a 10 ml culture tube. The cells were incubated at 37¡C for 45 minutes with shaking by adding 1 ml LB liquid medium. 200 µl of the cells were spread on the LB medium

containing 60 mg/l of gentamycin, and incubated the plate upside down at 37¡C overnight. Only the transformed cells containing gentamycin-resistance marker gene that obtained gene expression could grow on the plate, because the plasmid DNA replicated and expressed the gentamycin-resistance markers that allowed the transformed cells to survive in the presence of gentamycin. So the clones that could grow on this medium were the transformed cells.

3.4 DNA Extraction

The plasmid purification protocol provided by the Qiagen Inc (Chatsworth, CA). was optimized for both yield and ease-of-use based on the alkaline lysis method, as it is rapid, simple and free of all contaminants.

Bacterial cultures were always grown from a single clone picked from a 60 mg/l gentamycin selective medium plate and incubated in 5 ml of gentamycin selective liquid medium at 37¡C overnight. This miniculture was diluted 1:100 into a larger volume of 60 mg/l selective LB medium and regrew overnight. The bacterial cells were harvested by centrifugation at 4¡C for 15 minutes at 6000 rpm. Removed supernatant by inverting the open centrifuge tube until all medium had been drained. The bacterial pellet was resuspended in 10 ml of buffer P1 (100 mg/ml RNase A, 50 mM Tris/HCl, 10 mM EDTA, pH 8.0) and mixed with 10 ml of buffer P2 (200 mM NaOH, 1% SDS) and incubated at room temperature for 5 minutes. These cells were added to 100 ml of chilled buffer P3 (3.0 M KAc, pH 5.5), mixed, and incubated on ice for 20 minutes before centrifuging at 4¡C for 30 minutes at 16000 rpm. Equilibrated a Qiagen-tip 500 with 10 ml of buffer QBT (750 mM NaCl,

50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100) and filtered the supernatant through miracloth into the Qiagen-tip 500. After the supernatant was washed with 2·30 ml of buffer QC (1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0), the DNA were eluted with 15 ml of QF (1.25 M NaCl, 50 mM Tris/HCl, 15% ethanol, pH 8.5). Then the DNA was precipitated in 0.7 volume of isopropanol and centrifuged at 16000 rpm at 4C for 30 minutes. The DNA pellets were washed with 15 ml of cold 70% ethanol and centrifuged to get the DNA pellets. The DNA pellets were air dried for 5 minutes, and redissolved in a suitable volume (500-1000 µl) of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

Plasmid DNA concentrations were measured by using Spectrophotometer and identified by digestion of the DNA samples with HindIII enzyme. 8 µl of water mixed with 1.0 µl of plasmid DNA and 1 unit of HindIII, and the mixture was incubated at 37¡C for one hour. This digested mixture was electropheresised on 0.7% agarose gel contained 0.7 g agarose and 0.5 mg/ml ethidium bromide in 100 ml 1 × TBE buffer after adding 1 µl of dye. Coat protein gene fragment could be identified by comparing with molecular markers (Lambda DNA digested with HindIII).

3.5 DNA Delivery

The plasmid DNA was transferred into papaya tissue using the PDS-10000/HELIUM particle gun (BIO-rad) described by Klein et al (1988). Because of the large number of variables associated with the particle bombardment process, such as particle preparation, the size and shape of the particle, tissue mass and velocity, a model system was developed for the microprojectile bombardment.

We oriented the immature embryos with one cotyledon removed tightly together on the middle part of 1% of water agar medium in the petri dash and set them at the third level from the bottom of the machine. The plasmid DNA was coated with 1.6 nM diameter of gold particles, mixed with 2.5 M Calcium Chloride ($CaCl_2$) and 0.1 M spermidine, washed with 100% ethanol, and 60 µl of 100% ethanol was added. 10 µl of this solution was loaded on the front surface of the microprojectile, and kept it at the second level from the top. We used 1550 and 1800 pounds per square inches of rupture disk to control the pressure and shoot the tissue twice individually on each petri dish at 27 inches of mercury vacuum level. The transformation efficiency was examined by visualization of GUS activity at 24 hours after bombardment using the procedures described as below.

3.6 GUS Histochemical Assay

E. coli. beta-glucuronidase gene (GUS), is the gene fusion marker for the analysis of gene expression in transformed plants. Different stages of tissue following particle bombardment were examined for GUS expression using histochemical assay with 5-bromo-4-chloro-3-indolyl glucuronide (X-Glux) substrate. The assay produces a blue precipitate at the site of the enzyme activity provided a simple and sensitive method to detect the expression of the (GUS) transgene products (Jefferson et al, 1987).

10 mg of X-gluc was dissolved in 200 µl dimethyl formamide, and diluted to the final volume 10 ml with 0.1 M sodium phosphate buffer pH 7.0. Color development was recorded after one to forty eight hours of incubation at 37¡C depending on the different kind and stage of tissue.

The hypocotyls and green leaves needed to be destained with 100% ethanol to remove the green chlorophyll pigmentation until the background became clear, then the tissue was stored at 70% ethanol.

3.7 ELISA Assays for The Presence of NPTII

Neomycin phosphotransferase II (neo) is widely used as a selectable marker in plant transformation experiment. Here we evaluated its gene product (NPTII) by immunological detection using a kit obtained from 5 Prime-3 Prime, Inc (West Chester, PA, USA). The protocol we were using was according to the manufacturer's specifications with some modifications.

Sample preparation:

0.2 g of tissue samples were prepared by grinding the young hypocotyl seedling about twenty days following bombardments in 500 µl of PBST-PVP buffer (PBST buffer contains 8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2PO_4, 0.2 g KCl, 0.2 g NaN_3, per liter of aqueous solution, pH 7.4, then add 0.5 ml Tween-20 supplemented with 2% polyvinyl pyrrolidone PVP 40) (Lius and Manshardt, 1994). The extract was used in the ELISA assay and the material might be stored at -70¡C overnight.

Modifications to ELISA assay:

Before the experiment started, all 1×Blocking/Dilution buffer and 1×PBST washing buffer should be warmed to room temperature. The wells were coated with 100 µl of coating antibody at 1:1000 dilution in coating buffer provided by the manufacturer and incubated at 37¡C for 2 hours or at 4¡C overnight. The wells were washed three times for three minute

incubation individually between each time in PBST buffer, then wells were blocked using 200 µl of blocking and dilution buffer provided by the manufacturer. The plates were incubated for 30 minutes at room temperature, and wells were washed three times like above. 100 µl of plant tissue sample was loaded into each well and the plate was incubated at room temperature for two hours or at 4¡C overnight. Then the plate was washed for three times with PBST buffer before adding 100 µl of botinylated antibody at 1:1300 dilution in blocking buffer. The plate was incubated for one hour at room temperature, and washed for three times with PBST buffer. 100 µl of streptavidin conjugated alkaline phospatase at 1:1000 dilution in blocking buffer was added in each well and incubated for thirty minutes at room temperature.

Following the incubation, the wells were washed again and 100 µl of p-nitriopheny phosphate substrate at 2 mg/ml in substrate buffer (97 ml diethanilaminde in 1 liter aqueous solution, pH 9.8) was loaded into each well, then the plate was incubated thirty to forty minutes at 37¡C.

Analysis of ELISA:

Color development was recorded by scanning the optical density of each well of the microtiter plate with a multiscan spectrophotometer generally called the "ELISA Plate Reader" by absorbance at 405 nm wavelength (A_{405nm}). The absorbance was compared with that of NPTII enzyme standard curve to determine the concentration of NPTII enzyme present in transformed tissue.

3.8 ELISA Assays for the Presence of Coat Protein

An indirect double antibody sandwich enzyme-linked immunosorbent assay (ELISA) based on the description of Clark and Adams (1977) was used for the detection of the coat protein gene expression. Monoclonal antibody provided by Gonsalves (Department of Plant Pathology, Cornell University 1992) was used in this assay for coat protein expression in transformed papaya tissue. 100 µl of 1:1000 diluted coating antibody in coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ in 1 liter aqueous solution, pH 9.6) was added to each well and incubated at 4¡C overnight. The plate was washed for three times with PBST buffer with a three minute incubation each time, and blotted dry on paper towels. The hypocotyl seedling which base part and leaves were removed was ground in 500 µl extraction buffer (200 ml of 0.25 M K_2HPO_4 and 0.1 M EDTA solution to make pH 7.5 Buffer) (Lius and Manshardt. 1994), then 100 µl of the plant sample extract was loaded and incubated at 4¡C overnight. The plate was washed and 100 µl of 1:1000 dilution of monoclonal antibody-enzyme conjugate in enzyme conjugate buffer (PBST buffer supplemented with 2% polyvinyl pyrolidone PVP-40 and 0.2% ovalbumin) was added. Then The plate was incubated at 37¡C for three to four hours or 4¡C overnight. After incubation, the plate was washed and100 µl of p-nitriphinyl phosphate substrate at 1 mg/ml in substrate buffer (NPTII diethanilamine buffer) was added. Then the plate was incubated for 1.5 to 3 hours at 37¡C. The changes in color of the end products were recorded by scanning the optical density of each well of the microtiter plate or strips with a multiscan spectrophotometer by absorbance at 405 nm wavelength (A_{405nm}). The absorbance values which were at least three times as high as that of healthy untransformed papaya controls were regarded as positive reactions.

3.9 Tissue Culture Media

The half-strength MS salts (Murashige and Skoog 1962) with full strength MS vitamins, 3% sucrose, 0.6% Difco Bactoagar, and different concentration of IBA 0, 0.1, 0.5, 1.0, 5.0 and 10 μM were used as "rooting medium" (Drew et al. 1993). The half-strength MS containing 50 mg/l myo-inositol, full strength MS vitamins, 400 mg/l glutamine, 6% sucrose, and 1% Difco Bactoagar were used as 'maturation medium' (Fitch et al. 1990). The maturation with 10 mg/l 2,4-D or with 6 mg/l and 0.5 μM BA were referred as "induction medium" (Fitch and Manshardt 1990; Yang et al. 1992). The induction medium with different concentrations of kanamycin was used as "selective induction medium". The "germination medium" was half-strength MS combining with 0.5 mg/l of Kinetin, 0.5 mg/l BA, 3% sucrose, and 0.7% Difco Bactoagar to germinate the embryos. The full-strength MS salt medium containing 2% sucrose without growth regulator was used for plant enlargement.

The pH of all media was adjusted to 5.8 prior to autoclaving (21 lb and 20 minutes). About 25 ml of induction medium, selective induction medium, and maturation medium were dispensed into sterile 100×15 mm petri dishes; rooting medium and germination medium were dispensed into majentil boxes in 50 ml individually. All petri dishes and Majenta boxes were sealed with parafilm.

3.10 Tissue Culture Conditions

All immature zygotic embryos with one cotyledon removed were bombarded with gold particles coated with plasmid DNA, and also with uncoated particles for use as a negative control. After bombardment the

immature zygotic embryos were immediately transferred into rooting medium, and four experiments were conducted.

Experiment I: Effects of different concentrations of IBA on hypocotyl seedling development.

The bombarded immature zygotic embryos were placed on rooting medium with different concentrations of IBA (0, 0.1, 0.5, 1.0, 5.0, or 10 μM) under continuous illumination at 27¡C after three days, the embryos were transferred into germination medium. Twenty days after bombardment, the diameter and length of hypocotyl seedlings were measured to determine the best concentration of IBA for hypocotyl seedling development. Each IBA treatment consisted of two replicates, and each replicated consisted of a Majenta box containing 16 immature zygotic embryos. All immature zygotic embryos were from a single fruit.

Experiment II: Selective regeneration of transgenic tissues by embryogenesis from chimeric hypocotyl sections.

The immature zygotic embryos were put into 1.0 μM IBA rooting medium for three days after bombardment, then transferred into germination medium for about twenty days. The 2 to 4-cm long hypocotyl was sectioned into 2 to 3-mm lengths, each of which was numbered. Every other hypocotyl section was analyzed for GUS expression, while the remainder was kept in culture. When successive pieces were found to be GUS positive, the intervening section was considered to be chimeric transformed hypocotyl section and was cultured on induction medium containing 10 mg/l 2,4-D without kanamycin for eight weeks to induce callus. The chimeric sections were subcultured on induction medium

with 30 mg/l kanamycin for four weeks, after which the kanamycin concentration was doubled to 60 mg/l to inhibit growth of nontransformed embryogensis. When embryogensis occurred on the selective induction medium, the embryos were moved out of kanamycin and regenerated on germination medium.

Experiment III: Effect of two phytohormone formulation on callus induction from hypocotyl sections.

Two different kinds of induction media containing 10 mg/l 2,4-D (Fitch, 1993) or 6 mg/l 2,4-D and 5 mM BA (Yang, 1992) were tested for their ability to proliferate callus. Immature zygotic embryos were put into 1.0 μM IBA rooting medium for three days after bombardment, then transferred into germination medium for about twenty days as above. The 2 to 4-cm long hypocotyls were sectioned into 2 to 3 mm pieces and plated on the two kinds of induction media. After eight weeks, callus development on hypocotyl sections were scored. The callus was transferred into selective induction medium, containing 10 mg/l of 2,4-D and 30 mg/l kanamycin, for four weeks. Kanamycin concentration was increased to 60 mg/l for four to six weeks, and the number of embryogenic hypocotyl sections was recorded.

Experiment IV: Effect of duration of exposure to 10 μM IBA on rooting of shoots.

Shoots with one or two leaves from immature zygotic embryos, which had been cultured on germination medium for four weeks, were excised the base section and cultured on rooting medium containing 10 μM IBA for 0, 3, 6, 9, or 12 days, and transferred to MS germination

medium for about three weeks (Drew et al,1993). At the end of this period, root development was recorded as number of shoots with roots, number of roots per rooted shoot, and callus rating per rooted shoot. Shoots with root development from were cleaned of agar and sucrose, transplanted to vermiculite in plastic pots, and covered with transparent plastic bags that were opened gradually over a two-week period to adapt the plantlets to ambient conditions.

Overall, the objective of these experiments were to develop a procedure for obtaining chimeric transgenic hypocotyls first, then induce embryogenic callus from hypocotyl sections on induction medium followed by selection of transgenic embryos with media containing kanamycin.

Chapter 4. Results and Discussion

The embryo excision procedure was very easy to handle and free of contamination, and the immature zygotic embryos were not effected by Clorox solution. Within one day of placing the immature zygotic embryos on 1% water agar medium, almost 100% of cotyledons were opened (Fig. 1). About 300 immature zygotic embryos with one cotyledon removed were oriented tightly (2.5 cm in diameter) in the middle of the water agar medium in a petri dash (Fig. 2). The transformation efficiency test and tissue development experiments were carried out following particle bombardment.

4.1 Plasmid DNA Assay

Plasmid DNA of pGA482GG/cp PRV4 and pGA482GG/cp P208 were successfully transformed into competent cells of E. coli (DH5α). A volume of cell culture containing 200 µl PRV4 or P208 was spread on LB medium containing 60 mg/l of gentamycin, and the plates were incubated upside down at 37¡C overnight. There were three colonies containing the PRV4 construct and five colonies containing the P208 construct that survived and grew on the selective media. A single colony of each was picked, and subcultured on liquid selective LB medium. The plasmid DNA were extracted following the Qiagen plasmid purification protocol. Plasmid DNA was dissolved in 600 µl of water, the concentrations of the plasmid DNA were 0.8-1.2 µg/ ml measured by using spectrophotometer. Yields of 400 to 500 µg were obtained each time

and the quality of the samples was determined by electrophoresis following digestion with HindIII enzyme (Fig. 3). Compared with molecular markers (Lambda DNA digestion with HindIII enzyme), the coat protein gene fragments of PRV4 and P208 were the right size at about 1 kb.

Fig. 1. Cotyledons of immature zygotic embryos unfolded one day after the embryos were placed on 1% water agar medium.

Fig. 2. About 300 immature zygotic embryos with one cotyledon removed were oriented tightly in the middle of the water agar medium in a petri dish.

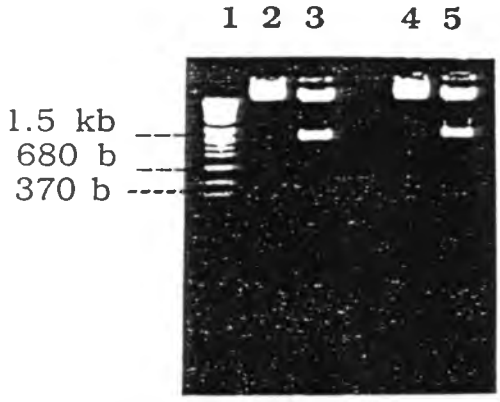

Fig. 3. Plasmid DNA samples, pGA482GG/cp PRV 4 and pGA482GG/cp P208, were digested with HindIII enzyme and showed the proper coat protein gene fragment size (1 kb). DNA molecular weight marker VII (line 1), PRV4 plasmid DNA (line 2), PRV4 plasmed DNA digested by Hind III enzyme (line 3), P208 plasmid DNA (line 4), P208 plasmid DNA digested by Hind III enzyme (line 5).

Fig. 4. GUS-positive spots were shown on the radicle of immature zygotic embryos one day after bombardment.

4.2 GUS Assay

On the day following bombardment, assay for GUS expression in the immature embryos commenced. About 10% of the total number bombarded, or 100 zygotic embryos were tested after two hours incubation, 72 dark blue, GUS-positive spots were observed on 59 zygotic embryos (Fig. 4), and some of them had more than a single blue spot. Transient expression of GUS was, therefore, noted in 59% of the bombarded embryos. Most of those blue spots were on the hypocotyls of embryos, and consequently these tissues had the best potential to give rise to chimeric transgenic tissues.

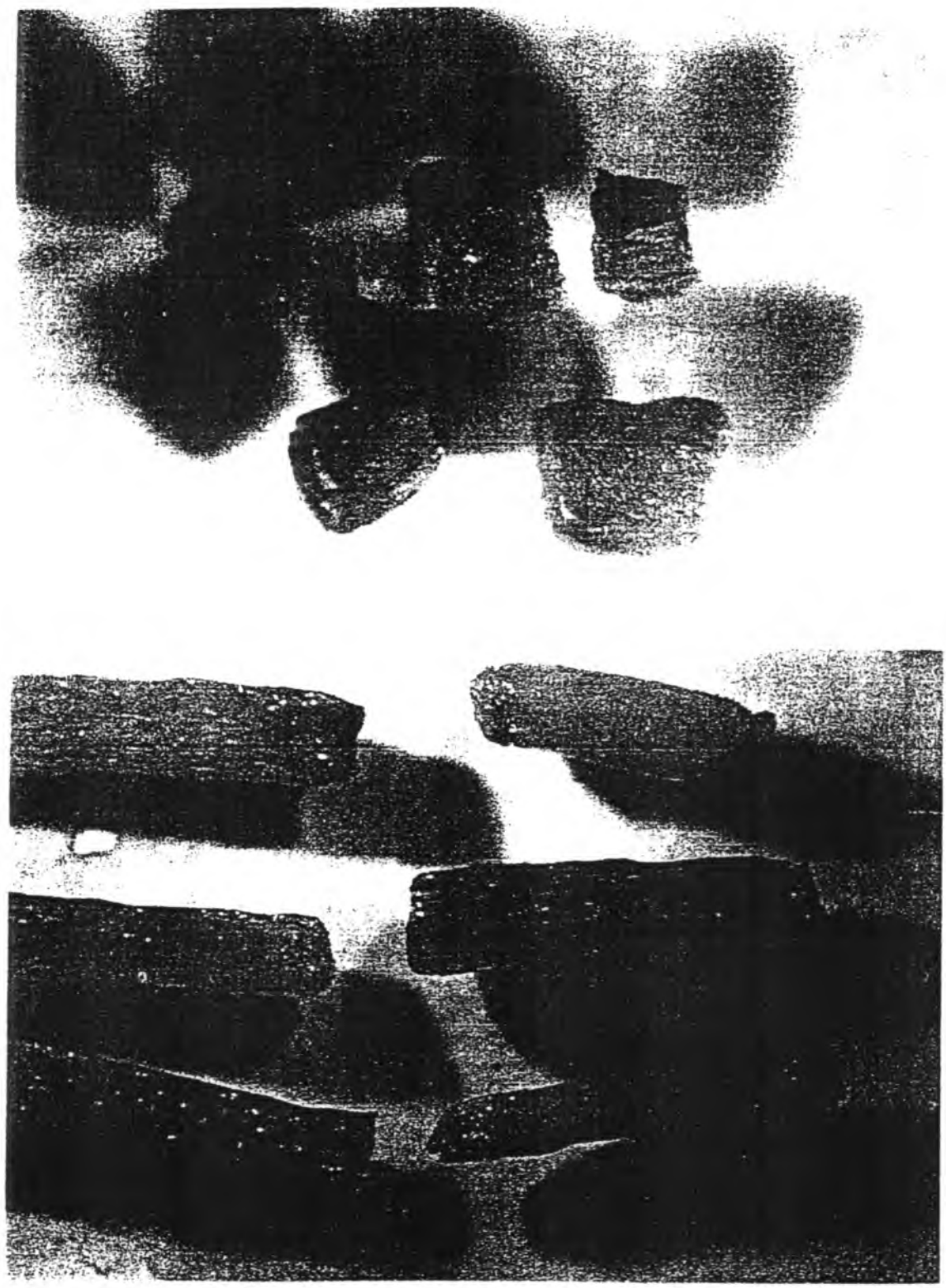

Fig. 5. Chimeric transformed hypocotyls showed strong and light GUS positive blue at 20 days following bombardment.

Fig. 6. GUS-positive transgenic embryo from chimeric transgenic hypocotyl section had been cultured for a total of nine months.

Fig. 7. Transgenic papaya seedling from the chimeric transformed hypocotyl section that had been cultured for a total 11 months showed a GUS-positive response.

At twenty days following bombardment, there were 12 (28%) seedlings deived from bombarded hapocotyls showing GUS-positive blue out of a total of 43 hypocotyls that had been cultured on the germination medium. Some expressed very strong color, and some only showed light blue (Fig. 5). These tissue were considered to be chimeric transgenic hypocotyls that had the potential to give rise to transgenic plants through somatic embryogenesis on selective induction medium with kanamycin.

Figure 6 showes a GUS-positive transgenic embryo, derived from a chimeric hypocotyl section, that had been in tissue culture for a total of nine months. This chimeric hypocotyl section had been cultured on the induction medium with 10 mg/l of 2,4-D for eight to ten weeks, and selective induction medium with 30 mg/l kanamycin for four weeks and 60 mg/l kanamycin for four to six weeks, then was transferred into maturation medium.

Figure 7 showes similar GUS-positive seedling pieces from chimeric hypocotyl sections after a total of eleven months of culture, including two months on maturation medium, and four months on germination medium.

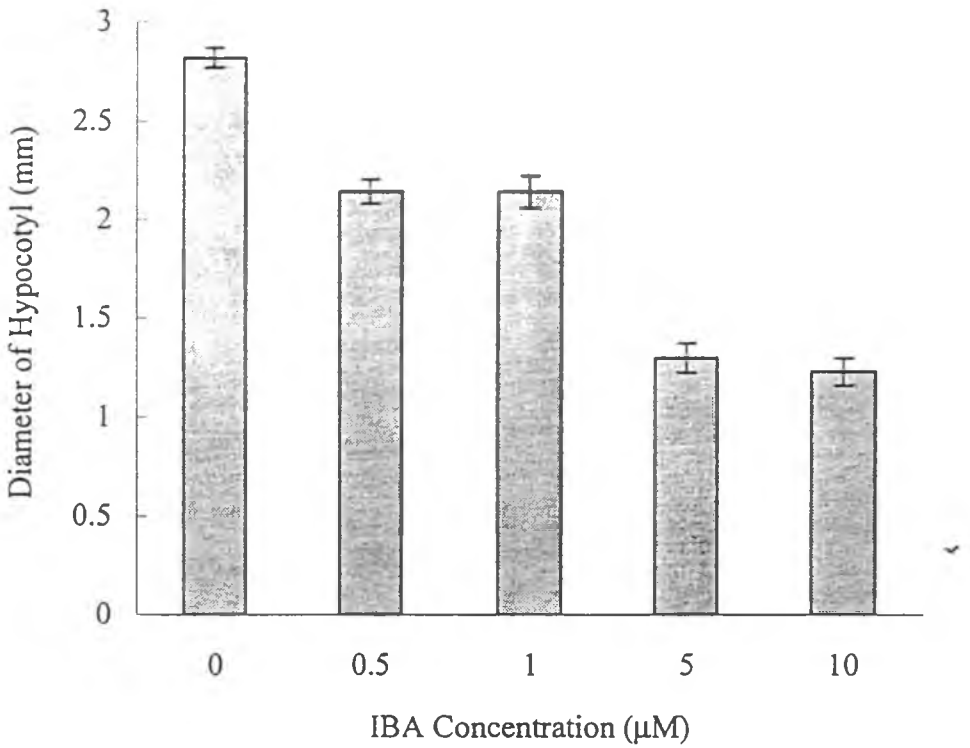

Fig. 8. Different concentrations of IBA effected the hypocotyl diameter development on germination medium.

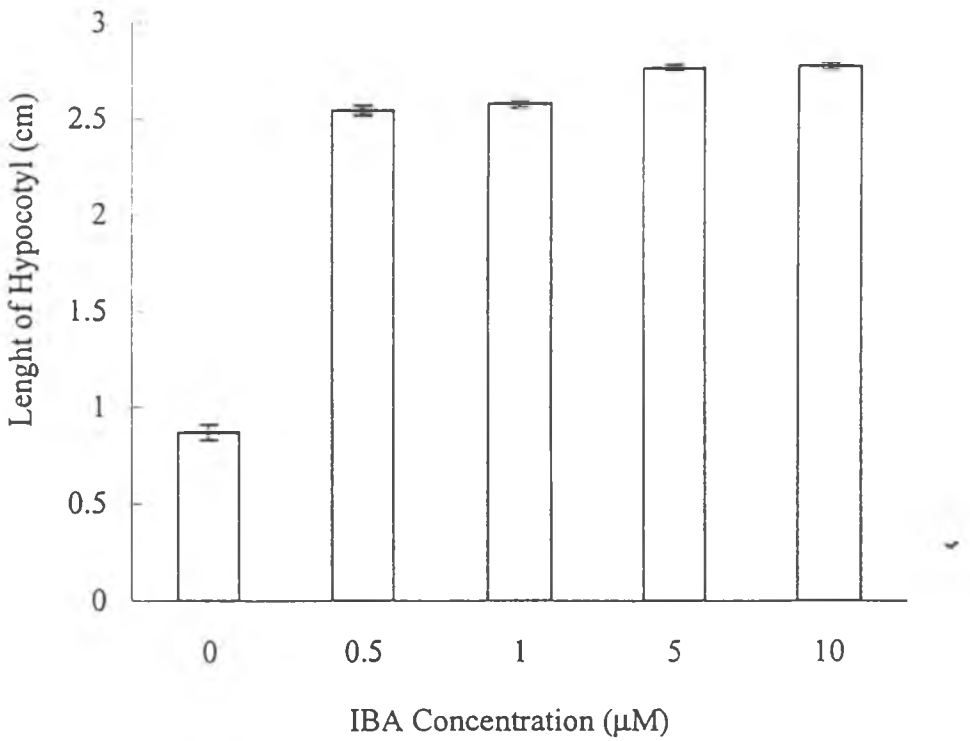

Fig. 9. Different concentrations of IBA effected the hypocotyl length on germination medium.

Fig. 10. Immature zygotic embryos cultured on 1.0 μM of IBA rooting medium for three days showed a good yield of hypocotyls for somatic embryogenesis 20 days after bombardment on germination medium.

4.3 Hypocotyl development

In this experiment, immature zygotic embryos were exposed to 0, 0.1, 0.5, 1.0, 5.0, or10 μM IBA rooting medium for three days after bombardment, then transfered into germination medium, and data were collected after 20 days of culture on germination medium.

Figure 8 showes that different concentrations of IBA effected the hypocotyl diameter development on germination medium. Figure 9 showes that different concentrations of IBA effected the hypocotyl length development on germination medium.

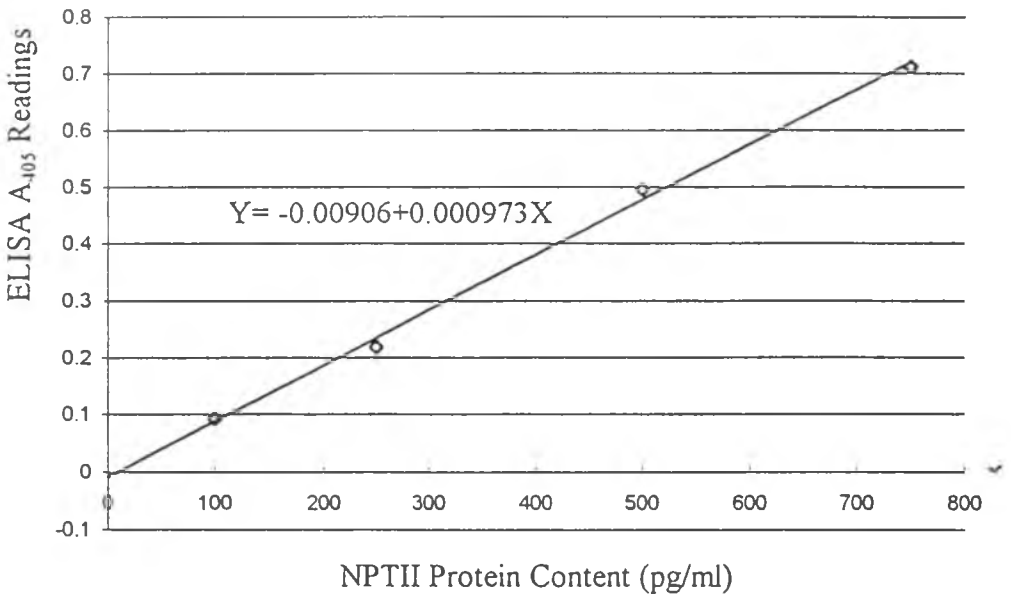

Fig. 11. Standard curve of ELISA assay for NPTII enzyme content of chimeric transformed hypocotyls.

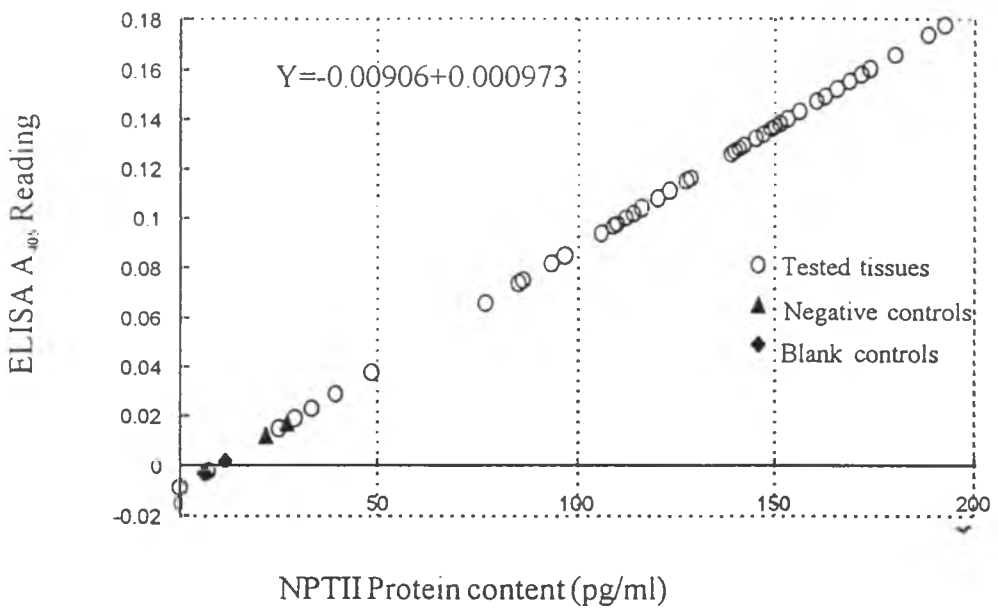

Fig.12. Expression of NPTII in chimeric transformed hypocotyls as determined by ELISA.

The mean hypocotyl diameter on 0 M IBA rooting medium was 2.82 mm – 0.22 (mean – SE), larger than that of other treatments, but the mean hypocotyl length was only 0.87 cm – 0.28, much shorter than the lengths in other IBA treatments. Hypocotyl growing on this medium without IBA were shorter, thicker, without much callus and lower yield of hypocotyl sections for somatic embryoginesis.

The mean hypocotyl length on 10 μM IBA medium was 2.78 cm – 0.39, the highest among all the treatments, but the mean hypocotyl diameter was only1.23 mm – 0.12. Hypocotyl sections from such culture were too small in diameter to produce embryogenic callus on induction medium.

The mean hypocotyl length and diameter on 0.5 μM IBA medium were 2.54 cm – 0.35 and 2.14 mm – 0.15, similar to 1.0 μM IBA medium. Except that on the former medium, there was more callus around the base of the tissue.

The hypocotyls on 5 μM IBA medium were also too small in diameter to undergo embryosgenesis. The best hypocotyl growth was observed when immature zygotic embryos were exposed to 1.0 μM IBA for three days before transfer to germination medium (Table 4.1). The mean hypocotyl diameter was 2.14 mm – 0.12, and the length of hypocotyl was 2.58 cm – 0.47 after twenty days of culture on germination medium (Fig. 10).

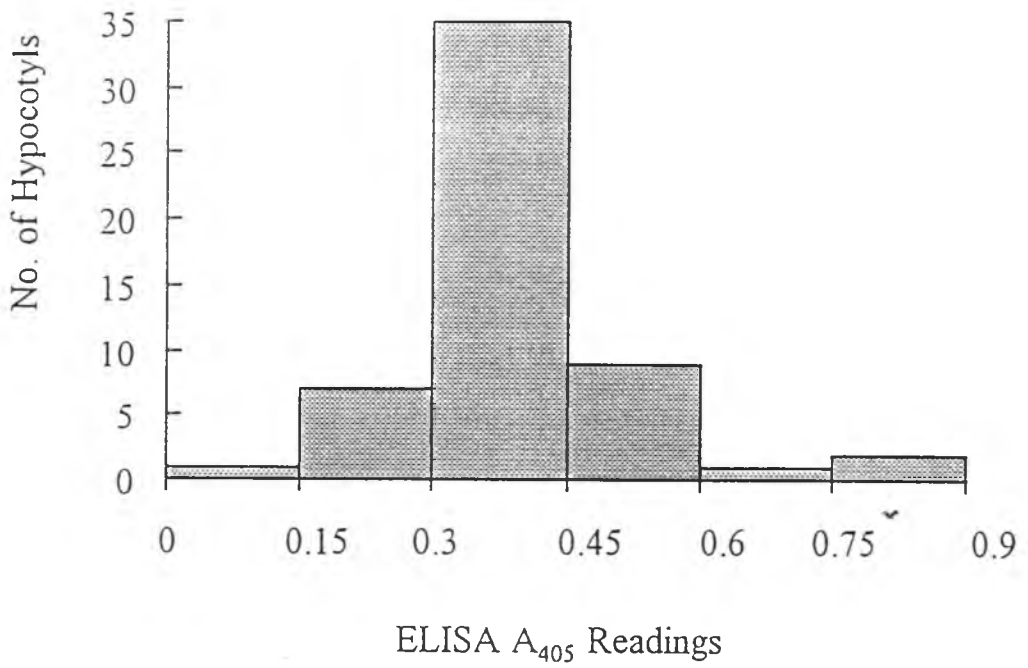

Fig. 13. Expression of PRV coat protein gene in chimeric transformed hypocotyls as determined by ELISA for coat protein. ELISA A_{405} for negative controls were 0.18 and 0.12, and ELISA A_{405} for positive controls were transgenic 'Sunset' papaya and the values were over 2.0.

Table 4.1. Effects of different concentrations of IBA on Hypocotyl Development#

IBA Conc.	Mean Hypocotyl Length	Mean Hypocotyl Diameter
0 μM	0.87 – 0.2	2.82 – 0.22
0.5 μM	2.54 – 0.35	2.14 – 0.15
1.0 μM	2.58 – 0.47	2.14 – 0.12
5.0 μM	2.77 – 0.42	1.30 – 0.10
10 μM	2.78 – 0.39	1.23 – 0.12

#Data were collected at twenty days after cultured on germination medium.

Fig. 14. The embryogenic callus from immature zygotic embryos showed GUS-positive expression the day after bombardment.

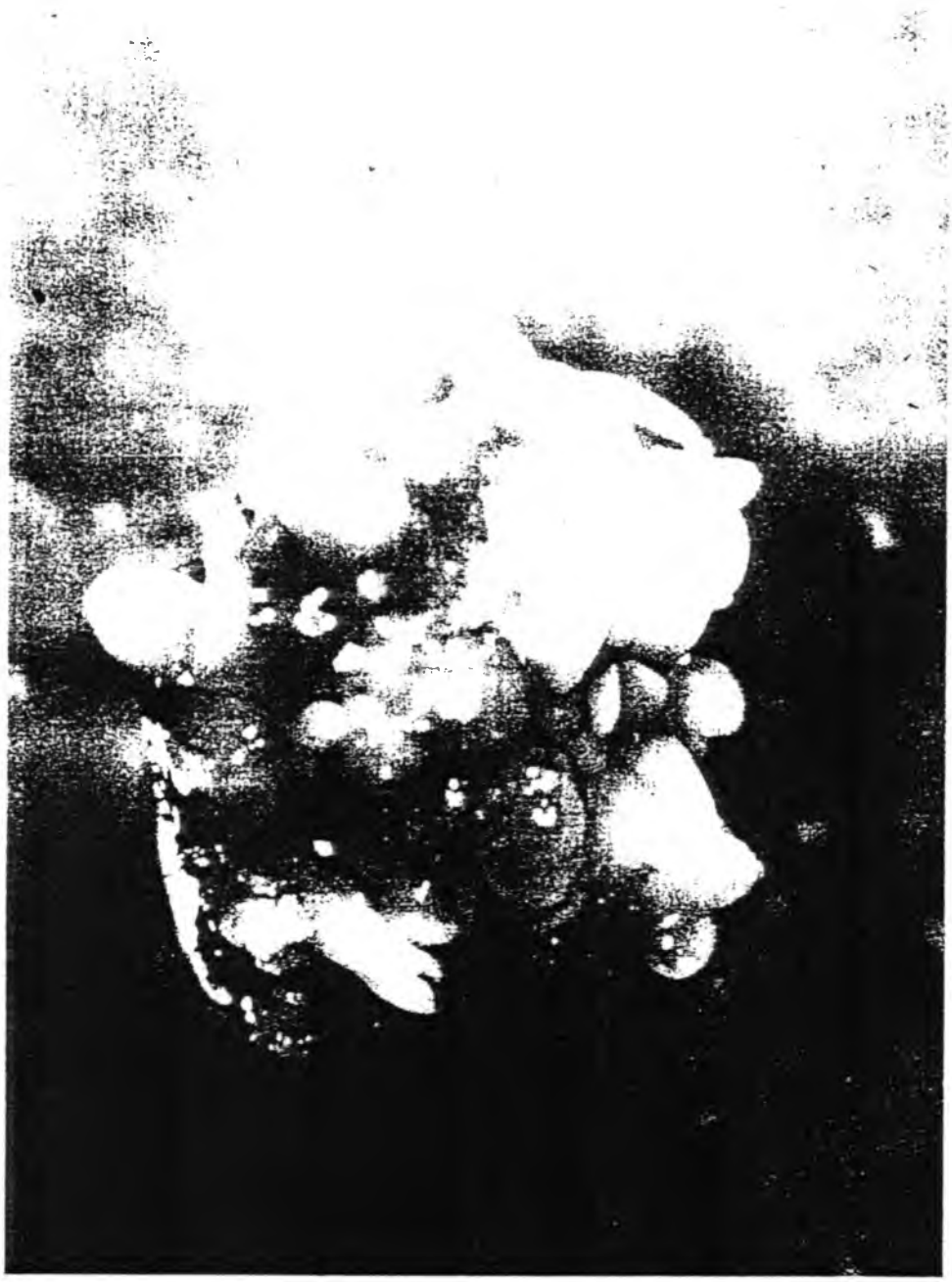

Fig. 15. The somatic embryos that showed GUS-positive blue stopped growth on maturation medium after they had been cultured on kanamycin selective medium for five months.

4.4 NPTII ELISA Assay

As mention previously, GUS assays indicated that 59% of bombarded zygotic embryos showed chimeric transient expression on the day following bombardment. After twenty days of culture on germination medium, 12 (28%) immature zygotic embryos developed into GUS-positive chimeric transgenic hypocotyl seedlings in total 43 bombarded immature zygotic embryos.

The chimeric hypocotyl seedlings were analyzed by NPTII ELISA, which NPTII ELISA assay was further study of chimeric transformed hypocotyls and confirmed that sections of chimeric transformed hypocotyls had embryogenic potential on selective induction medium containing kanamycin.

A four-point dilution series, 750 pg/ml, 500 pg/ml, 250 pg/ml and 100 pg/ml of NPTII enzyme, as established to produce a standard curve spanning the linear range of the assay, and the regression equation Y=-0.00906 + 0.004863X was obtained (Fig. 11). Seedling hypocotyls were produced by removing roots and apicotyls, and 0.2 g of hypocotyls was ground in 500 ml of extraction buffer for NPTII ELISA assay. The NPTII enzyme content was over 150 pg/ml to be considerd as NPTII ELISA positive, compared with standard curve. The results showed that 14 (28%) out of 50 hypocotyls were NPTII ELISA positive, and the A_{405} reading values were at least ten times greater than for untransformed negative controls (????). Positive ELISA tests had A_{405} value reading ranges were from 0.14 to 0.18 after 30 to 40 minutes of incubation at 37¡C, and NPTII concents of ELISA-positive hypocotyls ranged from 150 to 200 pg NPTII/ml, as determined from a standard curve (Fig. 11).

Compared to leaf petoile of transgenic Sunset 55-1, which previous research has shown to express NPTII at 420 pg/ml (Luis et al 1994), NPTII expression was lowin hypocotyls that tested positive by ELISA. However, this could be due to the chimeric nature of the hypocotyls, or to differences in developmental stage of the tissues tested. These chimeric transformed tissue had the potential to give rise to transgenic plants through somatic embryogenesis

4.5 Coat Protein ELISA Assay

Expression of coat protein as determined by ELISA assay with monoclonal antibody, showed positive results on 25% of the assayed tissues. The ELISA assay showed 12 out of 48 hypocotyl seedlings with A_{405} values in the range from 0.5 to 0.8 after three hours of incubation at 37¡C. These readings were at least ten times higher than untransformed negative controls, which had A_{405} values of (????). Purified coat protein of PRV was not available for generation a standard curve to obtain the coat protein amount, but A_{405} values of bombarded Kapoho hypocotyls were compared with those of transgenic Sunset papaya 55-1 and negative controls. The positive readings were two to three times lower than 55-1 'Sunset' papaya, but they were ten times higher than untransformed negative controls. Those tissues also had the potential ability to produce transgenic somatic embryos on selective induction medium with kanamycin. These results demonnstrated that a singnificant section of hypocotyl seedlings were transformed with the NPTII gene and PRV coat protein genes.

The GUS assay indicated a 59% (#GUS+/Total) transformation efficiency on the day following bombardment, and some hypocotyls had more than one GUS-positive blue spots. Twenty days after bombardment, GUS assay was of the hypocotyl yielded 28% positive expression, and ELISA assays revealed 28% positives for NPTII and 25% positives for coat protein. Lower positive expression in 20-day-old hypocotyls could be due to the failure of plasmid DNA to integrate with plant chromosomes in about half of the transiently expressing cells. Unincorperated plasmid

DNA is not stable, and although it could be expressed in early stages, it easily lost in subsequcent cell generations. Only the hypocotyls with cells containing integrated plasmid DNA give the potential to give rise to transgenic plants through somatic embryogenesis.

4.6 Somatic Embryogenesis from GUS-positive Hypocotyl Sections

The main part of our work was to get transgenic papaya plants from somatic embryogenesis. The first transgenic papaya plants were regenerated from somatic embryos bombarded with DNA-coated microprojectiles (Fitch et al. 1991). The excised immature zygotic embryos were plated on induction medium for about four weeks prior to bombardment to induce somatic embryogenic callus. After bombardment, embryogenic callus was cultured on selective medium with different concentrations of kanamycin to select the transgenic embryoids, which were subsequently regenerated without kanamycin to obtain the transgenic papaya plants.

We followed this procedure using the Kapoho papaya and obtained somatic embryogenic callus. On the day following bombardment, the somatic embryogenic callus showed GUS expression (Fig. 12). After culturing the embryogenic callus on selective medium with 75 mg/l kanamycin for one month and 150 mg/l kanamycin for four months, they stopped growth on nutrient medium without kanamycin and seemed dead (Fig. 13). We failed to regenerate any transformed tissue successfully. The possibility of this problem might be that we could not control the concentrations of kanamycin in induction medium. If the concentration of kanamycin was too high, all tissues stopped growing and died, even transformed tissue; if the concentration of kanamycin was

Fig. 16. Transgenic tissue developed embryos on maturation medium after they had been cultured on kanamycin selective medium for ten weeks.

Fig. 17. Transgenic papaya embryoids changed to green after they were exposed to light.

too low, all tissue grew up included untransformed tissue. The other problem was the amount of time that the bombarded tissue remained on kanamycin selective medium. We failed to regenerate the transformed tissue, which could be because the tissue had been in kanamycin selective medium over five month. It may be that the cultivar kapoho is more sensitive than other cultivars. Plant cells have the characteristic of totipotency, and transgenic plant regeneration is from the single-cell level (Kung et al. 1992). Cultivar Kapoho is slightly different from other cultivars, and the single-cell level could be more sensitive to kanamycin, so that it is necessary to try another approach in order to complete the experiment.

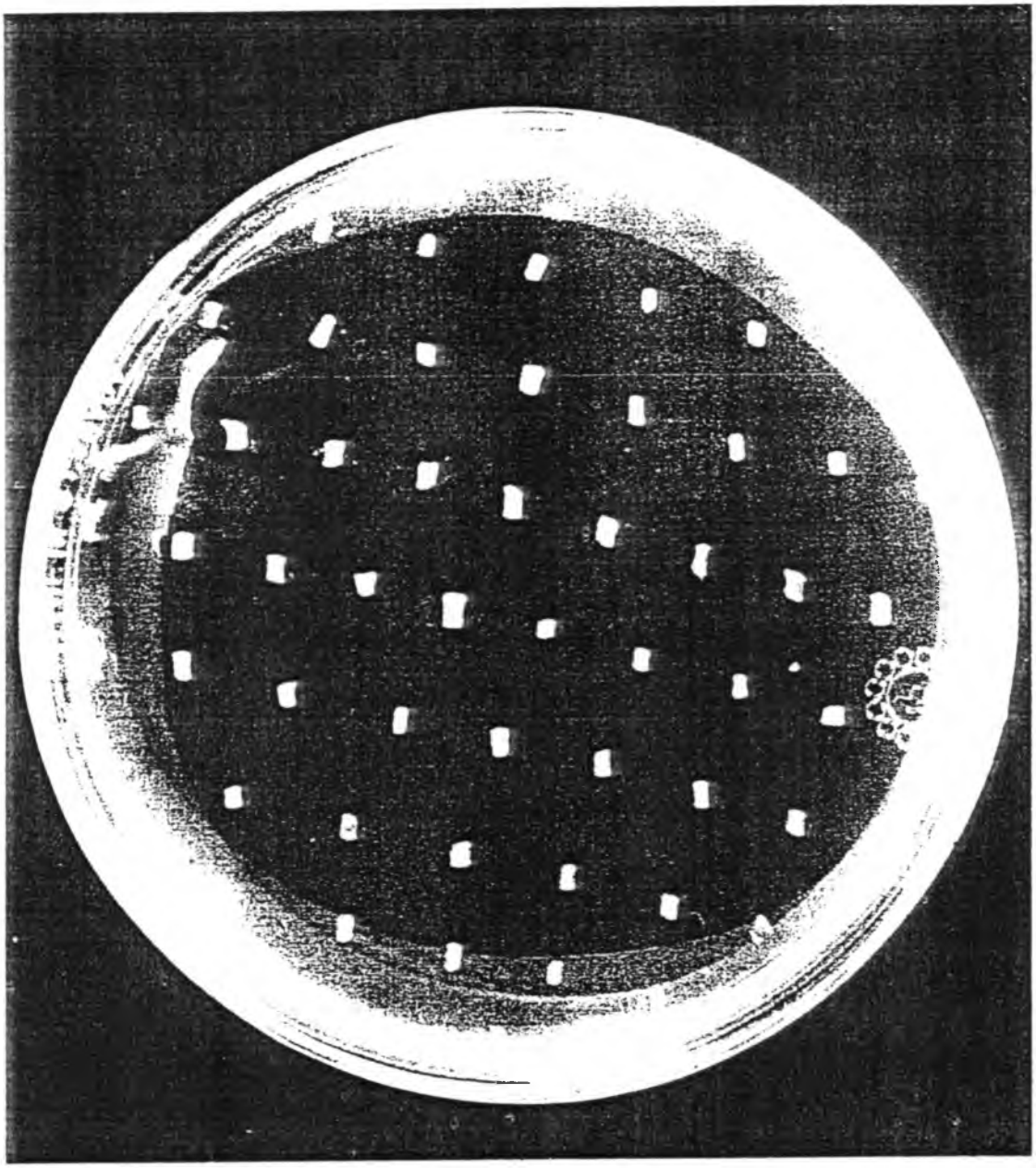

Fig. 18. 2-to-3 mm hypocotyl sections were cultured on induction medium.

Our first new approach was to use the epicotyl meristem of immature zygotic embryos as targets. The embryonic epicotyl, consisting mostly of meristematic tissue at the top of the axis between the two cotyledons (Esau, 1961), is a good target for bombardment. If plasmid DNA were introduced into the meristematic part of the embryos, it would be possible to get transgenic fruits and transgenic seeds after regenerating plants on selective medium. The next generations of plants obtained from those seeds, must be 100% transgenic papaya plants, no matter whether the original plants were chimeric transgenic plants or 100% transgenic plants. We could early assay the leaves for GUS activity or use ELISA metnoss to detect NPTII or coat protein to identify positive seedlings, then regenerate them to papaya plants.

Immature embryos with one cotyledon removed to expose the meristematic tissue were bombarded. On the day following bombardment, the immature zygotic embryos were assayed for GUS expression, and it was discovered that most GUS-positive blue spots were at the base part of the hypocotyl or radicle (Fig. 4). The meristematic targets of the epicotyl were apparently too small to obtain transformion, but from the hypocotyl or the radicle, new roots are produced from lateral meristems originating deep in the taproot in many plants (Esau 1961). Although the attempt to transform the epicotyl meristem failed, a high percentage of bombarded embryos expressed GUS in the meristematic region of the root. This suggested another approach based on regeneration via somatic embryogeniesis from root cultures (Chen et al. 1987). If bombarded immature zygotic embryos were plated on rooting medium following Drew et al. (1993), and subsequently transferred to germination medium

Fig. 19. Normal-looking roots developed on MS germination medium without growth regulator after they had been exposed to rooting medium with 10 μM IBA for nine days.

containing kanamycin, the transformed parts of the hypocotyls or the radicles might have the ability to produce transgenic lateral roots on the selective germination medium. Upon obtaining transgenic roots, the procedure of Chen et al. (1987) for inducing somatic embryogenesis from root explants to regenerate papaya plants could be followed in the medium without kanamycin..

The immature zygotic embryos were immediately placed on 1.0 μM IBA rooting medium without kanamycin for three days after bombardment, and then they were cultured on selective germination medium containing 30 mg/l kanamycin for four weeks, and 60 mg/l kanamycin for another four to six weeks in magenta boxes. Most tissues had died with two weeks of being placed on germination medium with 60 mg/l kanamycin, and at twenty days all tissues were dead. None of the embryos cultured under these conditions produced roots that were big enough for somatic embryogenesis. This was true also of the root systems produved by bombarded embryos that were cultured on germination medium without kanamycin selection. After twenty days on this medium, embryos developed into large (2-4 cm high) and robust (about 2 mm diameter) seedlings (Fig. 10). However, this experiment shows that these seedlings had poorly developed roots that were not suitable for induction of somatic embryogenesis. Nevertheless, when hypocotyls of these seedlings were assayed for GUS expression, three out of ten were GUS positive. Two of them expressed very strong color, and one showed light blue (Fig. 5). These GUS-positive hypocotyls were considered to be chimeric, probably involving the epidermal and/or cortical tissues only (Esan, 1961). An summary, transformation of, and regeneration from, primary roots of immature zygotic embryos was not successful, but from

this experiment we obtained chimeric transgenic hypocotyls had the potential to produce transgenic somatic embryos, if cultured on induction medium containing kanamycin.

Fitch (1993) demonstrated high frequency somatic embryogenesis and plant regeneration form papaya hypocotyl callus, and this work was used as a model for a third approach to producing transgenic Kapoho plants via regeneration from hypocotyl sections. Immature zygotic embryos with one cotyledon removed were bombarded with plasmid DNA and immediately placed on 1.0 μM IBA rooting medium. After three days, the embryos were transferred into germination medium. After these tissses had been cultured on germination medium for twenty days, the roots and epicotyls were removed and hypocotyls were cut into 2-to-3 mm sections and numbered. Half of the sections were assayed for GUS expression and other half were kept in culture. When any section was found to be GUS-positive, the adjacent sections were considered to be chimeric for transgenic tissue. Chimeric hypocotyl sections were cultured on 10 mg/l 2,4-D induction medium with 30 mg/l or 60 mg/l of kanamycin for four weeks, and during that period they grew very slowly. This could be the kanamycin problem again.

In order to obtain more rapid growth, we first placed the chimeric hypocotyl sections on induction medium without kanamycin for eight to ten weeks, subculturing monthly. When the tissues had developed enough callus for somatic embryogenesis, they were subcultured on the induction medium with 30 mg/l kanamycin for four weeks, the kanamycin concentration was subsequently doubled to 60 mg/l for four to six weeks to inhibit growth of nontransformed embryogenesis. When new embryogenesis occurred on hypocotyl sections on kanamycin

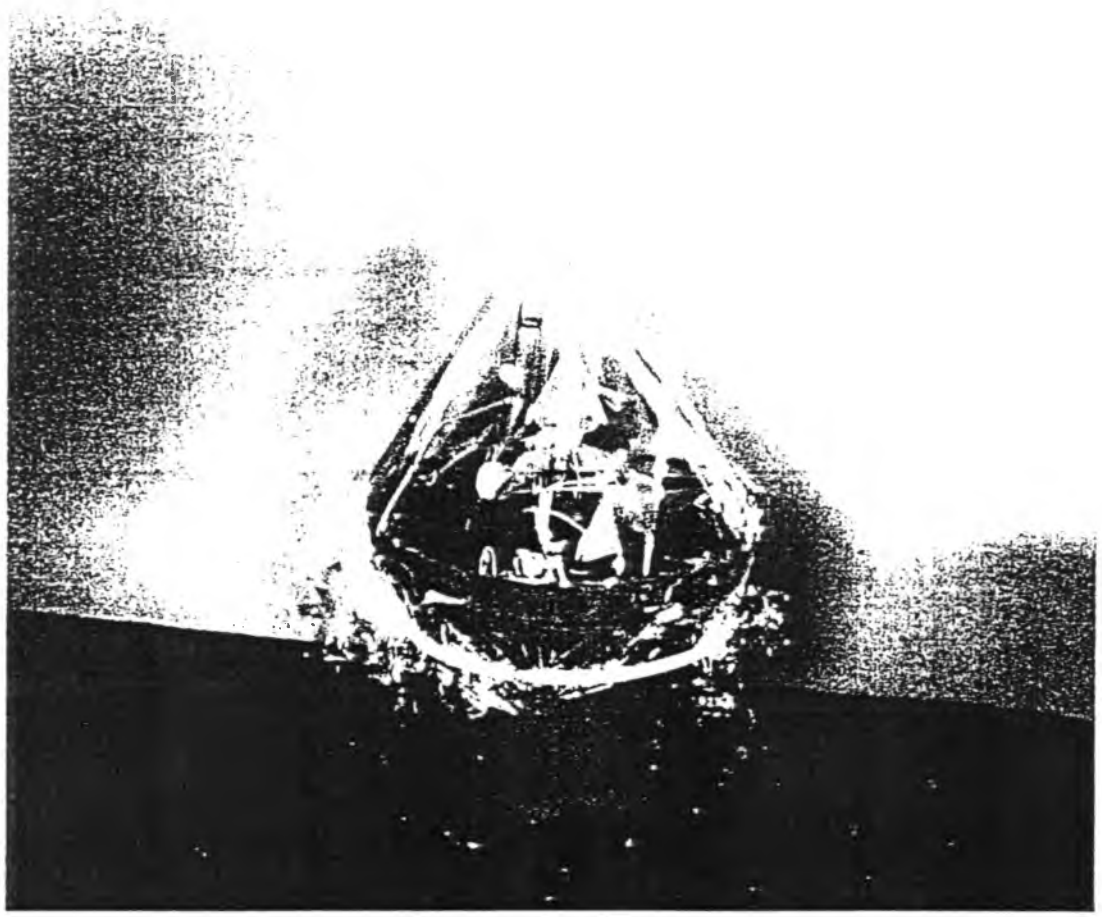

Fig. 20. The seedlings with roots and leaves were transferred successfully into vermiculite.

induction medium, the tissues were transferred to maturation medium without kanamycin for about two months. Tissue in maturation medium grew rapidly and formed embryoids (Fig. 14), which were moved to germination medium to enlarge.

A total of 233 transgenic hypocotyl sections from more than 1000 seedlings were cultured on induction medium, and 43% of these hypocotyl sections produced embryogenic callus for about two months of culture on induction medium without kanamycin.

Embryogenic calluses was transferred into selective induction medium with 30 mg/L kanamycin for four weeks, and then subcultured on induction medium with 60 mg/l of kanamycin.Under these conditions, tissues grew slowly, and some of them stopped growth. Grawth was scored weekly.

After two months of culture on selective induction medium, most embryogenic callus had turned brown and stopped growth. There were nine chimeric hypocotyl sections that yielded somatic embryos out of a total of 233 putative transgenic hypocotyl sections, after ten weeks of culture on selective induction medium.

Those nine pieces of tissue were moved to maturation medium without kanamycin and 2,4-D. When we transferred them under light, four of them were white and stopped growth on maturation medium, and the other five turned light green and grew rapidly on maturation medium (Fig. 15).

4.7 Somatic Embryogenesis from Hypocotyl Sections without GUS Assay

Using the GUS assay to identify potentially transgenic hypocotyl sections requied too much labor and time. Consequently, the GUS assay step was iliminated from the procedure to regenerate transgenic Kapoho plants via somatic embryogenesis from hypocotyl sections..

As described above, all hypocotyls were sectioned into 2 to 3-mm pieces (Fig. 16) and cultured on induction medium containing 10 mg/l of 2,4-D without kanamycin, subcultured monthly. In a total, only 55% of about 900 hypocotyl sections in thirty petri dishes developed embryogenic callus from the cut surfaces of the explants after two months of culture on induction medium. This result was not as successfrl as that reported by Fitch (1993), who obtained embryogenic callus from 81% of Kapoho hypocotyl sections on induction medium containing 45 μM 2,4-D. The initial IBA treatment to promote rapid hypocotyl growth may be partly responsible for this difference, as may a litter difference in 2,4-D concentation.

About 50% of hypocotyl sections cultured on 6 mg/l 2,4-D and 0.5 μM BA induction medium developed calluses from the cut surfaces of the explants. This result was similar to that reported by yang et al. (1992) on initiation of somatic embryogenesis petiole sections. The callus was seemed healthier, and the growth rate of hypocotyl sections was increased to induce more callus on 0.5 μM BA medium.

The hypocotyl sections with embryogenic callus were cultured for two month cultured on 10 mg/l 2,4-D induction medium, then transferred into selective induction medium containing 30 mg/l of kanamycin, and subsequently transferred into 60 mg/l of kanamycin to select the transgenic embryos. Up to this time, we found some embryogenesis occurring on 60 mg/l kanamycin selective induction medium..

4.8 Plant formation

After three month of culture on the maturation medium, We transferred the five remaining lines of somatic embryos (described in section 4.6) into half-strength MS germination medium, and subcultured monthly under continuous illumination at 27 C. There were more than thirty embryoids in each line, and these embryoids were spread on germination medium.

After four months of culture on germination medium, a shoot with one or two leaves from each line was assayed for GUS activity. Two of those lines was showed GUS positive (Fig. 7), one of them were GUS negative, and the other two were contaminated by bacteria but are still being kept in culture.

4.9 Root Production and Establishment in soil.

Tissue culture papaya plants are susceptible to shoot and root rots in the initial establishment stage in a nonsterile environment. Plants with poor roots in tissue culture did not survived in the transition to vermiculite.

The rooting procedure followed that of Drew (1993), in which the individual shoots (1.5 to 2.0 cm in height) with one to two leaves were severed at the base and exposed to the rooting medium containing10 μM IBA for 3, 6, 9, or 12 days to ditermine the best concentration for inducing root growth. Six replicates were in each treatment and root development was recorded as number of shoots with roots, number of roots per rooted shoot, and callus rating per rooted shoot. The shoots were then transferred into MS medium without growth regulator. where high quality roots were developed (Fig. 17).

The rooting tests showed that best treatment consisted of nine days of exposure to10 μM IBA rooting medium (Fig. 17 and Table 4.2). This result differed from Drew's (1993) recommended rooting method, which advised only 3 days of IBA exposure. All shoots were developed good quality roots after they were transferred into MS medium without growth regulator. Shoots with good roots were removed from culture to vermiculite in plastic pots and covered with transparent plastic bags that were opened gradually over a two week period to adapt the plantlet to ambient conditions. Right now, we transferred successfully three seedlings into vermiculite in plastic pots (Fig. 18).

Table 4.2. The effects of different duration of exposure to 10 μM IBA on rooting of papaya shoots

Duration day(s)	No. of shoots that initiated roots	Mean root number per rooted shoot#	Mean callus rating per rooted shoot##
0	0	0 a	1.0 a
3	2	2 b	3.5 b
6	3	2 b	4.0 b
9	6	4 c	4.0 b
12	6	4 c	4.0 b

Root and callus production was recorded after 20 days on germination medium.
#: Number of primary roots.
##: Callus was rated visually, on a scale of 0-5.
a, b and c differ significantly at $p<0.01$.

Chapter 5. Summary

Immature zygotic embryos are very good sources for somatic embryogenesis, and Fitch et al (1990) obtained transgenic 'sunset' papaya plants through somatic embryogenesis from immature zygotic embryos via particle bombardment. We tried to use the same protocol for cultivar kapoho papaya and obtained transformed tissues, but those tissues were failed to be regenerated. The reasons could be 1). It was hard to control the concentrations of kanamycin. 2). We did not find the best duration time on kanamycin selective medium for transformed tissue. 3). Cultivar kapoho papaya tissues were more sensitive to kanamycin compared to other cultivars.

In order to get rid of the kanamycin effects or decrease the kanamycin effects, the immature zygotic embryos with one cotyledon removed were bombarded with plasmid DNA. Twenty days after bombardment, we found that it was impossible to get rid of the kanamycin effects, but it was possible to decrease the kanamycin effects. These bombarded immature zygotic embryos were placed on rooting medium for three days, then transferred into selective germination medium to get transgenic roots. Our hypothesis was to obtain transgenic somatic embryogenesis from transformed root explants followed Chen et al (1987). Twenty days following bombardment, the tissues that were on the selective germination medium died. We did not obtain any root explants for somatic embryogenesis, even negative controls, but the

negative controls produced strong and green hypocotyl seedlings. These hypocotyl seedlings showed positive expressions of GUS assay and ELISA assay for NPTII and coat protein. These results indicated that the hypocotyl seedlings were chimeric transformed and the sections of chimeric transformed hypocotyls had the potential ability to rise to transgenic somatic embryogenesis on selective induction medium with kanamycin (Fitch. 1993).

The objectives of following experiments were 1). effectively transformed the forgein genes into immature zygotic embryos, 2). obtained chimeric transgenic hypocotyl yield from immature zygotic embryos, 3). produced embryogensis from callus of chimeric transgneic hypocotyl sections on kanamycin selective medium, 4). regenerated transgenic papaya embryoids.

The plasmid DNA, contained the NPTII selective marker gene, the reporter β-glucuronidase (GUS) gene, and the coat protein (cp) gene, was transformed into immature zygotic embryos and obtained GUS positive expression following the day of bombardment (Fig. 4). Chimeric transgenic hypocotyls, shown 28% of GUS positive expression (Fig. 5), 28% ELISA positive expression for NPTII and 25% ELISA positive expression for coat protein, were obtained good yield (Fig. 10). The embryogenic callus were induced from hypocotyl sections on induction medium without kanamycin and somatic embryogenesis were selected on kanamycin selective induction medium. The regeneration systems were exactly followed Fitch et al. (1990) and Drew et al. (1993). The transgenic seedlings were showed GUS positive results (Fig. 7).

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