Transformation and Regeneration of Taro with Two Plant Disease Resistance Genes: a Rice Chitinase Gene and a Wheat Oxalate Oxidase Gene

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

TROPICAL PLANT AND SOIL SCIENCES

DECEMBER 2006

BY

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ABSTRACT

Taro (*Colocasia esculenta*) is one of the most important crops in the Pacific Islands. However, taro yields have been declining in Hawaii over the past 30 years due to diseases caused by oomycete and fungal pathogens. Chitin, an important structural component of the cell wall of most fungi, is hydrolyzed by the action of the enzyme chitinase. Plants transformed with a chitinase gene have shown increased resistance to several fungal pathogens. Oxalate oxidase, which converts oxalic acid and O_2 to CO_2 and H_2O_2, has been shown to be involved in plant resistance to pathogens. Transformation of plants with an oxalate oxidase gene has increased their resistance to fungal pathogens.

An efficient regeneration and transformation system of the taro cultivar Bun Long has been developed. Regenerable calli were induced on MS medium with 2 mg L^{-1} BA and 1 mg L^{-1} NAA (M5 medium). Multiple shoots from these calli were induced on MS medium supplemented with 4 mg L^{-1} BA (M15 medium). The rice chitinase gene (*RICCHI11*) was introduced into taro calli using particle bombardment. Analyses using polymerase chain reaction (PCR) and Southern blot confirmed the presence of the rice chitinase gene in one transgenic line.

Agrobacterium tumefaciens-mediated transformation using two plant disease resistance genes: a rice chitinase gene (*RICCHI11*) and a wheat oxalate oxidase gene (*gf2.8*) were developed. Two hundred calli were infected with the supervirulent *A. tumefaciens* strain EHA105 harboring the plant transformation plasmid pBI121:*RICCHI11*. Six lines were shown to contain the rice chitinase gene using the PCR and Southern blot analyses. Transformation was also conducted using EHA105:pBI121:*gf2.8* that contains the wheat oxalate oxidase gene *gf2.8*. Two independent lines were shown to be positive for the specific *gf2.8* gene fragment using PCR, Southern blot and RT-PCR analyses, indicating the presence and transcription of *gf2.8* gene in the transformed lines. In a preliminary bioassay, six

transgenic taro lines with the rice chitinase gene exhibited tolerance to the fungal pathogen *Sclerotium rolfsii*. One transgenic taro line with the wheat oxalate oxidase gene showed tolerance to both the fungal pathogen *Sclerotium rolfsii* and oomycete pathogen *Phytophthora colocasiae*.

To our knowledge, this is the first report of taro transformed with disease resistance genes. In addition, this is the first report of genetically engineered taro that exhibited disease tolerance in preliminary bioassays.

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LIST OF ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AS	acetosyringone
A. t.	*Agrobacterium tumefaciens*
BA	6-benzylaminopurine
CaMV 35S	cauliflower Mosaic Virus 35S RNA
CIM	callus inducing medium
CM	coconut milk
CPM	callus proliferation medium
CTAB	hexadecyltrimethylammonium bromide
CV	cultivar
2, 4-D	2, 4-dichlorophenoxyacetic acid
EtOH	ethanol
FISH	Fluorescence in situ hybridization
GE	genetic engineering
G418	geneticin
gus	β-glucuronidase gene
GFP	green fluorescent protein
hpt	hygromycin phosphotransferase
HR	hypersensitive response
IAA	indoleacetic acid
KIN	kinetin
KM	Kao and Michayluk's organic substance
LB	left border
LS	Linsmaier & Skoog

MS	Murashige & Skoog
NAA	α-naphthaleneacetic acid
nptII	neomycin phosphotransferase II
n.t.	not tested
OA	oxalic acid
OXO	oxalate oxidase
PCM	protoplast culture medium
PDA	potato dextrose agar
PR protein	pathogenesis-related protein
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
RB	right border
SDS	sodium dodecyl sulfate
SIM	shoot inducing medium
SSR	simple-sequence repeat
TE	taro extract
TLB	taro leaf blight
TPR	taro pocket rot
2,4,5-T	trichlorophenoxyacetic acid
VA	V8 agar

CHAPTER 1

GENERAL INTRODUCTION

1.1 Importance of taro

Taro (*Colocasia esculenta* (L.) Schott) is a member of the Araceae family that originated in India and adjacent areas of Southeast Asia. It is one of the most important staple food crops in the Pacific Islands, and is widely cultivated throughout the South Pacific, South America, Asia, Africa, and the Caribbean (Caillon et al., 2006; Kreike et al., 2004; Lee, 1999; Quero-Garcia et al., 2006; Wang, 1983).

The corm, or underground stem, is consumed for its starch. In comparison with potato and other starchy crops, taro corms or cormels have much smaller starch grains that are fairly rich in the soluble starch called amylose. Amylose is excellent for people with digestive problems (Perez et al., 2005; Sefa-Dedeh and Sackey, 2002). Further, the taro corm has a higher proportion of protein (1.5-3.0%), calcium, and phosphorus (Gibson, 1999; Hussain et al., 1984; Shewry, 2003) compared to other starchy crops. In the traditional Maori diet, taro corms contain protective chemical constituents such as the anthocyanins cyanidin 3-glucoside, pelargonidin 3-glucoside and cyanidin 3-rhamnoside (Cambie and Ferguson, 2003). These anthocyanins were reported to have antioxidant and anti-inflammatory properties. Recently, Tanaka et al. (2005) showed that taro extracts, monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG), exhibited anti-hyperlipemia activity and anti-tumor-promoting activity.

In addition, taro leaves serve as a vegetable, providing good sources of dietary fiber and vitamin C (Ferguson et al., 1992; Pinto et al., 2000a, 2000b). Ferguson et al. (1992) revealed that the dietary fiber from taro leaves exhibited substantial adsorption of a hydrophobic mutagen. This finding provided one possible reason that people eating taro as a staple in the South Pacific had a much lower incidence of colorectal cancer than European

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populations. Moreover, Nair et al. (2005) evaluated the antibacterial activity of several plant extracts to determine their therapeutic potential. In their tests, taro leaf extracts showed the strongest antibacterial activity against the most resistant bacterial strain *Klebsiella pneumoniae.*

Due to its high starch and protein quality, the taro corm has become an important base for industrialized food such as taro flour, chips and baby food (Gibson, 1999; Huang, 2000; Hollyer et al., 2000; Nip et al., 1995). Taro flour is used in infant formulae and canned baby foods, and is good for people with allergies, such as lactose intolerance (Huang, 2000; Sefa-Dedeh and Sackey, 2002; Standal, 1983). In addition, taro ice cream products are appreciated by many consumers because they have unique flavor, smooth texture and special color (Lu et al., 2002). In Hawaii, taro, also called kalo (Hawaiian), has great cultural significance to indigenous peoples. Indigenous Hawaiian traditional food products include poi (cooked and mashed taro corm with water), kulolo (a pudding made with grated taro corm and shredded coconut), and laulau (a bundle of 5-6 taro leaves containing pork and fish) (Lee, 1999). Recently, Brown et al. (2005a) demonstrated that poi significantly inhibited growth of colon cancer cells and non-specifically activated lymphocytes, suggesting that poi has anti-cancer effects. Moreover, in another research project, Brown et al. (2005b) suggested that poi is a potential non-dairy probiotic (i.e. dietary supplement containing potentially beneficial bacteria or yeast) with more advantages than yogurt for therapeutic use in cancer patients.

1.2 Major diseases

In Hawaii, taro yields have been declining over the past 30 years with particularly steep decreases during the last five years partially due to diseases caused by various pathogens, especially oomycete and fungal pathogens. According to currently released data by the Hawaii Agricultural Statistics Service (2006), Hawaiian taro production in 2005 was only 4.0 million pounds, a decrease of 19 percent from 2004. This production level was the

lowest in records kept since 1946. The overall farm price of all taro products remained unchanged in 2005, resulting in the lowest farm value in 16 years for all type of taro valued at $2.2 million. The rainy weather, apple snail infestations, taro leaf blight, and taro pocket rot diseases were thought to be the major problems resulting in the sharp reduction of taro production (Hao, 2006; Hawaii Agricultural Statistics Service, 2006).

Many taro cultivars are susceptible to various pathogens. Taro pathogens include oomycetes, fungi, bacteria, nematodes, viruses and mycoplasmas (Ooka, 1994). As global transportation and economic cooperation have developed rapidly in modern times, Hawaii is no longer an isolated area that could avoid the invasion of taro pathogens. These pathogens probably caused much of the recent reduction of taro yields.

In Hawaii, possibly hundreds of taro cultivars were exterminated due to disease disasters (Ooka, 1994). Currently, in the Solomon Islands, the alomae-bobone viral complex destroyed 96% of the native taro varieties and heavily reduced taro yields by 95% (Kastom Gaden Association, 2005). Moreover, the insect transmitting this viral complex has been found in Hawaii, and all Hawaiian taro varieties are susceptible to it (S. Pacific Commission, 1978).

1.3 Oomycete and Fungal Diseases Reduce Yields of Taro

Oomycete and fungal diseases are the most economically significant among the taro disease classes (Ooka, 1994; Philemon, 1994). It is estimated that 25 to 50% of taro corms in Hawaii are lost due to oomycete and fungal diseases (Miyasaka et al., 2001; Takahashi, 1953; Trujillo, 1967). There is one fungicide registered in Hawaii to control fungal diseases, however, its restricted use is allowed only within six weeks after planting (Miyasaka et al., 2001). The cropping cycle of taro is typically nine to 13 months, therefore, the effectiveness of this fungicide is limited.

There are at least four oomycete and fungal diseases causing decreased taro yield in Hawaii. Taro leaf blight (TLB), caused by *Phytophthora colocasiae* Rac., usually occurs in rainy or overcast weather. It is the most commonly recorded oomycete disease and it can reduce yields quite substantially (Ooka, 1994). Pythium rot (soft rot, pala, palahi) is caused by *Pythium aphanidermatum* Fitzpatrick, *P. graminicola subramaniam*, *P. splendens* Braun, *P. irregulare* Buisman, *P. myriotylum* Drechsler, *P. carolinianum* Matthews, or *P. ultimum* Trow. This disease causes the taro corm to form a soft, often malodorous mass (Ooka, 1994). Sclerotium or southern blight, caused by *Sclerotium rolfsii* Sacc., is generally a problem of dryland taro and results in corm rot and stunted plants (Ooka, 1994). Recently, a new species of *Phytophthora* was found to cause taro pocket rot disease (TPR) (Uchida et al., 2002; Uchida, 2003).

Much research on taro oomycete and fungal diseases focused on taro leaf blight (TLB) and the taro pocket rot (TPR) (Brooks, 2000a; Hamasaki et al., 2000; Ooka, 1994; Philemon, 1994; Trujillo, 1967, 1996; Trujillo and Menezes, 1995; Trujillo et al., 2002; Uchida et al., 2002; Uchida, 2003). Taro leaf blight that is caused by *Phytophthora colocasiae* invaded Hawaii in about 1920. It probably resulted in the extinction of more than 270 traditional Hawaiian cultivars (Trujillo, 1996). In 1993, this disease was introduced to American and Western Samoa and devastated the traditional Samoan taro cultivars that were highly susceptible to this disease. This epidemic resulted in no taro production in Samoa from 1994-1998 until the introduction of disease-resistant cultivars (Brooks, 2000a; Trujillo and Menezes, 1995; Trujillo, 1996). Recently, taro pocket rot (TPR) was reported to be a major problem in most wetland taro fields in Kauai Island in Hawaii (Uchida et al., 2002; Uchida, 2003). This disease results in pockets of diseased tissue in the corm (Uchida et al., 2002; Uchida, 2003). According to Hawaii Agricultural Statistics Service (2006), one important

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reason for the taro production decline in 2005 was an increase of TPR fostered by unfavorable, rainy weather conditions.

1.4 Improvement by conventional breeding

Breeding for improved yields requires information on genetic variability of yield and yield enhancing traits. Genetic variability and heritability relating to certain yield traits were estimated in thirty accessions of taro (Dwivedi and Sen, 1997). The results indicated that individual plant selection for total number of petioles per clump, total number of side suckers per plant and girth of main sucker would be effective (Dwivedi and Sen, 1997).

Taro cultivars were tested for TLB resistance (Brooks, 2000b; Trujillo and Menezes, 1995; Trujillo, 1996), and several Palauan taro varieties were observed to be naturally resistant to *Phytophthora colocasiae* (Trujillo and Menezes, 1995; Trujillo, 1996). These Palauan cultivars were shown to have some level of TLB resistance, and were distributed to farmers in Hawaii and American Samoa (Brooks, 2000a). However, these TLB resistant, Palauan taro cultivars do not make a high quality poi (Hamasaki et al., 2000).

Use of disease resistant taro cultivars is a promising, non-chemical approach to increase yields and improve qualities. Conventional breeding of disease resistant cultivars with commercial taro cultivars is ongoing (Trujillo et al., 2002; J. Cho, personal communications). Recently, three promising new taro cultivars with resistance to the TLB were developed. One of them is a hybrid (cv. Pa 'lehua) developed between a Palauan taro cultivar with the TLB resistance trait (Ngeruuch) and a Hawaiian commercial cultivar (Maui Lehua) that has been shown to be TLB resistant. It has twice the yield potential of cv. Maui Lehua and its cooked corm results in good quality poi (Trujillo et al., 2002). However, preliminary observations of these three hybrids indicated that environmental conditions that are conducive to TLB could break down the disease resistance (Trujillo et al., 2002). In addition, cv. Pa 'lehua is more susceptible to *Pythium* soft rots, a serious disease of taro,

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particularly in acidic soils (Trujillo et al., 2002). Also, response of cv. Pa 'lehua to corm rots has been observed to produce an odd, unpleasant taste even in undiseased portions of corm (R. Yamakawa, personal communications). These three hybrids cultivars, Pa 'lehua, Pa 'akala, and Pauakea were patented by the University of Hawaii (UH), however, recently the UH has agreed to give up the rights over these patents due to a controversy over indigenous intellectual property rights (Bhattacharjee, 2006).

1.5 Improvement by genetic engineering

1.5.1 The advantages and safety of genetic engineering of taro

Compared with conventional breeding, genetic engineering has revolutionary advantages. Genetic engineering (GE) can improve crop yields, quality or disease resistance via transforming a single gene or several genes of interest into target plants (Manshardt, 2004). In principle, the genes of interest can come from any organism: plants, animals, or microorganisms. Therefore, genes can be transferred across the species barrier to enrich the gene pools of an individual species (Manshardt, 2004). In addition, unlike conventional breeding that transfers the total genome, GE only transfers one or a few genes of interest. Therefore, GE only alters certain characters of interest, retaining all other good characters (Manshardt, 2004). In practice, breeding via genetic engineering has achieved great progress throughout the world in recent decades (Singh et al., 2006). For example, genetic engineering of the papaya cv. Rainbow with the resistance gene against papaya ringspot virus has saved the Hawaii papaya industry from the devastating epidemic caused by the papaya ringspot virus (Goldstein, 2004).

People may be concerned whether: a) the transgene from GE plants will be transmitted to non-GE plants and cause genetic contamination of the native gene pool; or b) if GE will negatively affect the nutrition and other qualities of transgenic plants; or c) if GE will negatively affect human health. These risks of GE crops are very low (Singh et al., 2006). In

Hawaii, all GE crops including papaya, corn, soybeans, wheat and sunflowers have not been found to pollinate any Hawaiian native species (Goldstein, 2004). In this study, we chose the Chinese taro cultivar Bun Long to genetically transform. This cultivar rarely flowers in Hawaii. In addition, traditional Hawaiian cultivars rarely produce viable seeds in Hawaii (J. Cho, personal communication). Also, taro propagation is via hulis (suckers), corms or cormels but not via seeds. Therefore, the risk of transferring disease resistance genes from GE taro to native Hawaiian taro is not likely.

The possible health risk of GE crops is also very low. There is no scientific evidence to show that GE crops have negatively affected human health after decades of practice (Singh et al., 2006). In this study, two disease resistance genes were transformed into taro. One is the rice chitinase gene and another is the wheat oxalate oxidase gene. Rice and wheat are staple cereals that millions people have consumed for thousands of years. Chitinase breaks down chitin, a compound that does not exist in taro or other plants. Oxalate oxidase breaks down oxalate that is a notorious "anti-nutritive" compound. Therefore, GE taro with either of these two genes should have little negative effect on taro nutrition and human health.

1.5.2 Genetic mapping of taro and gene cloning

The genetic diversity of the Asian taro was analyzed by random amplified polymorphic DNA (RAPD) and isozymes (Ochiai et al., 2001; Xu et al., 2001). Recently, the genetic diversity of taro in Southeast Asia and the Pacific was studied using amplified fragment length polymorphism (AFLP) (Kreike et al., 2004). Moreover, Caillon et al. (2006) analyzed the genetic diversity in a Pacific island, Vanua Lava, Vanuatu using AFLP, and assessed the possible sources of variation. Recently, Quero-Garcia et al. (2006) presented the first taro genetic maps containing 161 AFLPs and 8 simple-sequence repeat (SSRs), and they identified several quantitative trait loci (QTLs) for corm yield and corm dimensions.

Only a few genes or their coding proteins that are related to disease resistance have been isolated and identified from taro. Tarin is a family of storage proteins that accounts for approximately 40% of the total soluble proteins in taro corms (Guimaraes et al., 2001; Monte-Neshich et al., 1995). A gene that encodes a tarin isoform was isolated and characterized with high homology to lectins that are thought to play a role in defense (Bezerra et al., 1995; Van Damme et al., 1995; Powell, 2001). Moreover, a novel phytocystatin gene was cloned from the fungal resistant taro cultivar Kaosiung no. 1, and the expressed cystatin exhibited significant resistance against the taro fungal pathogen, *Sclerotium rolfsii* (Yang and Yeh, 2005).

Other genes that have been isolated from taro include a novel, mannose-binding lectin gene from *Arisaema heterophyllum* (in the Araceae family) has been cloned and characterized (Zhao et al., 2003). In addition, Lin and Jeang (2005a; 2005b) isolated, expressed and characterized a soluble starch synthase I gene and a novel soluble starch synthase II gene. To better understand molecular mechanisms involved in hardiness and yield, it is important to develop both a genetic map of taro and to clone taro genes relating to their quality, nutrition, and disease resistance.

1.5.3 Genetic Transformation of Taro

There is only one report in the literature on genetic transformation of taro as summarized in Table 1.1. Fukino et al. (2000) inserted a reporter gene, ß-glucuronidase (*gus*) gene using particle bombardment into a triploid cultivar of taro, Eguimo, one of the major cultivars used in Japan. However, the efficiency of transformation was very low, less than 0.1% (only two transgenic lines were obtained) (Fukino et al., 2000). Furthermore, to our knowledge, there is no report of taro being transformed with any genes useful for improving yields, qualities or disease resistance.

Table 1.1 Genetic transformation of taro (*Colocasia esculenta*) in the literature

Cultivar	Transformed gene	Transformation method	Transformation efficiency	Reference
Triploid 'Eguimo'	*GUS* reporter gene *hpt* selection gene	Particle bombardment	<1%	Fukino et al. 2000

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CHAPTER 2

DEVELOPMENT OF AN EFFECTIVE REGENERATION SYSTEM

2.1 Literature review

Traditionally, taro is propagated vegetatively through corms or cormels (de la Pena, 1983). Approximately 10% of the yield from a previous crop is used for propagation. Different procedures for tissue culturing of various taro cultivars have been developed (Table 2.1; Hartman, 1974; Hain, 1991; Jackson et al., 1977; Malamug et al., 1992; Mapes and Cable, 1972; Murakami et al., 1995; Sabapathy, 1995; Yam et al., 1990). In addition, plantlet regeneration via callus (Malamug et al., 1992; Sabapathy, 1995) and via protoplasts (Murakami et al., 1995) also have been developed. However, certain cultivars are more difficult to tissue culture.

Dasheen mosaic virus (DsMV) decreases corm yield (Malamug et al., 1992; Ooka, 1994). The DsMV is carried by aphids (*Myzus persicae* Sulzer, *Aphis craccivora* Koch., *A. gossypii* Glov.) and there are no known taro varieties that are immune to this virus (Ooka, 1994; Philemon, 1994). Many protocols of shoot tip culture have been investigated to eliminate DsMV from infected taro (Hartman, 1974; Hain, 1991; Jackson et al., 1977; Mapes and Cable, 1972; Sabapathy, 1995; Yam et al., 1990).

2.2 Materials and methods

2.2.1 Materials

Taro cultivars Maui Lehua and Bun Long were chosen as plant materials. The cv. Maui Lehua is the most important cultivar grown in Hawaii for commercial "poi" production. The cv. Bun Long is the most important Chinese cultivar grown in Hawaii for consumption of leaves and production of chips. Corms of these two cultivars were obtained from the University of Hawaii's Waiakea Experiment Station.

2.2.2 Methods

2.2.2.1 Tissue culture of taro shoots

Shoot tips were explanted by modifying the method of Hartman (1974). Briefly, shoot tips (0.5-1.5 mm) in length were excised from taro corms under a dissecting microscope, surface-sterilized with 1.25% sodium hypochlorite (NaOCl) and 0.1% Tween 80 per 100ml solution for 16 s, rinsed successively in sterile water, then explanted into test tubes containing 3ml liquid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 4 mg L^{-1} BA (M15 medium) to produce multiple shoots. A single explant was placed in each test tube, and the culture tubes were maintained at 25 °C with a 16h photoperiod and shaken at 95 rpm.

Ten shoot lines were tested for the presence of DsMV using an ELISA test (Agdia PathoScreen Kit, Indiana). Shoot tips were excised from the plantlet lines that were free of DsMV for induction of calli.

2.2.2.2 Regeneration of taro calli and shoots

Genetic transformation involves the insertion of transgenes into totipotent cells (e.g. callus) that can regenerate into whole plants. To develop a method to produce highly regenerative calli, more than forty media containing MS with different concentrations and ratios of auxin NAA or 2, 4-dichlorophenoxyacetic acid (2, 4-D) and cytokinin (BA or kinetin) were tested for callus initiation and then regeneration. The five media tested for calli initiation are shown in Table 2.2. The three media tested for regeneration of shoots from calli are shown in Table 2.3.

Table 2.1 Various methods used for tissue culture of taro (*Colocasia esculenta*)

Cultivar	Explant type	Medium (Unit: mg L^{-1})	Reference
Eguimo	Shoot tips	CIM: LS+2BA+1NAA CPM: Liquid LS+2BA+1NAA SIM: LS	Fukino et al., 2000
___	Shoot tips	CIM: MS+1kinetin+15IAA SIM: MS+1kinetin+15IAA	Hartman, 1974
Dotare	Apical shoots	CIM: Nitsch+1 2,4-D+1BA SIM: Nitsch+1BA	Malamug et al., 1992
Eguimo	Etiolated stems	CIM: MS+2 2,4-D+2 2ip PCM: 1/2MS+KM+2BA SIM: MS+0.2NAA+2BA	Murakami et al., 1995
Keladi Birah	Shoot apices	CIM: LS+2 2,4,5-T+ 0.2kinetin SIM: LS+2kinetin	Sabapathy and Nair, 1995
Akalomamale	Axillary buds	CIM: MS+20TE+2NAA+0.2BA SIM: MS+20TE	Yam et al., 1990

- CIM : Callus Inducing Medium; CPM: Callus prolification medium; SIM : Shoot Inducing Medium; PCM: Protoplast culture medium

- MS: Murashige and Skoog medium (Murashige and Skoog, 1962); LS: Linsmaier and Skoog medium (Linsmaier and Skoog, 1965); Nitsch: Nitsch medium (Nitsch, 1969); KM: Kao and Michayluk's organic substances; TE: taro extract

- BA: benzyladenine; NAA: naphthaleneacetic acid; IAA: indoleacetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid; 2,4,5-T: trichlorophenoxyacetic acid

2.3 Results

2.3.1 Regeneration of taro cv. Bun Long

2.3.1.1 Tissue-culture of DsMV-free taro cv. Bun Long shoots

Explants of white shoot tips of cv. Bun Long, 0.5-1.5 mm in size, were grown in liquid M15 media for two weeks until they became light green in color and approximately 2-5 mm in size. After one month, light green multiple shoots (1-3 cm) were formed (Fig. 2.1a). Twenty-eight out of 30 tested explants formed multiple shoots. An average of 10 multiple shoots were obtained from each explant. After a second month, 3-5 green leaves were formed. The ELISA test that detects the presence of DsMV showed that all 10 shoot lines were negative, indicating that the tissue-culture procedure adapted from Hartman (1974) had eliminated DsMV successfully.

2.3.1.2 Induction and maintenance of cv. Bun Long calli

Soft, yellowish-white calli of cv. Bun Long were initiated after shoot tip explants were grown for eight weeks on M5 media: basic Murashige and Skoog (MS) medium with 2 mg L^{-1} benzyladenine (BA) and 1 mg L^{-1} napthaleneacetic acid (NAA) (Table 2.2; Fig. 2.2a). These calli were maintained on the same medium for 3-4 months in the dark with subculturing once each month. Multiple shoots could be induced later from these calli by manipulating phytohormone levels.

Calli of different colors and textures were induced on other types of media (Table 2.2). A greenish-yellow, compact callus was induced from shoot tip explants placed on MS medium supplemented with 0.2 mg L^{-1} BA and 0.5 mg L^{-1} 2,4-D (M23 medium), MS medium supplemented with 1 mg L^{-1} BA and 3 mg L^{-1} 2,4-D (M24 medium), and MS medium supplemented with 3 mg L^{-1} BA and 3 mg L^{-1} 2,4-D (M25 medium). However, it was difficult to regenerate shoots from these calli. A soft, yellowish-white callus was induced from shoot tip explants placed on MS medium supplemented with 1 mg L^{-1} kinetin and 1.5

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mg L^{-1} NAA (M35 medium). However, multiple shoots and roots also were induced simultaneously in this callus, making it a non-uniform and intractable transformation target.

2.3.1.3 Induction of multiple shoots of cv. Bun Long

The calli of cv. Bun Long were placed in various media to stimulate regeneration of shoots. Multiple shoots could be induced on calli formerly grown in M5 medium (Table 2.2) by transferring them to MS medium supplemented with 2, 4, or 8 mg L^{-1} BA (M14, M15, or M16, respectively) at 25 °C and a 16h photoperiod (Table 2.3). Growth of multiple shoots

was fastest on the medium with the highest level of BA; however, abnormal shoot morphology developed. Multiple shoots were induced on both solid and liquid M15 media, and growth was faster than on the M14 medium, indicating that the medium supplemented with 4 mg L^{-1} BA was optimal for multiple shoot production (Fig. 2.2c and Fig. 2.2d). Multiple shoots grew faster on liquid M15 media than on solid M15 medium. Alternating calli between M5 and M15 media every two weeks for the first two months increased multiple shoot production (Fig. 2.2b).

2.3.1.4 Summary of regeneration process of taro cv. Bun Long

The regeneration procedure of taro cv. Bun Long is summarized in the flow chart (Fig. 2.3). The tissue culturing of taro shoots is established using explants of shoot tips excised from taro corms. Then highly regenerative calli are induced from these tissue-cultured shoot tips on the medium M5 (MS + 2 mg L^{-1} BA + 1 mg L^{-1} NAA). Multiple shoots are induced from calli on the medium M15 (MS + 4 mg L^{-1} BA).

2.3.2 Regeneration of taro cv. Maui Lehua

2.3.2.1 Tissue-culture of DsMV-free taro cv. Maui Lehua shoots

The growth of explants of white shoot tips of cv. Maui Lehua in liquid M15 media was similar to that of cv. Bun Long. Light green, multiple shoots (1-3 cm) were formed after

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one month (Fig. 2.1b). However, the number of multiple shoots was less than that of cv. Bun Long. An average of five multiple shoots was obtained from each explant. After a second month, 3-5 green leaves were formed. The ELISA test result was the same as that of cv. Bun Long, indicating that the tissue-culture procedure adapted from Hartman (1974) had also eliminated DsMV successfully in cv. Maui Lehua.

2.3.2.2 Induction of calli and multiple shoots from cv. Maui Lehua

Either no callus or only a few calli were induced from explant shoot tips of cv. Maui Lehua on more than 40 media tested. Multiple shoots were not induced from calli. However, multiple shoots developed directly from shoot tip explants without going through a callus stage on the M15 medium (Fig. 2.4). Also, similar to cv. Bun Long, alternating calli between M5 and M15 media every two weeks increased multiple shoot production.

2.4 Discussion

An efficient, regeneration system of taro cv. Bun Long was developed from callus. However, cv. Maui Lehua could not be regenerated from callus under current research protocols.

This method of tissue-culturing DsMV-free taro plants differs from previously published methods. For example, 0.5-1.5 mm shoot tips were excised from the corms under the microscope first, and then surface-sterilized for very short time, 16s. Using this method there was no visible contamination observed and most explants remained healthy after surface-sterilization. Other methods of shoot tip culture (Hartman, 1974; Hain, 1991; Sabapathy, 1995; Yam et al., 1990) involved excising 1-1.5 cm sections from corms first, then surface-sterilizing for 1-5 minutes, and finally excising the 0.5-1.5 mm shoot tips under the microscope. We found that our new method was relatively easier, and resulted in microbial contamination-free explants.

In this study, we report that highly regenerable callus could be induced from shoot tips of cv. Bun Long on the MS medium supplemented with 2 mg L^{-1} BA and 1 mg L^{-1} NAA (Table 2.2), and that multiple shoots could be induced on the MS medium supplemented with 4 mg L^{-1} BA (Table 2.3). This protocol differs from previous published methods (Table 2.1), but is most similar to that reported by Fukino et al. (2000) with the following exceptions: a) these researchers used LS (Linsmaier and Skoog) medium; and b) their regeneration medium did not contain any hormones (Table 2.1). Our regeneration protocol is specific to cv. Bun Long, because attempts to produce regenerative calli from cv. Maui Lehua were not successful. Apparently, amounts and types of hormones necessary to produce regenerative calli differ between taro cultivars.

Table 2.2 Effects of various media on callus regeneration

Medium	Phytohormone (mg L^{-1})	Callus growth	Growth rate	Color	Texture	Regeneration ability
M5	BA(2)+NAA(1)	Good	Fast	Yellowish-white	Soft	High
M23	BA(0.2)+2,4-D(0.5)	Good	Slow	Greenish-yellow	Compact	No
M24	BA(1)+2,4-D(3)	Good	Fast	Greenish-yellow	Compact	No
M25	BA(3)+2,4-D(3)	Good	Fast	Greenish-yellow	Compact	No
M35	Kinetin(1)+NAA (1.5)	Non-uniform	Fast	Yellowish-white	Soft	Low

* BA: benzyladenine; NAA: naphthaleneacetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid

Fig. 2.1a: Multiple shoots of cv. Bun Long
Fig. 2.1b: Multiple shoots of cv. Maui Lehua
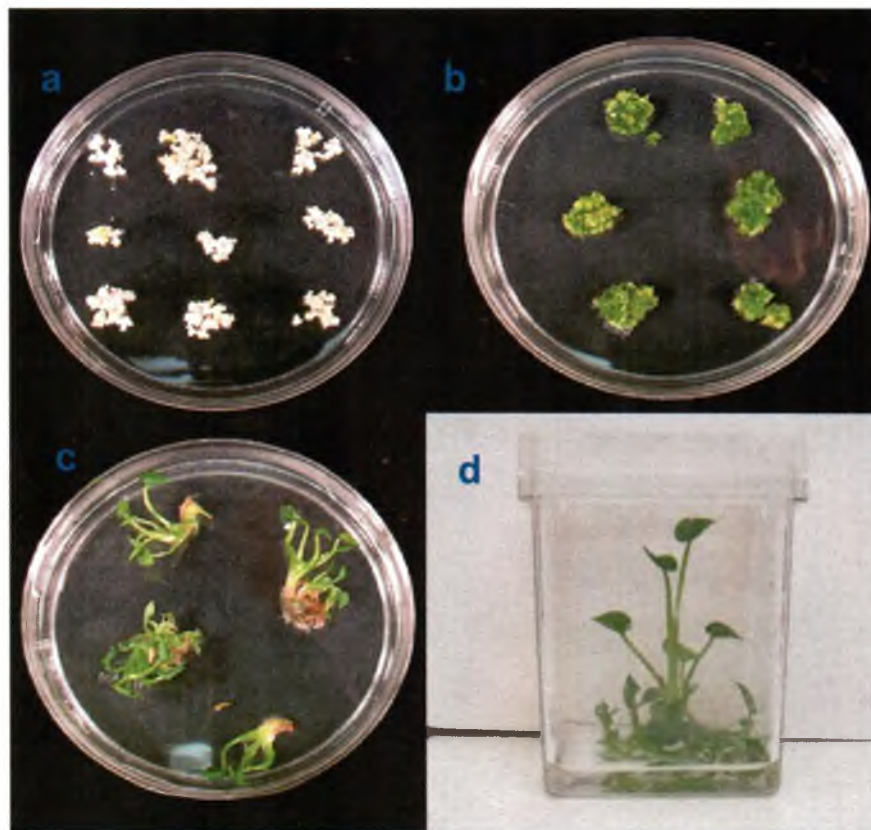
Fig. 2.2 Regeneration system of taro cv. Bun Long, **a** Highly regenerative calli induced on M5 medium. **b** Numerous shoot tips induced from calli alternating between M5 and M15 medium every two weeks. **c** Multiple shoots propagated on the solid M15 medium. **d** Multiple shoots propagated in the liquid M15 medium

Taro Bun Long corms

↓

Shoot tips excised under a dissecting microscope

↓

Surface sterilized and placed into liquid medium M15

↓

Multiple shoots induced and DsMV ELISA tested (2 months)

↓

Shoot tips excised from the DsMV free plantlet lines

↓

Highly regenerative calli induced on medium M5 (2 months)

↓

Shoots initiated from calli by alternating between M5 and M15 media (2 months)

↓

Shoots multiplied on M15 medium (3 months)

Fig. 2.3 The flow chart of the regeneration of taro cv. Bun Long

Table 2.3 **Effects of several media on shoot regeneration**

Media	Phytohormone $(mg\ L^{-1})$	Shoot quantity	Shoot size	Growth rate	Morphology
M15	BA(4)	Many	Medium	Fast	Normal
M16	BA(8)	Many	Large	Fast	Abnormal
M14	BA(2)	A few	Small	Slow	Normal

* BA: benzyladenine

Fig. 2.4 Multiplication of shoots of cv. Maui Lehua from shoot tip explants

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CHAPTER 3

TRANSFORMATION OF TARO WITH A RICE (*Oryza sativa* L.)

CHITINASE GENE

3.1 Literature review

The interaction of a plant and a pathogen can be designated as compatible (disease) or incompatible (no disease) (Lamb, 1994). No disease will occur when a plant that contains a specific resistance (R) gene recognizes a pathogen that contains the corresponding avirulence (Avr) gene (Staskawicz et al., 1995). Plant R genes mediate a plant's detection of pathogens (Keen, 1990), which could lead to a hypersensitive response (HR) in the plant (Dangl et al., 1996). This HR response in some cases is thought to trigger a nonspecific defensive response referred to as systemic acquired resistance (SAR) (Staskawicz et al., 1995). The SAR proteins produced in plants and associated with resistance belong to a group of proteins called pathogenesis-related (PR) proteins (Ryals et al., 1996). In response to infection of plants by pathogens and to other environmental stresses, plants are known to produce PR proteins and subsequently exhibit resistance to further pathogenesis (Linthorst, 1991).

Among the PR-proteins, chitinases (EC 3.2.1.14) have been among the most studied to clarify their roles in plant disease resistance. They have been found in over 41 monocotyledonous and dicotyledonous plants (Punja and Zhang, 1993). Various biotic factors including attack by pathogens (e.g. fungi, bacteria, and viruses) and pests, or abiotic factors (e.g. "stress" ethylene, or mechanical damage) can induce the production of chitinase (Clarke et al., 1998; Collinge et al., 1993; Linthorst, 1991; Punja and Zhang, 1993). Chitinases catalyze the hydrolysis of chitin, a linear homopolymer of β-1, 4-linked N-acetylglucosamine residues (Boller et al., 1983; Gooday, 1990; Ren and West, 1992). Chitin is a major structural component in insect exoskeletons, crustacean shells, egg shells of nematodes, and the cell walls of many fungi including Zygomycetes (e.g. *Mucor*, *Rhizopus*),

Pyrenomycetes (e.g. the powdery mildews), Discomycetes (e.g. *Sclerotinia*), Hyphomycetes

(e.g. *Botrytis*, *Penicillium*, and *Trichoderma*), Agonomycetes (e.g. *Sclerotium* and

Rhizoctonia), Chitridiomycetes (e.g. *Chytridiym*), Hymenomycetes (e.g. *Heterobsidium*),

Hemiascomycetes (*Taphrina*) and Deuteromycetes (e.g. *Ascochyta*) (Gooday, 1990; Riccardo

and Muzzarelli, 1999; Ruiz-Herrera, 1992). In contrast, no chitin has been found in higher

plants (Riccardo and Muzzarelli, 1999; Ruiz-Herrera, 1992). These results suggest that

chitinases may have an anti-fungal role, and chitinase genes may be outstanding candidate

genes for plant engineering to enhance fungal resistance without adversely affecting other

important qualities of plants.

A wide range of organisms including viruses, bacteria, fungi, animals and plants

contain various chitinases that play important roles in physiology, development, and disease

resistance (Ajit et al., 2006; Ali et al., 2003; Boot et al., 2005; Collinge et al., 1993; Hoster et

al., 2005; Itoh et al., 2006; Li, 2006). Chitinases are classified into family 18 or family 19 of

glycosyl hydrolases according to their amino acid sequences and their three-dimensional

structure (Henrissat, 1999).

In addition, plant chitinases can be categorized as acidic or basic, depending on their

isoelectric point. Acidic chitinases have been hypothesized to release cell wall fragments

from invading fungi during early stages of pathogenesis, which in turn may activate other

defense-related mechanisms (Bishop et al., 2000; Graham and Sticklen, 1994). Basic

chitinases are usually localized to vacuoles, and may affect the fungal hyphae following host

cell collapse when vacuolar contents are released into extracellular spaces (Boller, 1987;

Mauch and Staehelin, 1989).

Plant chitinases also can be classified as class I, II, III, IV, V or VI based on their

amino acid sequences (Brunner et al., 1998; Hamel et al., 1997; Margis et al., 1994; Melchers

et al., 1994; Nielsen et al., 1994; Sahai and Manocha, 1993). Class I chitinases are generally

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basic enzymes which contain two structural domains: a cysteine-rich amino terminal domain with chitin binding properties and a highly conserved chitinolytic catalytic domain (Collinge et al., 1993; Graham and Sticklen, 1994). Class II chitinases are generally acidic enzymes that have a high degree of sequence homology within the catalytic domain with class I chitinases but lack a chitin-binding domain (Collinge et al., 1993; Sahai and Manocha, 1993). Class III chitinases can be acidic or basic, and have no significant homology to class I chitinases and lack the chitin-binding domain (Sahai and Manocha, 1993). Classes IV, V, and VI chitinases have been reported to have significant homology to PR-4, stinging nettle (*Urtica dioica* L.) lectin, and bacterial exochitinase (Meins et al., 1994). The chitin binding domain that is found in class I chitinases is not necessary for catalytic and antifungal activities, but it can enhance fungal resistance function (Iseli et al., 1993). Therefore, among these classes, class I chitinases in general have the highest specific activities and strongest disease resistance abilities (Graham and Sticklen, 1994; Iseli et al., 1993; Sela-Buurlage et al., 1993; Truong et al., 2003).

Plant chitinases in class I, II, or IV and some chitinases in bacteria and fungi belong to the chitinase family 19 (Collinge et al., 1993; Henrissat, 1999; Itoh et al., 2006; Truong et al., 2003). Plant chitinases in class III or V and most chitinases in bacteria, fungi, viruses, and animals belong to the chitinase family 18 (Collinge et al., 1993; Henrissat, 1999).

The evidence indicates that plant chitinases are directly or indirectly associated with plant disease resistance against a wide range of fungal pathogens. Specific chitinases were markedly increased when infection occurred with fungal, bacterial and viral pathogens (Joosten and De Wit, 1989; Khan, 2003; Metraux and Boller, 1986; Robert et al., 2002). Class I and Class III chitinases were shown to accumulate in grapevine (*Vitis vinifera* L.) berries and leaves when infected by fungal or bacterial pathogens (Robert et al., 2002). Recently, Khan (2003) reported that chitinase activity in strawberry (*Fragaria* x *ananassa*

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Duchesne ex Rozier) leaves was markedly increased when plants were treated with fungal spores. Various chitinases have exhibited their antifungal activity both *in vitro* and *in vivo* (Chai et al., 2002; Collinge et al., 1993; El-Katatny et al., 2001; Huynh et al., 1992; Iseli et al., 1993; Moore et al., 2004; Shenoy et al., 2006; Truong et al., 2003). For example, Truong (2003) reported four family 19 chitinases from rice conferred antifungal activity with the highest antifungal activity found in class I chitinases. Recently, Moore et al. (2004) reported that chitinase activity in the kernels of maize (*Zea mays* L. subsp. mays) genotype had major antifungal activity. Class II chitinase identified from the bulbs of the plant Indian squill (*Drimia indica* (Roxb.) Jessop) exhibited antifungal activity against fungal pathogens, *Fusarium oxysporum* and *Rhizoctonia solani*, *in vitro* (Shenoy et al., 2006). Recently, Ajit et al. (2006) reported that the chitinases from the plant-growth-promoting rhizobacteria fluorescent pseudomonads exhibited antifungal activity *in vitro* by significantly inhibiting the mycelial growth of the fungal pathogen *Fusarium oxysporum* that causes carnation wilt.

Although cell walls of oomycetes, *Phytophthora* sp. and *Pythium* sp., do not contain chitin, zoospore germination was inhibited by chitinases (Young and Hwang, 1996). In addition, Carlson (2003) recently found that specific chitinase activity was involved in the tolerance to *Phytophthora* root rot (PRR) in soybean (*Glycine max* L. Merr).

Chitinase genes from various plants, bacteria (e.g. *Serratia marcescens* and *Streptomyces griseus*) and fungi (e.g. *Rhizopus oligosporus* and *Trichoderma harzionum*) have been transformed and expressed in many transgenic plants. Examples include apple (*Malus domestica* Borkh) (Bolar et al., 1997; Bolar et al., 2001), cacao (*Theobroma cacao* L.) (Maximova et al., 2006; Maximova et al., 2003), canola (*Brassica juncea*) (Broglie et al., 1991; Grison et al., 1996), carrot (*Daucus carota*) (Punja and Raharjo, 1996), cotton (*Gossypium hirsutum* L.) (Emani et al., 2003), creeping bentgrass (*Agrostis palustris* Huds) (Chai et al., 2002), cucumber (*Cucumis sativus*) (Kishimoto et al., 2002; Raharjo et al., 1996;

Tabei et al., 1998), grapevine (*Vitis vinifera* L.) (Yamamoto et al., 2000), papaya (*Carica papaya* L.) (Zhu et al., 1999), poplar (*Populus nigra*) (Boyle et al., 2005), potato (*Solanum tuberosum* L.) (Chang et al., 2002; Chye et al., 2005), rice (*Oryza sativa* L.) (Itoh et al., 2003; Kim et al., 2003; Kumar et al., 2003; Lin et al., 1995), rose (*Rosa gallica* L.) (Dohm et al., 2001), ryegrass (*Lolium multiflorum* Lam.) (Takahashi et al., 2005), silver birch (*Betula pendula*) (Pasonen et al., 2004), sorghum (*Sorghum bicolor* L.) (Liang et al., 2000), soybean (*Glycine max* L.) (Salehi et al., 2005), tobacco (*Nicotiana tabacum* L.) (Broglie et al., 1991; Carstens et al., 2003; Fung et al., 2002; Linthorst et al., 1990; Terakawa et al., 1997, Vierheilig et al., 1993; Zhu et al., 1994), tomato (*Lycopersicon esculentum*) (Van Den Elzen et al., 1993), and wheat (*Triticum aestivum* L.) (Anand et al., 2004; Anand et al., 2003; Chen et al., 1999; Wu et al., 2001).

Several transgenic plants with inserted chitinase genes have significantly increased resistance to fungal disease (Table 3.1). For example, transformed apple (*Malus domestica*) plants with the endochitinase and exochitinase genes of *Trichoderma atroviride* exhibited enhanced disease resistance against the pathogen, *Venturia inaequalis*, that causes apple scab disease (Bolar et al., 2001). Transgenic cacao (*Theobroma cacao*) with over-expression of a cacao class I chitinase showed significantly increased antifungal activity against the fungal pathogen *Colletotrichum gloeosporioides* (Maximova et al., 2006). In addition, canola (*Brassica juncea*) was transformed with a chitinase gene from tomato (*Lycopersicon esculentum*), and transgenic plant progeny showed a reduction of symptoms ranging from 23% to 79% against pathogens *Cylindrosporium concentricum*, *Phoma lingam*, and *Sclerotinia sclerotiorum*, under field conditions (Grison et al., 1996). Also, transgenic cotton (*Gossypium hirsutum*) expressing an endochitinase gene showed significant resistance to two pathogens, *Rhizoctonia solani* and *Alternaria alternata* (Emani et al., 2003).

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Kishimoto et al. (2002) developed a trangenic cucumber (*Cucumis sativus*) with a Class I chitinase cDNA (RCC2) of rice, and demonstrated increased disease resistance of this transgenic plant to gray mold (*Botrytis cinerea*). A class I chitinase, rice chitinase gene RCC2, was introduced into grapevine (*Vitis vinifera*); transgenic plants showed enhanced resistance against *Uncinula necator* (powdery mildew pathogen) by suppression of mycelial growth on the leaf surface. Also, transgenic grapevine showed a slight resistance against *Elisinoe ampelina* that induces anthracnose (Yamamoto et al., 2000). Recently, Boyle et al. (2005) reported that poplar (*Populus nigra*) transformed with an endochitinase gene of *Trichoderma harzianum* exhibited enhanced disease resistance against poplar fungal pathogens *Melampsora medusae* and *Melampsora laricipopulina*. Recently, a unique chitinase BjCHI1 from *Brassica juncea* with two chitin-binding domains was successfully expressed in potato (*Solanum tuberosum*) (Chye et al., 2005). This transgenic potato exhibited antifungal activity against the fungal pathogen *Rhizoctonia solani* (Chye et al., 2005).

Transgenic rice (*Oryza sativa*) with class I rice chitinase gene *CHI 11* showed increased resistance to *Rhizoctonia solani*, the rice sheath blight pathogen (Lin et al., 1995). Recently, Kumar et al. (2003) developed a simple and high throughput method for functional assessment of resistance against *Rhizoctonia solani*, and further confirmed that transgenic rice with the *CHI 11* gene exhibited enhanced fungal disease resistance. Kim et al. (2003) co-expressed a modified maize ribosome-inactivating protein gene (*MOD1*) and a rice basic chitinase gene (*RCH10*) in transgenic rice plants. Sheath blight disease severity was 25% lower in transgenic rice plants compared to controls; the authors suggested that *MOD1* and *RCH10* genes worked synergistically to protect against the fungal pathogen.

Recently, Takahashi et al. (2005) introduced the rice chitinase RCC2 gene into Italian ryegrass (*Lolium multiflorum*), and transgenic plants showed enhanced resistance to crown rust disease caused by the fungal pathogen *Puccinia coronata*. Pasonen et al. (2004) inserted

a sugar beet (*Beta vulgaris*) chitinase IV gene into silver birch (*Betula pendula*); the transgenic plants exhibited enhanced resistance to birch rust caused by the fungal pathogen *Melampsoridium betulinum*. Recently, Salehi et al. (2005) successfully transformed soybean (*Glycine max*) with the bean chitinase gene *CHN*, and they reported that transgenic plants exhibited enhanced fungal resistance against *Rhizoctonia solani* that causes root rot disease.

Zhu et al. (1994) developed transgenic tobacco (*Nicotiana tabacum*) plants that constitutively expressed a basic chitinase from rice and demonstrated enhanced resistance against *Cercospora nicotiana*. Carstens et al. (2003) transformed tobacco with a chitinase gene from yeast (*Saccharomyce cerevisiae*). This chitinase inhibited *Botrytis cinerea* spore germination and hyphal growth by up to 70% in a quantitative *in vitro* assay (Carstens et al., 2003). Zhu et al. (1999) reported that the protein extracts of papaya (*Carica papaya*) transformed with a rice chitinase gene completely inhibited mycelial growth of *Phytophthora palmivora* during *in vitro* assays.

Apparently, transformation of various plants with a chitinase gene is an effective method for enhanced resistance against a broad range of fungal pathogens. In this study, a class I rice chitinase gene *RICCHI11* has been transformed into taro cv. Bun Long.

3.2 Transformation via particle bombardment

3.2.1 Introduction

Various plant transformation methods have been developed successfully to improve the quality, yield, disease resistance, and other agronomic factors in a wide range of plant species (Taylor and Fauquet, 2002; Veluthambi et al., 2003). These transformation methods can be divided into two major methods: a) *Agrobacterium*-mediated transformation; and b) direct DNA delivery transformation. Of the direct gene transformation methods, particle bombardment (also called microprojectile bombardment or biolistic) transformation is the

most widely used and developed tool to date (Taylor and Fauquet, 2002; Veluthambi et al.,

2003). The major advantage of particle bombardment transformation is that there is no

Table 3.1 Plants transformed with a chitinase gene exhibited enhanced disease resistance in the following literature

Transgenic plants	Sources and types of transgene	Diseases (pathogens) defended against	References
Apple (*Malus domestica*)	Endochitinase and exochitinase of *Trichoderma atroviride*	Apple scab (*Venturia inaequalis*)	Bolar et al., 2001
Cacao (*Theobroma cacao*)	Cacao class I chitinase gene, *TcChi1*	(*Colletotrichum gloeosporioides*)	Maximova et al., 2006
Canola (*Brassica juncea*)	Bean chitinase gene, *CH5B*	root rot (*Rhizoctonia solani*)	Broglie et al., 1991
Canola (*Brassica juncea*)	Tomato chitinase gene	(*Cylindrosporium concentricum*), (*Phoma lingam*), and (*Sclerotinia sclerotiorum*)	Grison et al., 1996
Cotton (*Gossypium hirsutum*)	Endochitinase gene from *Trichoderma virens*	(*Rhizoctonia solani*) and (*Alternaria alternate*)	Emani et al., 2003
Creeping bentgrass (*Agrostis palustris*)	Elm chitinase-like gene, *hs2*	Brown patch disease (*Rhizoctonia solani*)	Chai et al., 2002

Table 3.1 Plants transformed with a chitinase gene exhibited enhanced disease resistance in the following literature (Continued)

Transgenic plants	Sources and types of transgene	Diseases (pathogens) defended against	References
Cucumber (*Cucumis sativus*)	Class I rice chitinase gene, *RCC2*	Gray mold (*Botrytis cinerea*)	Tabei et al., 1998
Cucumber (*Cucumis sativus*)	Class I rice chitinase gene, *RCC2*	Gray mold (*Botrytis cinerea*)	Kishimoto et al., 2002
Grapevine (*Vitis vinifera*)	Class I rice chitinase gene, *RCC2*	Powdery mildew (*Uncinula necator*) and anthracnose (*Elisinoe ampelina*)	Yamamoto et al., 2000
Poplar (*Populus nigra*)	Endochitinase gene of *Trichoderma harzianum*, *ech42*	(*Melampsora medusae*) and (*Melampsora larici-populina*)	Boyle et al., 2005
Potato (*Solanum tuberosum*)	*Brassica juncea* chitinase gene, *BjCHI1*	Rhizoctonia canker (*Rhizoctonia solani*)	Chye et al., 2005
Rice (*Oryza sativa*)	Class I rice chitinase gene, *CHI 11*	Rice sheath blight (*Rhizoctonia solani*)	Lin et al., 1995
Rice (*Oryza sativa*)	Family 19 chitinase gene of *Streptomyces griseus*, *ChiC*	Leaf blast (*Magnaporthe grisea*)	Itoh et al., 2003

Table 3.1 Plants transformed with a chitinase gene exhibited enhanced disease resistance in the following literature (Continued)

Transgenic plants	Sources and types of transgene	Diseases (pathogens) defended against	References
Rice (*Oryza sativa*)	Rice basic chitinase gene, *RCH10*	Rice sheath blight (*Rhizoctonia solani*)	Kim et al., 2003
Rice (*Oryza sativa*)	Class I rice chitinase gene, *CHI 11*	Rice sheath blight (*Rhizoctonia solani*)	Kumar et al., 2003
Ryegrass (*Lolium multiflorum*)	Class I rice chitinase gene, *RCC2*	Crown rust disease (*Puccinia coronata*)	Takahashi et al., 2005
Silver birch (*Betula pendula*)	Sugar beet chitinase IV gene	Birch rust (*Melampsoridium betulinum*)	Pasonen et al., 2004
Sorghum (*Sorghum bicolor*)	Rice chitinase gene, *G11*	Sorghum stalk rot (*Fusarium thapsinum*)	Liang et al., 2000
Soybean (*Glycine max*)	Bean chitinase gene, *CHN*	Soybean root rot (*Rhizoctonia solani*)	Salehi et al., 2005

Table 3.1 Plants transformed with a chitinase gene exhibited enhanced disease resistance in the following literature (Continued)

Transgenic plants	Sources and types of transgene	Diseases (pathogens) defended against	References
Tobacco (*Nicotiana tabacum*)	Bean chitinase gene, *CH5B*	Seedling blight and root rot (*Rhizoctonia solani*)	Broglie et al., 1991
Tobacco (*Nicotiana tabacum*)	Basic rice chitinase gene	(*Cercospora nicotiana*)	Zhu et al., 1994
Tobacco (*Nicotiana tabacum*)	*Brassica juncea* chitinase gene, *BjCHI1*	(*Trichoderma viride*)	Fung et al., 2002
Tobacco (*Nicotiana tabacum*)	Yeast *Saccharomyces cerevisiae* chitinase gene, *CTS1-2*	(*Botrytis cinerea*)	Carstens et al., 2003
Wheat (*Triticum aestivum*)	Class IV acidic chitinase	Fusarium head blight (*Fusurium graminearum*)	Anand et al., 2004

incompatibility problem as occurs between *Agrobacterium* and certain plant species. Particle bombardment transformation is the major alternative to *Agrobacterium*-mediated transformation when the transformation target is a monocotyledonous plant species, since *Agrobacterium* typically does not infect monocots (Taylor and Fauquet, 2002; Veluthambi et al., 2003). Recently many monocotyledonous plants have been transformed successfully by *Agrobacterium* due to improvements in strains, vectors, addition of a signal elicitor such as acetosyringone (AS), and co-cultivation time. However, particle bombardment transformation still is widely used in transformation of monocotyledonous plants (Taylor and Fauquet, 2002; Veluthambi et al., 2003).

Taro is a monocotyledonous plant. Since the only report on transformation of taro in the literature used particle bombardment (Fukino et al., 2000), we used this method to transform taro with a rice chitinase gene (He et al., 2004).

3.2.2 Materials and methods

3.2.2.1 Plant material

Taro cultivar Bun Long was chosen, because earlier (Chapter 2) we developed methods to produce highly regenerative calli of this cultivar. These highly regenerative calli were selected as the target of particle bombardment.

3.2.2.2 Plasmid construct

The plasmid pBI121/*RICCHI11* (Fig. 3.1) contains: a) the rice chitinase gene, *RICCHI11*, driven by the cauliflower mosaic virus (*CaMV*) 35S promoter; b) the selection gene neomycin phosphotransferase II (*NPT* II); and c) the reporter gene, ß-glucuronidase (*GUS*). The pBI121 plasmid was obtained from Clontech (Mountain View, CA. http://www.clontech.com). The rice chitinase gene, *RICCHI11*, was obtained from Dr. S. Muthukrishnan at Kansas State University.
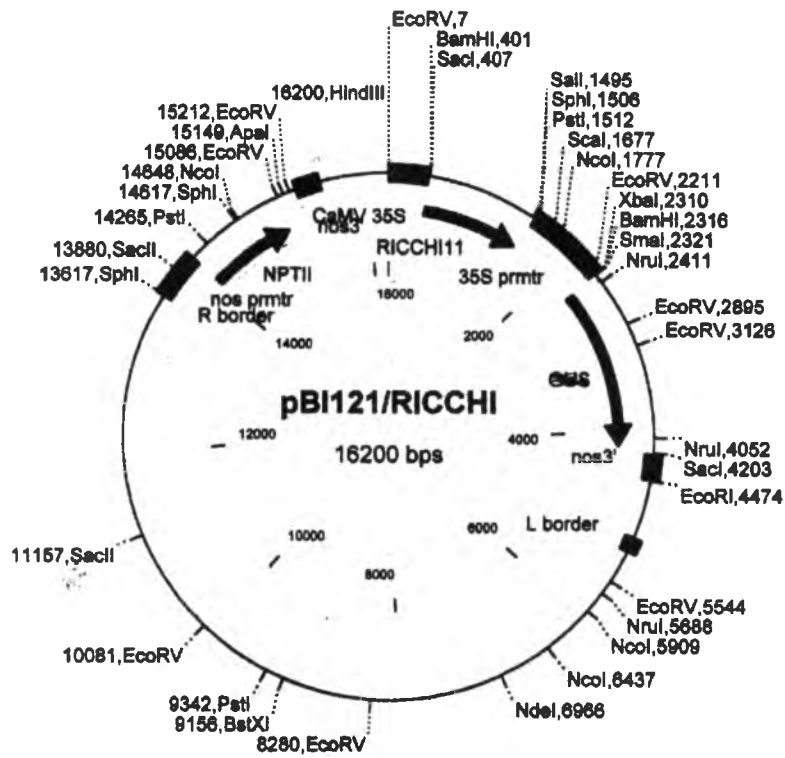
Fig 3.1. Map of the transformation plasmid pBI121/*RICCHI11*

3.2.2.3 Optimization of geneticin concentration

The toxin geneticin (G418) is used to kill all non-transformed plant cells, leaving only cells transformed with the selection gene *NPTII*. To determine the optimal concentration of geneticin on non-transformed taro callus, a total of 135 calli were placed on the M5 mediim (Chapter 2, Table 2.2) containing 0, 25, 50, 100 and 150 mg L^{-1} G418 (9 calli per plate, and 27 calli per concentration). After 30 days of culture, the surviving calli were subcultured onto the fresh medium containing 50 mg L^{-1} G418. After another 30 days of culture, the surviving calli were subcultured onto the fresh medium containing 50 mg L^{-1} G418. Finally, after 30 days of further culture, callus survival was examined.

3.2.2.4 Particle bombardment

Transformation of cv. Bun Long callus through particle bombardment was similar to the method of Fukino et al. (2000). Soft yellowish-white calli that were grown on M5 medium (Chapter 2, Table 2.2) were selected for particle bombardment. These calli (approximately 2 g) were cut into small pieces and cultured in the center of a petri-dish containing solid M5 medium at 25 °C for 24h (Fig. 3.2). Plasmids were coated onto 1.6 μm gold particles (Bio-Rad, Hercules, Calif.). Biolistic bombardment equipment (PDS-1000/He, Bio-Rad, Hercules, Calif.) was used to insert plasmids into calli at a pressure of 1100 psi. A total of 3 bombardments per plate were made for 30 plates. After bombardment, the calli were cultured on the same plates for 2 days at 25 °C, then cut into small pieces (approximately 45 pieces) and transferred to fresh media (approximately 9 pieces per plate) without antibiotics for 4 days at 25 °C. Selection of transformed lines was conducted in the presence of 50 mg L^{-1} geneticin (G418) subcultured once every month.

3.2.2.5 PCR (Polymerase Chain Reaction) analysis

To determine the presence of the transgene, genomic DNA was extracted from approximately 100 mg of callus or leaf tissues using the modified CTAB (hexadecyltrimethylammonium bromide) method described by Murray and Thompson (1980). The primer pair used to amplify a 540-bp fragment of the integrated RICCHI11 gene was: G11U (21 mer) 5'-CCGCGCTAAGGGCTTCTACAC-3' and G11L (20 mer) 5'-CACTCCACACCGCCGTTGAT-3'). The PCR reactions were performed in 50 μl volume consisting of 1 μl template DNA (20-30 ng), 2 μl of each primer (20 μmol), 5 μl dNTPs (2 mM), 5 μl 10x Taq buffer (Promega, Madison, WI), 1 unit Taq polymerase (Promega, Madison, WI), and 36.5 μl H_2O. Amplification of RICCHI11 fragments was performed for 30 cycles at 94 °C for 30 sec, 55 °C for 45 sec and 72 °C for 45 sec, for denaturing, annealing and primer extension, respectively. Primers and PCR conditions are summarized in Table 3.2.

3.2.2.6 Southern blot analysis

To further confirm the presence of the transgene, genomic DNA was isolated from fresh leaf tissue according to the CTAB method of Murray and Thompson (1980) as modified by Y. Suzuki (personal communication). Thirty μg of genomic DNA were digested overnight with the restriction enzyme *Bam*HI to release a 1.9kb fragment containing the *RICCHI11* gene which was flanked by two *Bam*HI sites. Digested DNA was fractionated by electrophoresis in a 1% agarose gel, then alkali-blotted onto a Hybond N + membrane (Amersham, UK) according to the manufacturer's instructions. The PCR amplification product (a 540-bp fragment of the integrated *RICCHI11* gene) was labeled with AlkPhos Direct (Amersham, UK) according to the manufacturer's instructions, and used as a chemifluorescent probe. Hybridization and detection were performed according to the manufacturer's protocol.

3.2.3 Results

3.2.3.1 Optimization of geneticin concentration

The soft, yellowish-white calli that were induced on M5 medium were selected for further study based on their ability to regenerate shoots. These non-transformed calli were used in this experiment on optimization of geneticin concentration. None of the non-transformed

Fig. 3.2 Target calli for particle bombardment

Table 3.2 Primers and PCR conditions used in amplification of the rice chitinase gene *RICCHI11*

Primer ID and sequences	PCR conditions
G11U: 5'-CCGCGCTAAGGGCTTCTACAC-3' G11L: 5'-CACTCCACACCGCCGTTGAT-3'	94 °C 3 min (94 °C 30 sec, 55 °C 45 sec, 72 °C 45 sec)x30 72 °C 5 min

calli placed on M5 medium with 50 mg L^{-1} G418 (subcultured every 30 days) survived after 90 days of selection (Table 3.3), indicating that growth of non-transformed cells was effectively suppressed. Based on these results, bombarded calli were grown for 90 days on 50 mg L^{-1} G418 with subculturing every 30 days. A total of 30 pieces of calli (approximately 2% of the original calli) survived and grew well after selection.

3.2.3.2 PCR analysis

Genomic DNA was extracted from these 30 surviving calli for PCR screening using primers specific for the *RICCHI11* gene. Two PCR-positive lines from these 30 lines showed the unique 540bp *RICCHI11* gene fragment (Fig. 3.3). Both callus lines were transferred to shoot multiplication media. Genomic DNA was extracted from these shoot lines for PCR analysis. Only one shoot line had a PCR-positive result of the 540 bp RICCHI11 gene fragment (Fig. 3.3), indicating a successful transformation rate of <0.1%.

3.2.3.3 Southern blot analysis

Genomic DNA from shoots derived from these two lines was analyzed by Southern blot hybridization. Digestion of the extracted DNA with the restriction enzyme *Bam*HI released a 1.9-kb fragment with homology to the *RICCHI11* gene from the transformation plasmid pBI121/RICCHI11 and PCR-positive line-1 plants. No gene fragment was observed in the non-transformed control plants or the PCR-negative shoot line-2 plants (Fig. 3.4).

3.2.4 Discussion

In the optimization of geneticin concentration, the growth of non-transformed taro calli showed a clear suppression at a concentration of 50 mg L^{-1}. Over 20 bombarded calli survived on geneticin 50 mg L^{-1}, but only two callus lines showed positive PCR results, and only one of these transformant lines showed positive PCR results in the induced shoots. These results indicated that at the callus level, some transformants were chimeric, escaping from selection stress but then reverting back to a non-transformed state.

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To confirm that the *RICHI11* gene had been successfully introduced as intact into the plant genome, both PCR and Southern blot analyses were conducted on plants. Since transformation efficiency of biolistic bombardment was very low, <0.1% (only one transgenic line was obtained from 45 x 30 = 1350 calli). *Agrobacterium*-mediated transformation was initiated.

To our best knowledge, this is the first report of the transformation of taro with an anti-fungal gene, the rice chitinase gene *RICHI11*. Similar to the only other report on transformation of taro (Fukino et al. 2000), we used a particle bombardment method, and confirmed its low transformation efficiency. However, Fukino et al. (2000) only transformed taro with a GUS reporter gene, whereas we have transformed taro with a disease-resistance gene. More detailed studies need to be conducted to enhance the efficiency of genetic transformation and to produce more lines of taro transformed with the rice chitinase gene.

3.3 *Agrobacterium*-mediated transformation

3.3.1 Introduction

Agrobacterium-mediated transformation transfers a segment of DNA (T-DNA) from *Agrobacterium* and integrates it into the target plant genome. The tumor-inducing (Ti) plasmid in the *Agrobacterium* contains T-DNA, that is delimited by right and left borders (25 bp direct repeats), and the virulence (*vir*) genes. Expression of *vir* genes is induced by phenolics (e.g., acetosyringone) or sugars. Products of the *vir* genes generate the transfer of an intermediate T-complex which is comprised of a single-strand copy of T-DNA (T-strand) covalently bonded to two Vir proteins. This *vir* system will process and transfer the T-complex from the bacterium into the plant. Inside the plant cell, the T-complex is imported into the nucleus, where the T-strand is stably integrated into the plant chromosome (Veluthambi et al., 2003; Zupan et al., 2000).

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Table 3.3 Effect of G418 concentration on the growth of non-transformed taro callus

Concentration of G418 (mg L-1)	Surviving calli (%) after 30 days	Surviving calli (%) after 60 days	Surviving calli (%) after 90 days
0	100	--	--
25	48.1	25.9	7.4
50	33.3	7.4	0
100	18.5	0	0
150	0	--	--
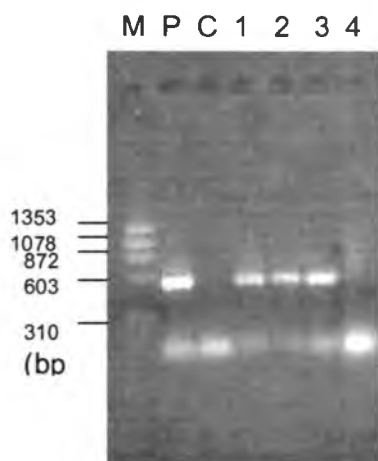
Fig. 3.3 Agarose gel electrophoresis showing PCR amplification product of the *RICCHI11* **unique 540bp fragment**

Lanes: **M** Molecular size marker, **P** plasmid control, **C** non-transformed callus control, **1-2** two transformed taro cv. Bun Long callus lines (**1**-Line 1: T1 callus, **2**-Line 2: T2 callus), **3-4** two shoot lines respectively derived from T1 and T2 callus (**3**-Line 1 shoot derived from T1 callus, **4**-Line 2 shoot derived from T2 callus)
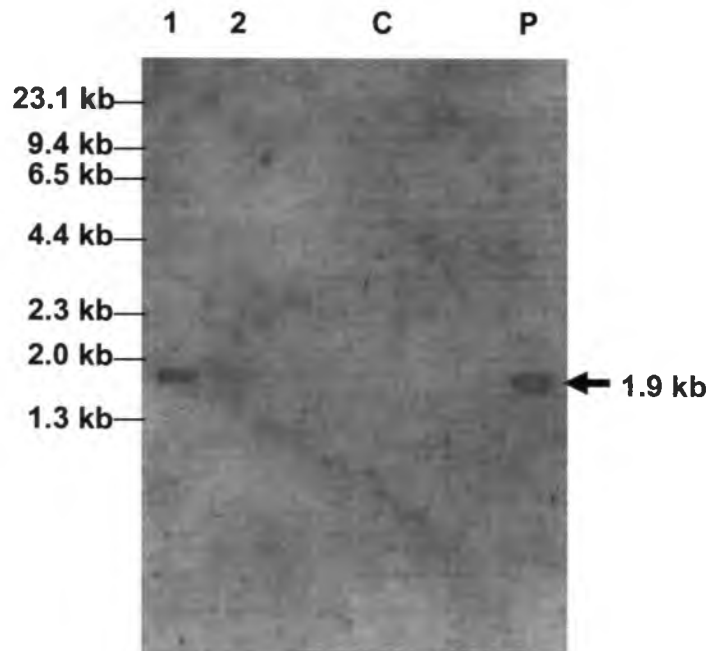
Fig. 3.4 Southern blot analysis of *Bam*HI-digested genomic DNA extracted from two transformed taro cv. Bun Long shoot lines that were analyzed by PCR amplification

Lane: **M** Molecular markers **1** Digestion of the extracted DNA with restriction enzyme *Bam*HI released a 1.9-kb fragment with homology to the *RICCHI11* gene from the PCR-positive T1 plants. **2** PCR-negative T2 plants. **C** non-transformed control taro plant. **P** plasmid pBI121/*RICCHI11* DNA.

A range of transformation *Agrobacterium* strains and binary transformation vectors have been developed. *Agrobacterium* strains can be divided into types based on opines (i.e. amino-acid like compounds) that each strain is able to catabolize. The enzymes for catabolism (break-down) of specific opines are encoded on the Ti-plasmid and complement the enzymes for opine biosynthesis that are encoded on the T-DNA (Zupan et al., 2000). Examples of strains are: octopine-type *vir* helper strains, nopaline-type *vir* helper strains, and L, L-succinamopine-type *vir* helper strain (Veluthambi et al., 2003). The commonly used strain LBA4404 is an octopine-type. Other commonly used strains, EHA101 and EHA105, belong to the L,L-succinamopine-type *vir* helper strain that harbour the so-called 'supervirulent' *vir* genes. As a result of these 'supervirulent' *vir* genes, EHA101 and EHA105 strains generally can infect a broader range of host plants and result in a greater transformation efficiency (Veluthambi et al., 2003).

Agrobacterium-mediated transformation has many unique advantages over particle bombardment transformation. It is simpler and less expensive than particle bombardment transformation (Veluthambi et al., 2003). In addition, *Agrobacterium*-mediated transformation results in a single copy or a low copy number of the transgene, resulting in lower incidences of gene rearrangement and gene silencing (Luo et al., 2004; Veluthambi et al., 2003). In contrast, particle bombardment transformation usually results in insertion of multiple copies of the transgene into plant genomes resulting in greater risks of gene silencing (Luo et al., 2004; Veluthambi et al., 2003).

Formerly, a major limitation of *Agrobacterium*-mediated transformation stemmed from the fact that most monocotyledonous plants are not natural hosts of *Agrobacterium* and do not secrete the molecular signal phenolics, such as acetosyringone, that are needed to induce *vir* genes (Veluthambi et al., 2003). However, acetosyringone can be applied in the transformation system (Veluthambi et al., 2003). Also, more efficient *Agrobacterium* strains,

transformation vectors and transformation conditions have been developed (Veluthambi et al., 2003). To date, a wide range of monocotyledonous plants including rice, maize, wheat, barley have been successfully transformed by *Agrobacterium*-mediated transformation (Luo et al., 2004; Veluthambi et al., 2003).

In this study, first, we compared the efficiency of *Agrobacterium*-mediated transformation of taro using LBA4404 and EHA105 that harbor the binary vector pCNL65 which contains the intron β-glucuronidase (*gus*) reporter gene and the *nptII* selection gene. Then, we selected the more efficient strain to transform taro with the rice chitinase gene *RICCHI11*.

3.3.2 Materials and methods

3.3.2.1 Plant materials

Taro cultivar Bun Long was chosen as the plant transformation material because highly regenerative calli of this cultivar have been developed. The highly regenerative calli were selected as the transformation materials of *Agrobacterium*-mediated transformation.

3.3.2.2 Plasmids and *Agrobacterium* strains

The plasmid pCNL65 and *Agrobacterium* strains LBA4404 and EHA105 were used to test the efficiency of *Agrobacterium*-mediated transformation of taro cv. Bun Long. This plasmid carried *gus* reporter gene with an intron driven by the CaMV 35S promoter and a *nptII* selection gene. The intron in *gus* gene can eliminate leaky expression of *gus* gene in *Agrobacterium* because *Agrobacterium* lacks mRNA splicing. Later, the plasmid pBI121/*RICCHI11* and the *Agrobacterium* strain EHA105 were used to introduce the *RICCHI11* gene into taro.

3.3.2.3 Transformation of *Agrobacterium* strain EHA105 with the plasmid pBI121/*RICCHI11*

The plasmid pBI121/*RICCHI11* was transformed into the *Agrobacterium* strain EHA105 using the freezing and thawing method (Holsters et al., 1978). A single colony of *Agrobacterium* strain EHA105 was selected and cultured overnight in 5 ml YEB medium (5 g L^{-1} tryptone, 1 g L^{-1} yeast extract, 5 g L^{-1} beef extract, 4 ml of 0.5M MgSO4, sucrose 5 g L^{-1}, pH 7.0-7.2) containing 25 µg mL^{-1} rifampicin at 250rpm and 28 °C. Then 2 mL of this culture was added to 50 mL YEB medium and cultured for another 4-5 hrs until the OD$_{600}$ value reached 0.5-1.0. The cultured cells were then chilled on ice, centrifuged and re-suspended in 1 mL of ice-cold 20mM CaCl$_2$ solution. Approximately 1 µg of plasmid DNA was added into 100 µL of these ice-cold cells and then frozen in liquid nitrogen for 2 minutes. The frozen cells were thawed at 37 °C for 5 min. Then 1 mL of YEB medium was added to the cells and cultured for another 2-4 hr. The cells were spread on a YEB agar plate containing 50 µg mL^{-1} kanamycin and 25 µg mL^{-1} rifampicin. The plate was incubated at 28 °C, and transformed colonies appeared after 2-3 days.

3.3.2.4 Verification of EHA105:pBI121/RICCHI11 by enzyme digest and PCR analysis

To verify the transformation of EHA105 with pBI121/*RICCHI11* using this freezing and thawing method, the plasmid pBI121/RICCHI11was extracted from *Escherichia coli* strain DH5α and *Agrobacterium* strain EHA105 using a DNA miniprep kit (Invitrogen, San Diego, CA). Then, DNA was digested with the enzyme *BamH*I to release a 1.9 kb fragment containing the *RICCHI11* gene which is flanked by two *Bam*HI sites. The PCR primers, reaction system and conditions were described earlier in the section on particle bombardment transformation (Chapter 3.2.2.5), with the exception of a lower concentration of DNA

template (approximately 200 pg). Gel electrophoresis (0.8% agarose) was performed to verify the sizes of the digested DNA and PCR products.

3.3.2.5 *Agrobacterium*-mediated transformation method

Agrobacterium-mediated transformation of taro cv. Bun Long with the RICCHI11 gene or intron GUS gene was modified from the method of Chen and Kuehnle (1996) for anthurium. A single colony of the *Agrobacterium* strain (either EHA105 or LBA4404) carrying a binary vector with the selection gene *nptII* (for geneticin and kanamycin resistance) was selected and cultured in 3 mL YEB medium containing 50 μg mL^{-1} kanamycin and 25 μg mL^{-1} streptomycin (for LBA4404) or rifampicin (for EHA105). The medium was shaken at 250 rpm and 28 °C for 1-2 nights until the bacterial suspensionS became turbid (OD$_{600}$ = 0.5-1.0). The 2 μL 0.3 *M* acetosyringone (AS) was added to the turbid bacterial suspension and mixed well. The bacteria-containing AS suspension was diluted tenfold with YEB liquid medium (final concentration of 20 μ*M* AS). Calli or shoots of cv. Bun Long were cut, immersed in the *Agrobacterium* suspension for approximately 10 minutes, then transferred to the co-cultivation medium (hormone-free MS) and co-cultivated with *Agrobacterium* for four days. The co-cultivated calli or shoots were transferred onto a M5 or M15 medium containing 50 mg L^{-1} geneticin and 250 mg L^{-1} cefotaxime or 500 mg L^{-1} carbenicillin and cultured for 60 to 90 days.

Explants were transferred monthly onto fresh selection medium, and examined at least once a week for regrowth of *Agrobacterium*. The explants were rinsed with sterile water and cultures with over-grown *Agrobacterium* were discarded or subcultured in an attempt to prevent the regrowth of *Agrobacterium*. Healthy, light yellow and green calli were selected for shoot and root induction on M15 selection medium with 50 mg L^{-1} geneticin and 250 mg L^{-1} cefotaxime or 500 mg L^{-1} carbenicillin. Induced multiple shoots were sub-cultured on

M15 selection medium with 50 mg L^{-1} geneticin and 125 mg L^{-1} cefotaxime or 250 mg L^{-1} carbenicillin. When 2-3 leaves emerged, the geneticin level was reduced to 35 mg L^{-1}. Geneticin-resistant plantlets were transferred into Magenta boxes with liquid M15 selection medium. Leaves were analyzed for the presence of the transgene by amplification in PCR analysis and southern blotting at this stage.

3.3.2.6 Selection using geneticin (G418)

For selection of transformed lines, co-cultivated calli were grown for 90 days on 50 mg L^{-1} G418 with subculturing every 30 days. In addition, 50 mg L^{-1} G418 was added to shoot-inducing media M15 for 90 days, and then G418 concentration was reduced to 35 mg L^{-1}.

3.3.2.7 Assay for presence of the *GUS* gene

The following protocol was used in the *Agrobacterium*-mediated transformation of taro cv. Bun Long with the *RICCHI11* gene or the intron *gus* gene. In a 96-well tissue culture plate, approximately 50% of the calli or shoot cuttings were tested for the presence of the *gus* gene using X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide) as a substrate. The plate was incubated for 1 to 2 hours at 37 °C. The calli or shoot cuttings were observed under the microscope for the blue spots that indicate the presence of GUS activity.

3.3.2.8 Comparison of strains EHA105 and LBA4404

Two *Agrobacterium* strains, EHA105 and LBA4404, were selected to test the efficiency of transformation. Both strains harbored the plasmid pCNL65. Thirty calli or 30 shoots were tested for each strain and experiment. Each agar plate contained 10 calli. After 1 week and 2 weeks of G418 selection, a GUS assay was conducted and the number of positive blue calli or shoots was counted.

3.3.2.9 Effect of co-cultivation time

Both *Agrobacterium* strains, EHA105 and LBA4404, were co-cultivated for varying times (2d, 4d, 6d). For each strain and experiment, 30 calli or 30 shoots were tested. Each selection plate contained 10 calli. After 1 week and 2 weeks of selection using G418, a GUS assay was conducted, and the number of blue calli or shoots was counted.

3.3.2.10 Effect of acetosyringone (AS)

Varying levels of acetosyringone (0 μM, 20 μM, 60 μM, 100 μM of AS) were respectively applied to transform calli with *Agrobacterium* strain LBA4404 and plasmid pCNL65. For each trial, 30 calli were tested. Each agar plate contained 10 calli. After 1 week and 4 weeks of selection using G418, a GUS assay was conducted, and the number of positive blue calli was counted.

3.3.2.11 Verification of presence of the *RICCHI11* gene using PCR analysis

Genomic DNA was extracted from approximately 30 mg (fresh weight) of induced shoot tissues using the modified sodium dodecyl sulfate (SDS) method described by Lin et al. (2001). The primers specific for the *RICCHI11* gene and the PCR reaction system and conditions were described previously in the section on particle bombardment transformation (Chapter 3.2.2.5).

3.3.2.12 Verification of transcription of the *RICCHI11* gene using reverse transcription-PCR (RT-PCR) analysis

Total RNA was isolated from approximately 10 mg of fresh shoot tissue according to a method described by Berendzen et al. (2005). Reverse transcription (RT) was conducted with oligo-dT primers and reverse transcriptase in the RT-PCR kit (Promega, Madison, WI). The reverse transcripts were used as templates for PCR. The PCR primers for the the *RICCHI11* gene and the PCR reaction system and conditions were described previously in the section on particle bombardment transformation (Chapter 3.2.2.5). As a control, PCR was

also conducted using a total RNA without reverse transcription to confirm that the PCR product was derived from mRNA and not from the contaminating DNA.

3.3.2.13 Verification of presence of the *RICCHI11* gene using Southern blot analysis

Genomic DNA was extracted from approximately 80-100 mg of fresh induced shoot tissues using the modified SDS method described by Lin et al. (2001). DNA (15 µg) was digested with the enzyme *Not*I that has a single digestion site in the T-DNA. The DNA blotting, probing and hybridization were described earlier in the section on particle bombardment transformation (Chapter 3.2.2.6).

3.3.3 Results

3.3.3.1 Verification of transgene in EHA105:pBI121/*RICCHI11* using an enzyme digest and PCR analysis

The expected 1.9kb band was found for the *BamH*I digested plasmid DNA extracted from both *E. coli* strain DH5α: pBI121/*RICCHI11* and *Agrobacterium* strain EHA105:pBI121/*RICCHI11* (Fig. 3.5). The expected 540bp PCR product was amplified in plasmid DNA extracted from both *E. coli* strain DH5α: pBI121/*RICCHI11* and *Agrobacterium* strain EHA105:pBI121/*RICCHI11* (Fig. 3.5). These results confirmed that the binary transformation plasmid pBI121/*RICCHI11* had been successfully transformed into *Agrobacterium* strain EHA105 (Fig. 3.5).

3.3.3.2 The effect of *Agrobacterium* strains, co-cultivation time and acetosyringone (AS) on *Agrobacterium*-mediated transformation

To obtain an effective *Agrobacterium*-mediated transformation system for taro, the effects of *Agrobacterium* strains, co-cultivation times, and acetosyringone levels were tested in preliminary studies using the plasmid pCNL65 containing the *gus* gene with an intron. Based on GUS transient expression results after 1 week and 2 weeks of selection on G418,

the supervirulent strain EHA105 appeared to be efficient for both callus transformation (Fig. 3.6) and shoot transformation (Fig. 3.7).

The highest transient expression level was achieved at 4 days of co-cultivation with *Agrobacterium* strain EHA105:pCNL65. At 4 days of co-cultivation, 28 co-cultivated calli tested positive for GUS after 1 week of selection using G418 out of a total of 30 tested co-cultivated calli, indicating a 93% transient expression level (Table 3.4). After 2 weeks of selection using G418, 18 out of 20 co-cultivated calli showed a positive GUS assay, indicating a 90% transient expression level. Non-transformed calli tested negative in the GUS assay (did not exhibit a blue color) (Fig. 3.6).

At 4 days of co-cultivation with strain EHA105:pCNL65, 20 co-cultivated shoots out of a total of 30 tested shoots were positive in the GUS assay after 1 week of selection using G418, indicating a 67% transient expression level (Table 3.4). At 4 days of co-cultivation, 10 out of 20 shoots showed a positive GUS assay after 2 weeks of selection using G418, indicating a 50% transient expression level. The non-transformed shoots were negative in the GUS assay (Fig. 3.7).

Results of the GUS assay of co-cultivated calli and shoots with *Agrobacterium* strain EHA105: pCNL65 are summarized in Table 3.4. Co-cultivation with *Agrobacterium* for 2 days and 6 days showed 75% and 80% transient expression level, respectively, after 2 weeks of selection using G418. However, there was a severe problem of overgrowth of *Agrobacterium* in the 6 day co-cultivation.

Transformation with *Agrobacterium* strain LBA 4404:pCNL65 showed no GUS activity at all. Number of co-cultivation days and acetosyringone concentration used in the transformation system did not alter this result. Therefore, *Agrobacterium* strain LBA4404 is considered ineffective for *Agrobacterium*-mediated transformation of taro using the current transformation protocols.

Based on these results, 4 days of co-cultivation with the supervirulent strain EHA105 and 20 μ*M* acetosyringone were selected for the highest transformation efficiency. Transformation of taro with the rice chitinase gene and wheat oxalate oxidase gene were conducted using these protocols.

3.3.3.3 Geneticin selection of taro transformed with *Agrobacterium* strain EHA105:pBI121/*RICCHI11*

For the first five transformation experiments, 500 calli of taro cv. Bun Long were used in total, with 100 calli per experiment. After 2 weeks of selection using G418, 120/150 calli were positive in the GUS assay, indicating an 80% transient expression level.
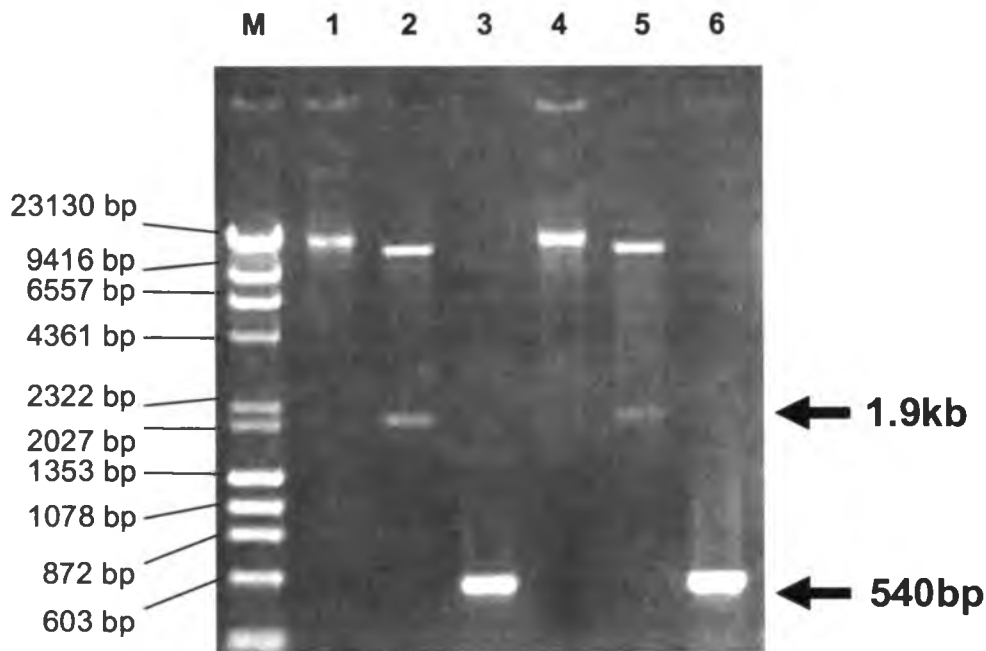
Fig. 3.5 Verification of pBI121/*RICCHI11* extracted from both *E. coli* and EHA105 by

enzyme digest and PCR analysis

M: molecular marker λ/HindIII and ΦX174/HaeIII mix

1: plasmid pBI121/*RICCHI11* extracted from *E. coli*

2: *BamH*I digest of pBI121/*RICCHI11* extracted from *E. coli* shows presence of 1.9kb

  chitinase gene

3: PCR analysis of pBI121/*RICCHI11* extracted from *E. coli* shows presence of 540bp

  chitinase fragment

4: pBI121/*RICCHI11*extracted from EHA105

5: *BamH*I digest of pBI121/*RICCHI11* extracted from EHA105 shows presence of 1.9kb

  chitinase gene

6: PCR analysis of pBI121/*RICCHI11* extracted from EHA105 shows presence of 540bp

  chitinase fragment

Non-co-cultivated calli
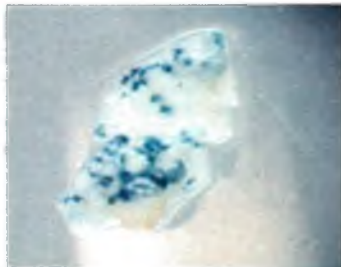
Co-cultivated calli
(at one week)
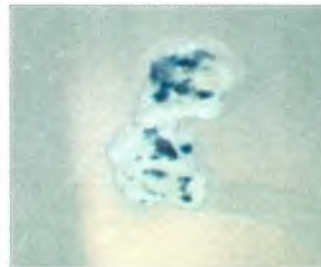
Co-cultivated calli
(at two weeks)

Fig 3.6 Results of the GUS assay of calli co-cultivated with *Agrobacterium* strain EHA105:pCNL65

Non-co-cultivated shoot

Co-cultivated shoot
(at one week)

Co-cultivated shoot
(at two weeks)

Fig. 3.7 Results of the GUS assay of shoots co-cultivated with *Agrobacterium* strain EHA105:pCNL65

Table 3.4 The GUS expression in calli and shoots co-cultivated with *Agrobacterium* strain
EHA105:pCNL65 at 1 or 2 weeks after a co-cultivation period of 2, 4, or 6 days

	GUS positive number/ Tested number (%)		
Calli	2days	4days	6days
1 week	25/30 (83%)	28/30 (93%)	27/30 (90%)
2 weeks	15/20 (75%)	18/20 (90%)	16/20 (80%)
Shoot			
1 week	12/30 (40%)	20/30 (67%)	12/30 (40%)
2 weeks	5/20 (25%)	10/20 (50%)	3/10 (30%)

However, after 4 weeks of selection using G418, most calli were overgrown by *Agrobacterium*. After 10 weeks of selection using G418, overgrowth by *Agrobacterium* killed all 500 calli of the calli in the five experiments. The antibiotic used to control the overgrowth of *Agrobacterium* was 500 mg L^{-1} carbenicillin. This problem of overgrowth of *Agrobacterium* could not be solved by washing with sterile water or 500 mg L^{-1} carbenicillin. In fact, washing with sterile water worsened the contamination problem. Using 250 mg L^{-1} cefotaxime instead of 500 mg L^{-1} carbenicillin controlled the overgrowth of *Agrobacterium*. In addition, pressing the calli into the medium appeared to eliminate the overgrowth problem with *Agrobacterium*.

An additional four transformation experiments were performed using the same transformation process and parameters as before with the exception that 250 mg L^{-1} cefotaxime was used in the selection medium. In total, 200 calli were co-cultivated with the super-virulent strain EHA105: pBI121/*RICCHI11*. Of these, 90 calli survived on the 50 mg L^{-1} G418 selection media after 90 days. Twenty independent lines were induced to produce shoots after transferring the calli to shoot-inducing media for 60 days (Fig. 3.8).

3.3.3.4 GUS assay in co-cultivated taro with EHA105: pBI121/*RICCHI11*

Forty co-cultivated calli were selected for the GUS assay after 1 week of selection on 50 mg L^{-1} G418. Of these calli, 30 were positive for GUS assay, indicating a 75% transient expression level at this stage (Fig. 3.9). Twenty independent shoot lines were induced from the calli and survived on the 50 mg L^{-1} G418 selection media. Of these 20 shoot lines, 10 lines showed a positive GUS assay, indicating a 50% transformation rate at this stage (Fig. 3.10).

3.3.3.5 Verification of presence and transcription of *RICCHI11* gene in transformed taro using PCR analysis and RT-PCR analysis

The expected 540 bp PCR product specific for the *RICCHI11* gene fragment was found from total DNA extracted from 6 independent taro cv. Bun Long shoot lines and the plasmid pBI121/*RICCHI11* positive control, indicating that the *RICCHI11* gene had been successfully transformed into these six lines (Fig. 3.11). Also, the expected 540 bp PCR product specific for the *RICCHI11* gene fragment was amplified in reverse-transcription of total RNA extracted from these 6 independent shoot lines (Fig. 3.12). No 540bp PCR product was obtained from the total DNA of the non-transformed control plant. Similarly, the reverse-transcription product of total RNA extracted from the non-transformed control plant did not result in the 540 bp PCR product.

3.3.3.6 Verification of the presence of the RICCHI11 gene in transformed taro using Southern blot analysis

*Not*I restriction digests of genomic DNA extracted from the six transgenic taro cv. Bun Long lines yielded various bands larger than the 1.9 kb of the *RICCHI11*. A single band was found in five transgenic lines, and two bands were found in one transgenic line (Fig. 3.13). Since *Not*I only has one digestion site in the T-DNA containing the *RICCHI11* gene, a single band in the southern blot indicated a single-copy transgene insertion into the taro genome and two bands indicated a two-copy insertion of the transgene. Therefore, five independent lines contained a single-copy insertion with no apparent gene rearrangement, and one independent line contained a two-copy insertion. In addition, the six different sized bands demonstrate that the transgene was integrated into different sites in the taro genome. This result confirmed the presence of the intact transgene *RICCHI11* into the taro genome.

3.3.4 Discussion

In preliminary studies, the GUS gene with an intron from Agrobacterium strain EHA105: pCNL65 was successfully introduced into taro cv. Bun Long calli and shoots with very high transient expression levels of 90% for calli and 50% for shoots (based on a GUS assay after two weeks of selection using G418). While the strain *A. tumefaciens* EHA105 resulted in high transient expression levels, in contrast, *Agrobacterium* strain LBA 4404 appeared to be ineffective.

Methods developed in these preliminary studies were used to transform taro cv. Bun Long with the rice chitinase gene *RICCHI11*. Analysis using PCR and RT-PCR confirmed the presence and expression of the *RICCHI11* gene in six transformed lines. Southern blot analysis of the six independent transformed lines indicated that five out of six (83%) had integrated a single copy of the transgene, and the other one line had two copies of the transgene. Six stable transgenic lines with the rice chitinase gene *RICCHI11* have been achieved via *Agrobacterium*-mediated transformation.

Compared to the particle bombardment transformation of taro, this *Agrobacterium*-method obtained a higher transformation efficiency (6/200=3%) vs the efficency of lower 0.1% for particle bombardment. In addition, this transformation via *Agrobacterium* may be more effective for transgene expression as a result of a single-copy or a low-copy insertion of the transgene. To our knowledge, this is the first report on *Agrobacterium*-mediated transformation of taro with a disease resistance gene.
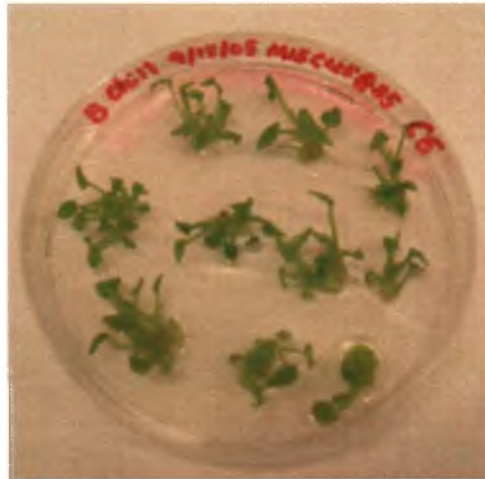
Fig. 3.8 Transgenic taro cv. Bun Long shoot line C6 with the rice chitinase gene
RICCHI11 growing on G418 selection medium
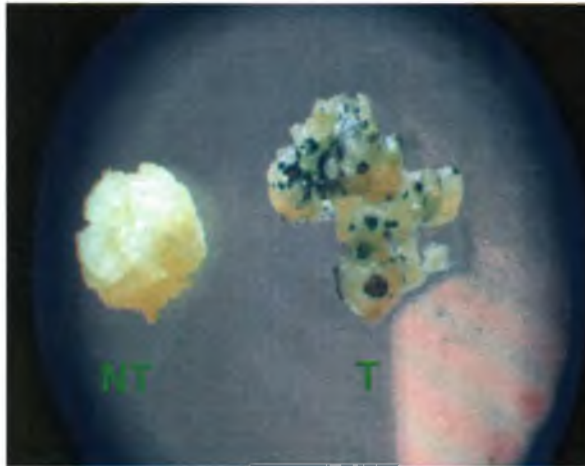
Fig. 3.9 GUS Assay of taro cv. Bun Long calli co-cultivated with *RICCHI11* gene (T) and non-co-cultivated control (NT)
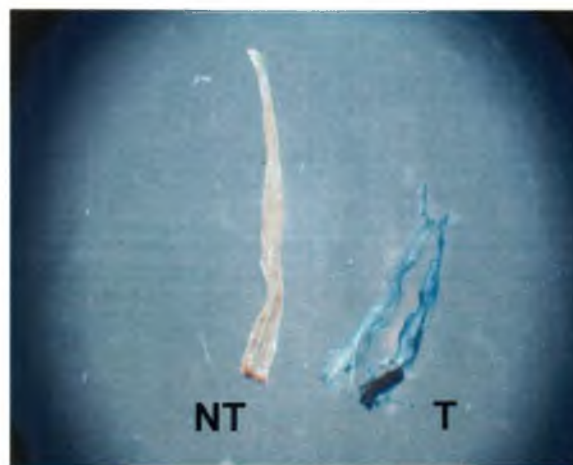
Fig. 3.10 GUS Assay of taro cv. Bun Long shoot induced from callus transformed with *RICCHI11* gene (T) and non-transformed control (NT)
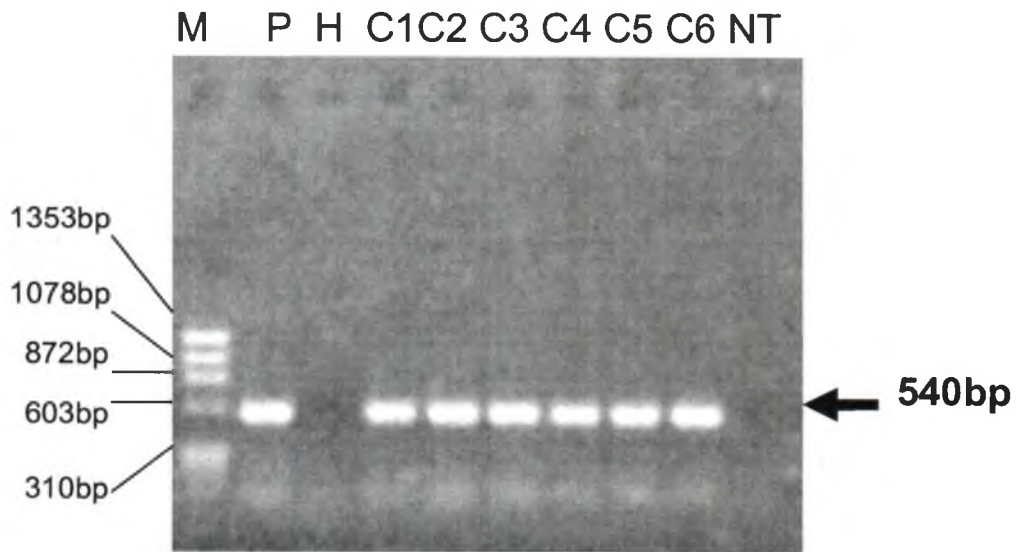
Fig. 3.11 PCR analysis of taro cv. Bun Long lines transformed with

EHA105:pBI121/*RICCHI11*

M: molecular weight marker ΦX174/HaeIII

P: Plasmid pBI121/*RICCHI11* control

H: Water control

NT: Non-transformed plant control

C1-C6: Transgenic lines1-6
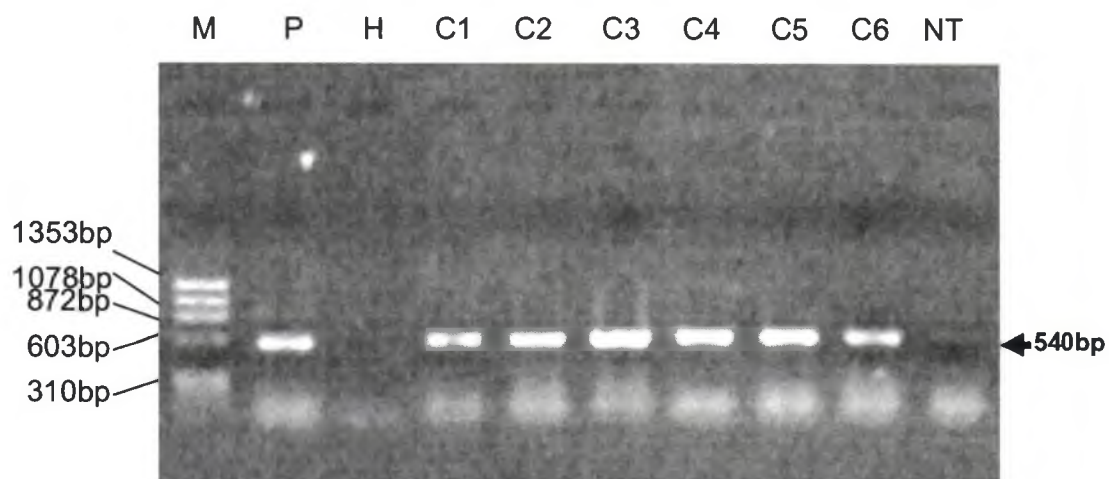
Fig. 3.12 RT-PCR analysis of taro cv. Bun Long lines transformed with

EHA105:pBI121/*RICCHI11*

M: molecular marker ΦX174/HaeIII

P: Plasmid pBI121/*RICCHI11* control

H: Water control

NT: Non-transformed plant control

C1-C6: Transgenic lines1-6
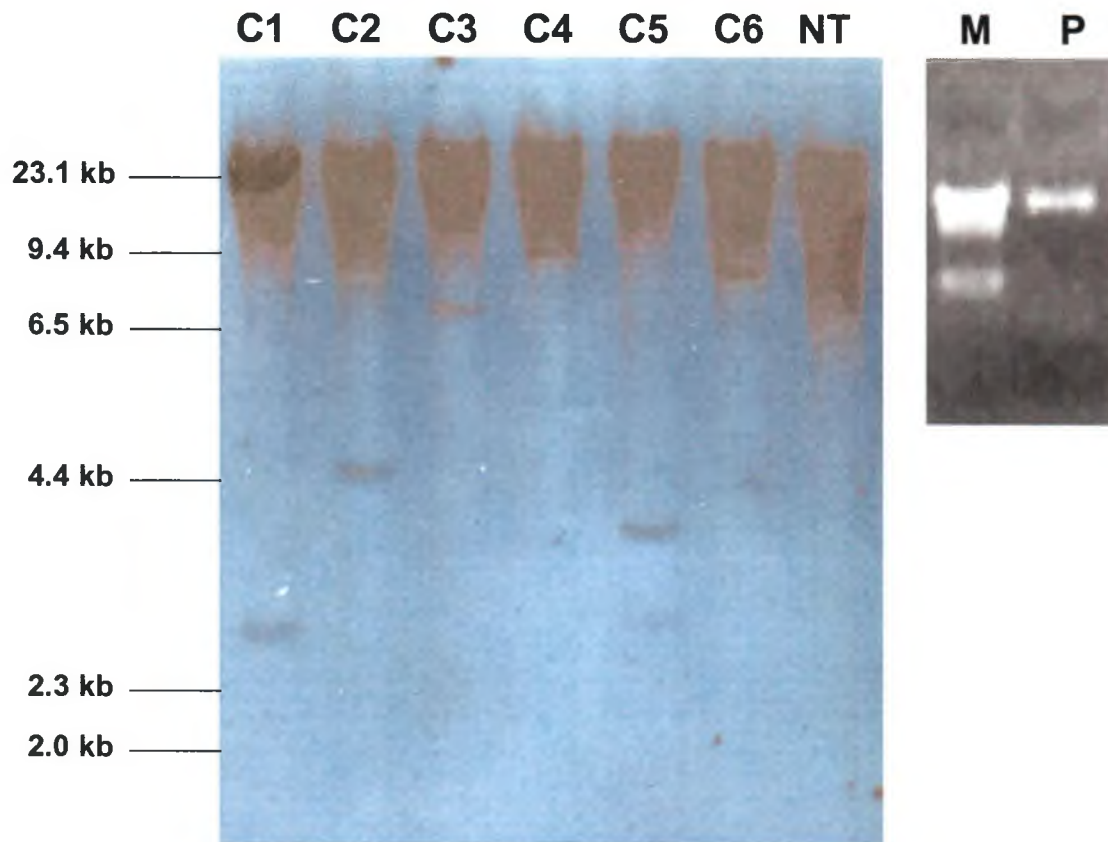
Fig. 3.13a (left) Southern blot analysis of six independent lines (C1-C6)

transformed with the rice chitinase gene, *RICCHI11* and a non-transformed

control (NT). A 15 μg aliquot of the genomic DNA extracted from leaves was

digested with *Not*I, which cuts only once within the T-DNA region. Blots were

hybridized with the PCR product of the rice chitinase gene labeled with the

AlkPhos direct labeling and detection system (Amersham non-radio active kit)

Fig. 3.13b (right) Agarose gel electrophoresis showing *Not*I digestion cuts only

once within the plasmid p BI121/ *RICCHI11*

M: Molecular marker, λ/*Hind*III P: Plasmid pBI121/ *RICCHI11* digested by *Not*I

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CHAPTER 4

TRANSFORMATION OF TARO WITH A WHEAT (*Triticum aestivum*) OXALATE

OXIDASE GENE

4.1 Literature review

Oxalate oxidase (OXO) was first isolated and characterized from barley and wheat (Lane et al., 1993; Kotsira and Clonis, 1997). The OXO gene is found in the cupin superfamily and was known formerly as the germin gene in the "true cereals" (maize, oat, rice, rye). The OXO gene was found to be involved in the defense responses to invasion by fungal pathogens in cereals (Dunwell et al., 2000). The enzyme OXO catalyzes the oxidation of oxalic acid by molecular oxygen to form carbon dioxide and hydrogen peroxide (H_2O_2). However, the exact biological significance and defense mechanism of OXO in plants remains unknown.

There are several hypotheses to explain the mechanism of OXO in fungal resistance (Lane, 2002), a) H_2O_2 generated by OXO elicits hypersensitive cell death and is directly toxic to microbes (Peng and Kuc, 1992), b) H_2O_2 -mediated lignification of cell walls forms effective barriers against fungal penetration (Bolwell et al., 1995; Wei et al., 1998; Schweizer et al., 1999), c) H_2O_2 generated by OXO possibly has a role in signal transduction cascades that coordinate various defense responses, such as induction of synthesis of PR proteins and phytoalexins (Greenberg et al., 1994; Hammond-Kosack and Jones, 1996), and d) oxalic acid (OA)-generating fungi such as *Sclerotinia sclerotiorum* secrete high concentrations of OA as a toxin, and OXO is able to break down OA (Donaldson at al., 2001; Liang et al., 2001).

A lot of evidence exists that OXO is associated with antifungal activities in wheat and barley (Hurkman and Tanaka, 1996; Zhang et al., 1995; Zhou et al., 1998). Schweizer et al. (1999) found transient expression of the pathogen-induced wheat germin *gf2.8* gene that reduced the penetration of the fungus *Blumeria graminis*.

Of increasing interest is improving fungal resistance in plants through genetic transformation with OXO genes. Several successful transformations have been reported to improve resistance to fungal pathogens (Cober et al., 2003; Donaldson et al., 2001; Hu et al., 2003; Liang et al., 2001; Livingstone et al., 2005) (Table 4.1). For example, Liang et al. (2001) reported that a transgenic hybrid poplar with a wheat germin-like oxalate oxidase gene enhanced disease resistance against the fungal pathogen *Septoria musiva*. Cober et al. (2003) investigated a transgenic soybean line with the transgene wheat oxalate oxidase gene *gf-2.8* and demonstrated that the transgenic plants exhibited partial resistance to the white mold disease caused by the fungal pathogen *Sclerotinia sclerotiorum*. In addition, Hu et al. (2003) transformed a wheat oxalate oxidase gene, *gf2.8*, into sunflower. The transgenic plants showed high levels of oxalate oxidase activity which were associated with hypersensitive response-like lesions and enhanced the resistance to disease. Recently, transgenic peanut with a barley oxalate oxidase gene also showed enhanced resistance to *Sclerotinia minor* that causes *Sclerotinia* blight of peanut (Livingstone et al., 2005). Apparently, genetic transformation with an oxalate oxidase gene has potential for increasing plant disease resistance.

In this study, we transformed taro cv. Bun Long with a wheat oxalate oxidase gene. The transgenic plants showed increased resistance against the taro fungal pathogen *Sclerotium rolfsii* and the oomycete pathogen *Phytophthora colocasiae* in a preliminary bioassay.

Evidence indicates that oxalate can act as an undesired anti-nutrient or toxin in animal and human nutrition (Betsche and Fretzdorff, 2005; Bhandari and Kawabata, 2005; Bohn et al., 2004; Franceschi and Nakata, 2005; Palgi et al., 2005). Taro corms and leaves have abundant levels of oxalate and calcium oxalate (Sakai et al., 1984; Sefa-Dedeh et al., 2004). Sakai et al. (1984) demonstrated that in the edible aroid genera, the oxalate raphide

microstructure was related to irritation when consumed by humans. Transformation of taro

plants with an oxalate oxidase gene could reduce the concentration of oxalate in the corms

and leaves, and could reduce anti-nutritive qualities while increasing disease resistance.

Table 4.1 Transgenic plants with an oxalate oxidase gene exhibited an enhanced disease
resistance in the literature

Transgenic plants	Sources of transgene	Diseases (pathogens) defended against	References
Hybrid poplar *Populus* x *euramericana*	Wheat germin-like oxalate oxidase gene	(*Septoria musiva*)	Liang et al., 2001
Soybean *Glycine max*	Wheat oxalate oxidase gene, *gf2.8*	Sclerotinia stem rot (*Sclerotinia sclerotiorum*)	Donaldson et al., 2001
Soybean *Glycine max*	Wheat oxalate oxidase gene, *gf2.8*	Sclerotinia stem rot (*Sclerotinia sclerotiorum*)	Cober et al., 2003
Sunflower *Helianthus annuus*	Wheat oxalate oxidase gene, *gf2.8*	Sclerotinia stem rot (*Sclerotinia sclerotiorum*)	Hu et al., 2003
Peanut *Arachis hypogaea*	Barley oxalate oxidase gene	Sclerotinia blight (*Sclerotinia minor*)	Livingstone et al., 2005

4.2 Materials and methods

4.2.1 Plant materials

Taro cultivar Bun Long was chosen as the plant transformation material because earlier we developed earlier an efficient regeneration system for this cultivar. Highly regenerable calli were selected as plant materials for *Agrobacterium*-mediated transformation.

4.2.2 Plasmids and *Agrobacterium* strains

The plasmid pEMBL18:*gf2.8* was provided by Dr. Francois Bernier at I.B.M.P. (Institut de Biologie Moleculaire Des Plants) in France. Enzyme digestion followed by PCR analysis and partial sequencing verified that this plasmid contained the intact 2.8kb *gf2.8* gene including the promoter and terminator.

4.2.3 Sub-cloning the intact *gf2.8* gene into a plant binary vector and transformation of *Agrobacterium* strain EHA105

The intact 2.8kb OXO *gf2.8* gene fragment was successfully introduced into each of the binary vectors, pBI121 and pCambia1303. The intact 2.8kb OXO *gf2.8* gene was extracted from the plasmid pEMBL18:*gf2.8* with the enzyme *Eco*RI. It was inserted into the *Eco*RI site of either vector pBI121 or pCambia1303. The sub-cloning process is based on standard methods (Sambrook and Russell, 2001). Figure 4.1 summarizes the process of sub-cloning the *gf2.8* gene into the binary vector pBI121.

The plasmid pBI121:*gf2.8* contains the *nptII* selection gene, the *gus* report gene, and the intact *gf2.8* gene. The plasmid pCambia1303:*gf2.8* contains a hygromycin resistance plant selection gene, *gus* and green fluorescent protein (*gfp*) fusion genes, and the intact *gf2.8* gene. Table 4.2 summarizes the construction of plant transformation plasmid pBI121:*gf2.8* and pCambia1303:*gf2.8*.

The plasmids pBI121:gf2.8 and pCambia1303:*gf2.8* were transformed individually into the *Agrobacterium tumefasciens* strain EHA105 using the freezing and thawing method

pBI121 (Vector)

Intact wheat oxalate oxidase gene **gf2.8** (Insert)

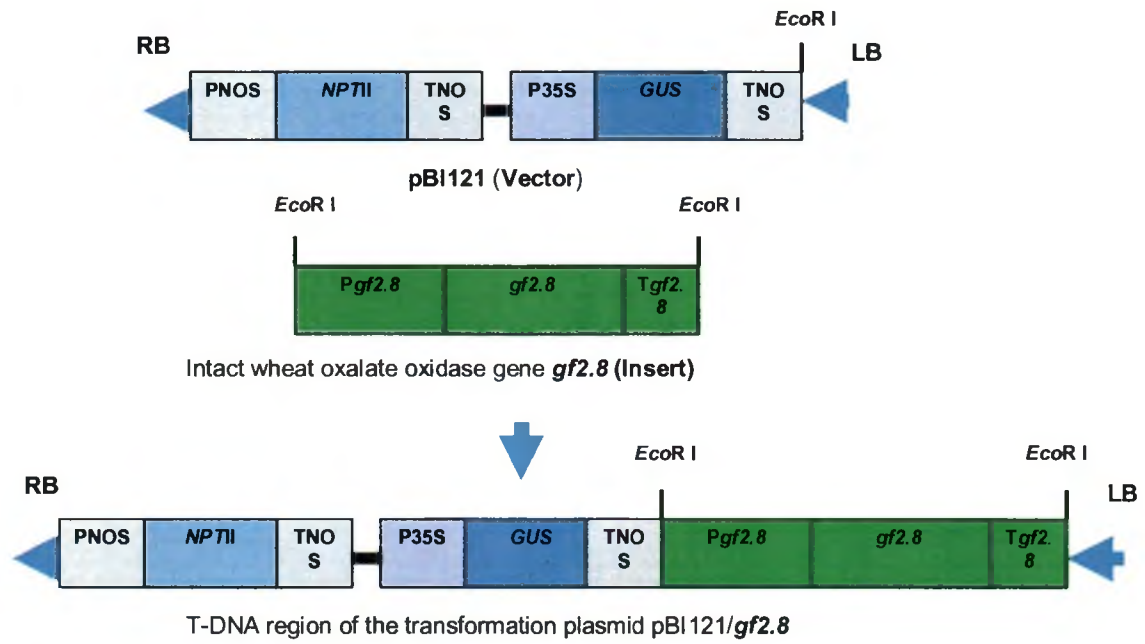T-DNA region of the transformation plasmid pBI121/**gf2.8**

Fig. 4.1 Sub-cloning the intact 2.8kb *gf2.8* gene into binary vector pBI121

Table 4.2 Construction of plant transformation plasmid pBI121/*gf2.8* and pCambia1303/*gf2.8*

Plasmid	Selection gene (Antibiotic resistance)	Reporter gene	transgene
pBI121:gf2.8	*NptII* Geneticin (G418)	*gus*	Intact *gf2.8*
pCambia1303:gf2.8	*Hpt* Hygromycin	*gus* and *gfp* fusion	Intact *gf2.8*

that was described earlier in the section of *Agrobacterium*-mediated transformation with the rice chitinase gene *RICCHI11* (Chapter 3.3.2.3).

4.2.4 PCR primers design for *gf2.8*

The PCR primers specific for *gf2.8* were designed using the Clone Manager software (Cary, NC), and based on the *gf2.8* gene sequence in Gene bank (Lane et al., 1991). A pair of primers specific for amplifying the 755 bp core gene fragment of *gf2.8* was designed with the Clone Manager software.

4.2.5 PCR analysis for *gf2.8*

The primer pair used to amplify a 755-bp fragment of *gf2.8* gene was: gfF (20 mer) 5'-GCTTAGCAGCAGCAACAACC-3' and gfR (20 mer) 5'-GCGGCAAACTTGGACTTGAG-3'). The PCR reactions were performed in 50 µl volume consisting of 1 µl template DNA, 2 µl of each primer (20 µmol), 5 µl dNTPs (2 mM), 5 µl 10x Taq buffer (Promega, Madison, WI), 1 unit Taq polymerase (Promega, Madison, WI), and 36.5 µl H_2O. Amplification of the *gf2.8* fragment was performed for 30 cycles at 94 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s, for denaturing, annealing and primer extension, respectively. Primers and PCR conditions are summarized in Table 4.3.

Table 4.3 Primers and PCR conditions used in the amplification of the OXO gene *gf2.8*

Primer ID and sequences	PCR conditions
gfF: 5'- GCTTAGCAGCAGCAACAACC -3' gfR: 5'- GCGGCAAACTTGGACTTGAG -3'	94 °C 3 min (94 °C 30 s, 55 °C 45 s, 72 °C 45 s)x30 72 °C 5 min

4.2.6 Verification of EHA105: pBI121/*gf2.8* and EHA105: pCambia1303/*gf2.8* by enzyme digestion and PCR analysis

To verify the transformation of EHA105 with pBI121:*gf2.8* or pCambia1303:gf2.8, the plasmid pBI121/*gf2.8* or pCambia1303/*gf2.8* was extracted from *E. coli* strain DH5α and *Agrobacterium* strain EHA105 using a DNA miniprep kit (Invitrogen company, San Diego, CA). Then DNA was digested with *EcoRI* to release a 2.8 kb fragment containing the intact 2.8 kb OXO gf2.8 gene that is flanked by two *EcoR*I sites. Gel electrophoresis in 0.8% agarose was performed to examine the digested DNA and PCR products.

4.2.7 *Agrobacterium*-mediated transformation method

The same method was used as described earlier for the chitinase gene transformation (Chapter 3.3.2.5). Briefly, Calli of cv. Bun Long were cut, immersed in the *Agrobacterium* suspension for approximately 10 minutes, then transferred to the co-cultivation medium (hormone-free MS) and co-cultivated with *Agrobacterium* for four days. The co-cultivated calli were transferred onto the M5 medium containing 50 mg L^{-1} geneticin and 250 mg L^{-1} cefotaxime, and cultured for 60 to 90 days.

4.2.8 Geneticin (G418) selection and GUS assay

The same protocol was used as described earlier for *Agrobacterium*-mediated transformation with the chitinase gene (Chapter 3.3.2.6 and Chapter 3.3.2.7). In summary, for

G418 selection of transformed cells, transformed calli were grown for 90 days on 50 mg L^{-1}

G418 with subculturing every 30 days. In addition, 50 mg L^{-1} G418 was applied to shoot-

induction medium M15 for 90 days, and the G418 concentration was reduced to 35 mg L^{-1}

thereafter. Approximately 50% of the calli or shoot cuttings were tested for the presence of

the GUS gene using X-Gluc as a substrate in a 96-well tissue culture plate. The plate was

incubated overnight at 37 °C. The calli or shoot cuttings were observed under the microscope

for blue spots that indicated GUS activity.

4.2.9 Hygromycin selection of the transformation with EHA105: pCambia1303:*gf2.8*

Hygromycin concentration for selection of transformants was 25 mg L^{-1} based on the

protocol described by Fukino et al. (2000). Transformed calli were grown for 60 days on 25

mg L^{-1} hygromycin with subculturing every 30 days, and the hygromycin concentration was

reduced to 15 mg L^{-1} thereafter. In addition, 15 mg L^{-1} hygromycin was added into shoot-

induction mediium M15 (Chapter 2.2.2.1).

4.2.10 Verification of the presence of *gf2.8* using PCR analysis

Genomic DNA was extracted from approximately 30 mg (fresh weight) of shoot

tissues induced from transformed calli using the modified SDS method described by Lin et al.

(2001). Approximately 200 ng total DNA was used to perform a PCR reaction. The primers

specific for the *gf2.8* gene, the PCR reaction reagents, and the PCR conditions were the same

as described earlier in PCR analysis for *gf2.8* (Chapter 4.2.5).

4.2.11 Verification of transcription of *gf2.8* gene using RT-PCR analysis

Total RNA was isolated from approximately 10 mg of fresh shoot tissue according to

the Sucrose Prep method described by Berendzen et al. (2005). Reverse transcription (RT)

was conducted with oligo-dT primers and reverse transcriptase in the RT-PCR kit (Promega,

Madison, WI). The reverse transcripts were used as templates for PCR analysis using primers

specific for the amplification of *gf2.8* gene fragment. The PCR reaction system and conditions were described previously in the section on transformation of taro with the chitinase gene (Chapter 3.2.2.5). As a control, PCR was also conducted using total RNA without reverse transcription to confirm that the PCR product was derived from mRNA and not from the contaminating DNA.

4.2.12 Verification of the presence and copy number of *gf2.8* using Southern analysis

Genomic DNA was extracted from approximately 80-100 mg of freshly induced shoot tissues using the modified SDS method described by Lin et al. (2001). Fifteen μg of DNA was digested with the enzyme *BamH*I which has a single digestion site in the T-DNA. The DNA blotting, probing and hybridization methods were the same as described earlier in the section on chitinase gene transformation (Chapter 3.2.2.6).

4.3 Results

4.3.1 Verification of EHA105: pBI121/*gf2.8* by enzyme digestion and PCR analysis

The expected 2.8kb band was found for the *EcoR*I digested plasmid DNA extracted from both the *E. coli* strain DH5α: pBI121/*gf2.8* and the *Agrobacterium* strain EHA105: pBI121/*gf2.8* (Fig. 4.2). The PCR product of the plasmid DNAs extracted from the *E. coli* strain DH5α: pBI121/*gf2.8*, and the *Agrobacterium* strain EHA105: pBI121/*gf2.8* were the expected 755 bp band. These results confirmed the binary transformation plasmid pBI121/*gf2.8* was successfully transformed into the *Agrobacterium* strain EHA105 (Fig. 4.2).

4.3.2 Hygromycin selection of transformation with EHA105: pCambia1303/gf2.8

A total of 700 calli were used with 100 calli per experiment. After 2 weeks of selection using hygromycin selection, 100 calli out of 150 tested calli showed a positive GUS result, indicating a 67% transient expression level (Fig. 4.3). After 2 weeks of selection, 50 calli out of 150 tested calli showed a positive GFP result, indicating a 33% transient expression level (Fig. 4.4). After 4 weeks of hygromycin selection, 40 calli survived but they

appeared brown. The concentration of hygromycin was reduced from 25 mg L^{-1} to 15 mg L^{-1} and the 40 calli were cultured for 60 days with subculturing every 30 days. Ten lines survived and shoots were induced on M15 selection medium. Three lines were selected and leaves and shoots were excised for both GUS and GFP assays. The leaves and shoots of 2 lines showed positive GUS results (Fig. 4.5), but no lines showed a positive GFP result. Unfortunately, after aother 3 month selection on M15 medium containing 15 mg L^{-1} hygromycin, no shoots survived.

4.3.3 GUS assay for transformed taro with EHA105: pBI121/gf2.8

In total, 200 calli were transformed. Out of 30 calli selected for the GUS assay, 20 calli (approximately 65%) showed positive GUS results after 30 days of selection on 50 mg L^{-1} G418 (Fig. 4.6). Of the remaining 170 calli, 50 calli survived after 90 days of selection, and were transferred to the shoot inducing medium M15 containing the 50 mg L^{-1} G418 with subculturing every 30 days. After 90 days, 30 independent shoot lines were initiated (Fig. 4.7). After another 60 days, 10 lines survived. Shoots of these ten lines were excised for GUS assay, and nine of them showed a strong GUS expression (Fig. 4.8).

4.3.4 PCR analysis and RT-PCR analysis for transformed taro with EHA105: pBI121/*gf2.8*

Leaves of the ten survived lines were selected for isolation of DNA for PCR amplification and of RNA for reverse transcriptase PCR (RT-PCR) of the specific *gf2.8* gene fragment. The expected 755 bp PCR product specific for the *gf2.8* gene fragment was found from 8 GUS-positive lines and the plasmid pBI121/*gf2.8*, indicating that the *gf2.8* gene had been successfully transformed into the 8 lines (Fig. 4.9). Also, the expected 755 bp PCR product specific for the *gf2.8* gene fragment was found from the reverse-transcription product of total RNA extracted from the 8 lines and the DNA of the plasmid pBI121/*gf2.8* (Fig. 4.10). As expected, there was no PCR product obtained from the total DNA PCR amplification or

the reverse-transcription product of total RNA extracted from the non-transformed control plants.

4.3.5 Southern blot analysis

Restriction digests using the enzyme, *BamH*I, of genomic DNA extracted from the 8 transgenic lines yielded various bands larger than the *gf2.8* gene size of 2.8 kb. line1 to line6 (g1-g6) appeared to have the same integration pattern and line7, 8 (g7, g8) appeared to have the same integration pattern, indicating g1-g6 derived from one independent transformation event, and g7, g8 derived from another independent transformation event. A single band was found in g1-g6 and four bands were found in g7 and g8 (Fig. 4.11). Since *BamH*I only has one digestion site in the T-DNA, a single band in the southern blot analysis indicated a single-copy transgene insertion into the taro genome and four bands suggested a four-copy insertion of the transgene. Therefore, one independent line (1/2 = 50%) contained a single-copy insertion with no apparent gene rearrangement, and the another independent line contained four-copy insertions. This result confirmed the presence and intactness of the trangene *gf2.8* in the taro genome.

4.4 Discussion

Out of 700 calli transformed with EHA105: pCambia1303/*gf2.8*, none survived in the hygromycin (25 mg L^{-1} or 15 mg L^{-1}) selection medium. This result indicated that hygromycin is much more toxic to taro cv. Bun Long than geneticin. The selection of transformants by hygromycin was not successful, but should be attempted using lower concentration of the antibiotic.

In transformation with EHA105: pBI121/*gf2.8*, PCR analysis indicated the presence of the *gf2.8* gene and RT-PCR indicated the expression of the *gf2.8* gene in transformed lines. Based on the homologous fragment patterns in Southern blot analysis, two independent transgenic lines with the wheat oxalate oxidase gene *gf2.8* have been produced *via*

Agrobacterium-mediated transformation. Southern blot analysis of the transformed lines also indicated that one out of two independent lines (50%) had integrated a single copy of the transgene, and the other trangenic line had four copies of the inserted gene. Compared with particle bombardment transformation of taro, *Agrobacterium*-mediated transformation resulted in higher transformation efficiency (2/200 = 1% vs 1/1350 < 0.1% for bombardment). These transformants may show more effictive transgene expression as a result of single-copy insertions of the transgene in 50% of transformed lines. To our knowledge, this is the first report on *Agrobacterium*-mediated transformation of taro with a disease resistance gene.
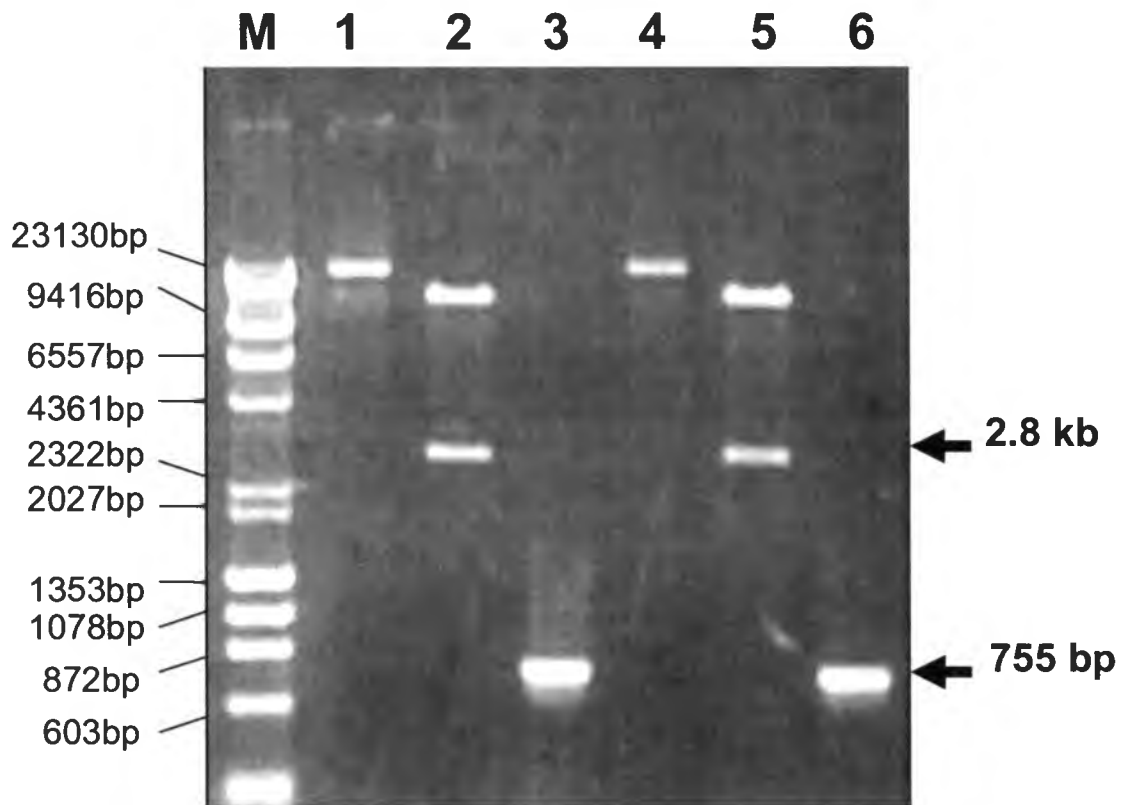
Fig. 4.2 Verification of the integrity of pBI121/*gf2.8* extracted from both *E. coli* and

EHA105 by enzyme digest and PCR analysis

M: molecular weight marker λ/HindIII and ΦX174/HaeIII mix

1: Plasmid pBI121/*gf2.8* extracted from *E. coli*

2: *EcoR*I digest of pBI121/*gf2.8* extracted from *E. coli* shows presence of the 2.8 kb *gf2.8*

  gene

3: PCR analysis of pBI121/*gf2.8* extracted from *E. coli* shows presence of the 755 bp

  gf2.8 gene fragment

4: Plasmid pBI121/*gf2.8* extracted from EHA105

5: *EcoR*I digest of pBI121/*gf2.8* extracted from EHA105 shows presense of the 2.8 kb *gf2.8*

  gene

6: PCR analysis of pBI121/*gf2.8* extracted from EHA105 shows presence of the 755 bp

  gf2.8 gene fragment

Non-co-cultivated control   Co-cultivated callus
                              (at two weeks)

Fig. 4.3 Results of a GUS assay for calli co-cultivated with EHA105: pCambia1303/ *gf2.8*

and non-co-cultivated control

Non-co-cultivated control   Co-cultivated callus
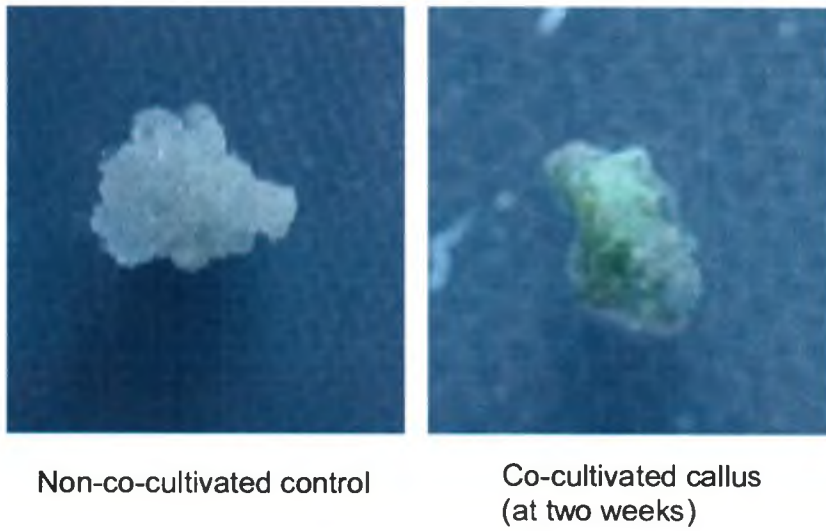   (at two weeks)

Fig. 4.4 Test for GFP using calli co-cultivated with EHA105:

pCambia1303/*gf2.8* and a non-co-cultivated control
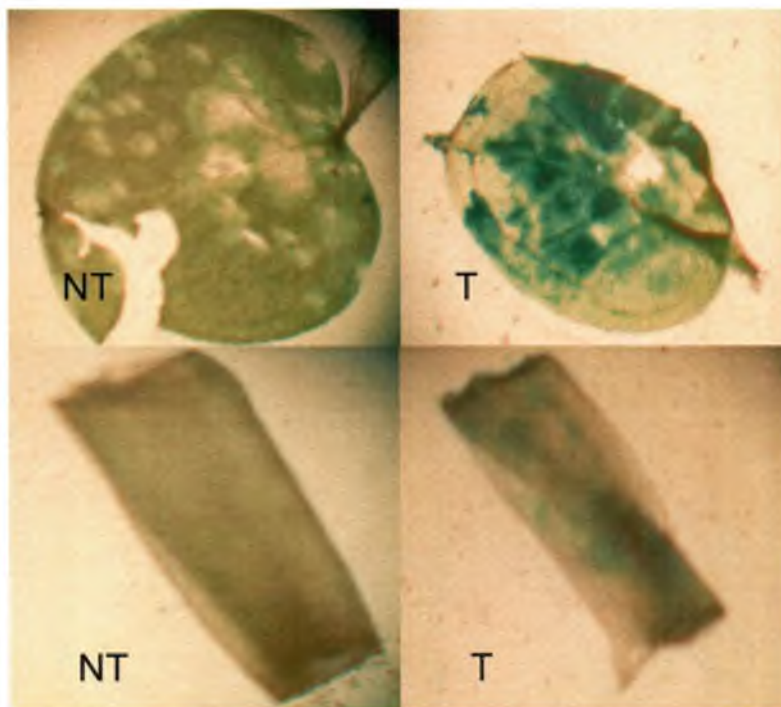
Fig. 4.5 Assay for GUS using leaves and shoots of taro cv. Bun Long transformed with
EHA105: pCambia1303/*gf2.8* (T) as well as a non-transformed control (NT)
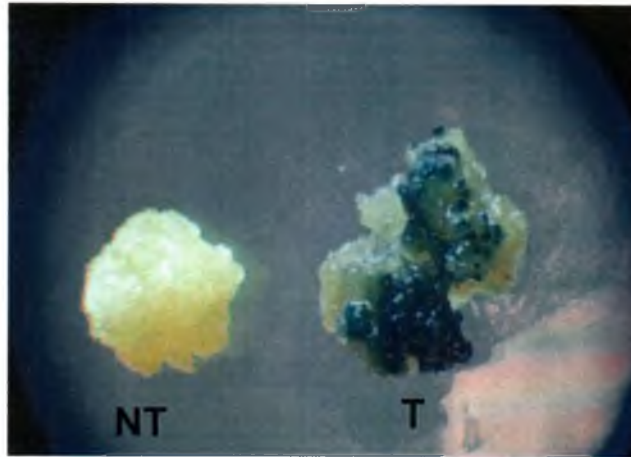
Fig. 4.6 Results of a GUS Assay for taro cv. Bun Long calli co-cultivated with EHA105:pBI121/*gf2.8* (T) after 30 days of selection on 50 mg L^{-1} G418 and non-co-cultivated control (NT)

Fig. 4.7 Transgenic taro cv. Bun Long shoot line g5 with *gf2.8* gene survived on G418 selection medium
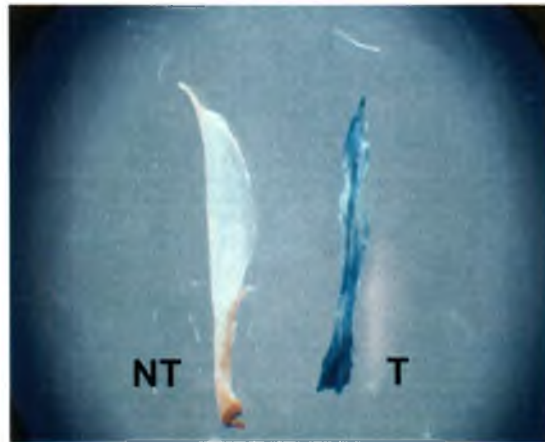
Fig. 4.8 Results of a GUS assay of a taro cv. Bun Long shoot induced from callus transformed with *gf2.8* gene (T) and a non-transformed control (NT)
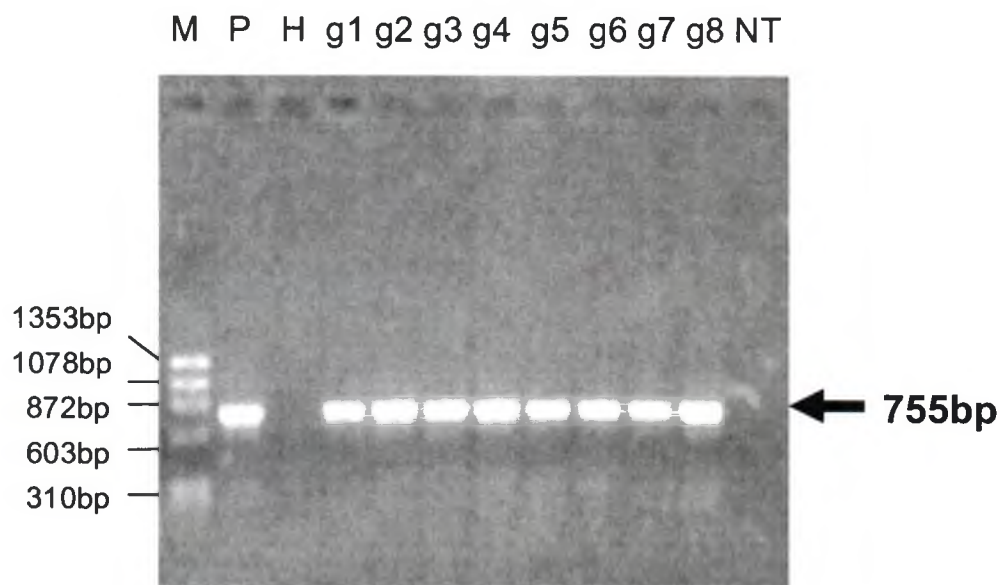
Fig. 4.9 Results of PCR analysis of taro cv. Bun Long lines

transformed with EHA105:pBI121/*gf2.8*

M: molecular weight marker ΦX174/HaeIII

P: Plasmid pBI121/*gf2.8* control

H: Water control

NT: Non-transformed plant control

g1-g8: Transformed lines1-8

M H P NT g1 g2 g3 g4 g5 g6 g7 g8

←755bp
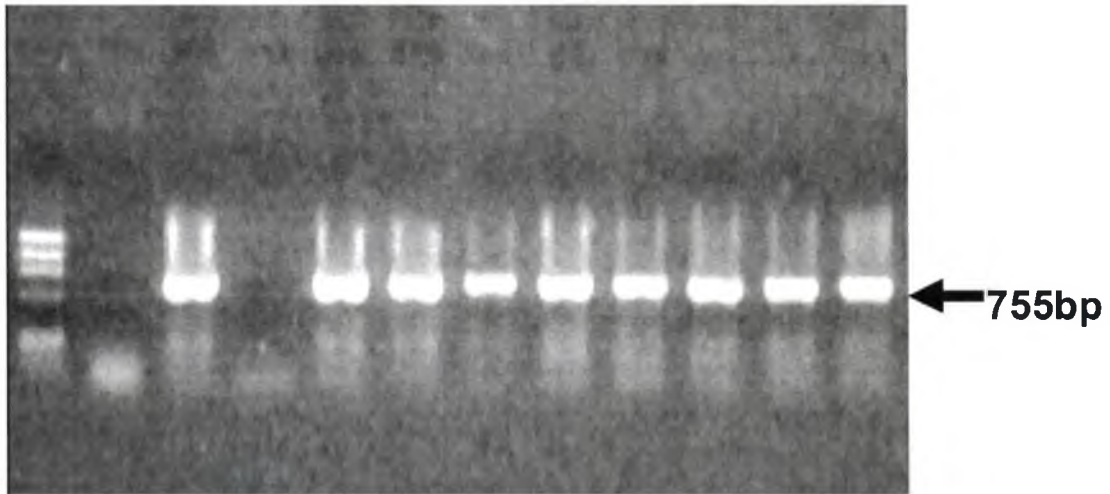
Fig. 4.10 Results of RT-PCR analysis of taro cv. Bun Long lines

transformed with EHA105:pBI121/*gf2.8*

M: molecular weight marker ΦX174/HaeIII

P: Plasmid pBI121/*gf2.8* control

H: Water control

NT: Non-transformed plant control

g1-g8: Transformed lines1-8
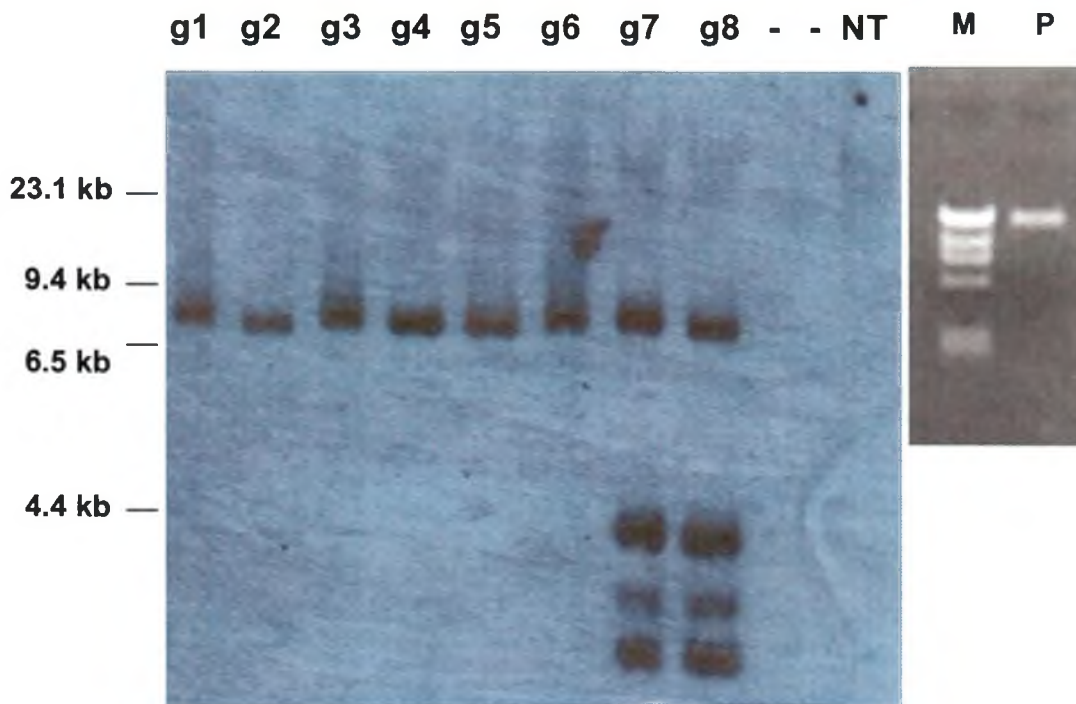
Fig. 4.11a (left) Southern blot analysis of 8 taro cv. Bun Long lines transformed with EHA105:pBI121/*gf2.8* (g1-g8) and a non-transformed control (NT). A 15 μg aliquot of the genomic DNA extracted from leaves was digested with *BamH*I, which cuts only once within the T-DNA region. Blots were hybridized with the PCR product of the wheat oxalate oxidase gene labeled with the AlkPhos direct labeling and detection system (Amersham non-radio active kit)

Fig. 4.11b (right) Agarose gel electrophoresis showing single digest site of *BamH*I digestion of the plasmid pBI121/ *gf2.8* containing the wheat oxalate oxidase gene, *gf2.8*

M: Molecular weight marker, λ/*Hind*III. P: Plasmid pBI121/*gf2.8* digested by *BamH*I

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CHAPTER 5

TRANSGENIC PLANTS AND PATHOGENS

5.1 Introduction

Plant fungal and oomycete pathogens attack plants through hyphae that penetrate into plant cells secreting enzymes, peptide elicitors or toxins that degrade and/or kill the cells (Okubara and Paulitz, 2005). Plants also have developed corresponding strategies to defend against and control plant diseases caused by fungal or oomycete pathogens (Greenberg and Yao, 2004; Okubara and Paulitz, 2005; Thevissen et al., 2003; West et al., 2003). Based on an understanding of the molecular mechanisms involved in plant defense responses to fungal and oomycete pathogens, plants have been transformed with disease resistance genes to enhance disease resistance (Chye et al., 2005; Cober et al., 2003; Hu et al., 2003; Livingstone et al., 2005; Lin et al., 1995; Salehi et al., 2005; Zhu et al., 2004). Transgenic plants express a single or several transgenes to produce compounds that could: a) be directly toxic to pathogens (Zhu et al., 2004); b) degrade the cell structural compounds or toxins of pathogens (Chye et al., 2005; Cober et al., 2003; Lin et al., 1995; Livingstone et al., 2005; Salehi et al., 2005); c) induce the plant hypersensitive response causing cell death to arrest pathogens and also cause the death of pathogens (Cober et al., 2003; Hu et al., 2003; Livingstone et al., 2005); or d) mediate signal transduction to induce other disease resistance genes or enhance plant defenses (Cober et al., 2003; Hu et al., 2003; Livingstone et al., 2005).

In this study, one transgenic taro cv. Bun Long plant line with a disease resistance gene, the rice chitinase gene (*RICCHI11*), was obtained by particle bombardment transformation. In addition, six independent transgenic taro cv. Bun Long plantlet lines with the rice chitinase gene (*RICCHI11*) were obtained via *Agrobacterium*-mediated transformation. Eight independent transgenic taro cv. Bun Long plantlet lines containing the wheat oxalate oxidase gene *gf2.8* were obtained via *Agrobacterium*-mediated transformation.

Several fungal pathogens were isolated and identified from diseased taro corms. The transgenic plants or plantlets were challenged with these fungal pathogens as well as the oomycete pathogen *Phytophthora colocasiae* to evaluate disease resistance.

5.2   Isolation and identification of taro fungal pathogens

5.2.1 Materials and methods

5.2.1.1 Materials

Corms of taro cv. Bun Long thought to exhibit symptoms characteristic of *Sclerotium rolfsii* were obtained from Kamuela, Hawaii (Randy Hamasaki, extension agent). One diseased corm exhibited yellowish and brownish lesions; however, there were no severely rotten lesions. Mature, brownish sclerotia were observed. Other rotten corms of taro cv. Maui Lehua with unknown pathogens were obtained from the Kona Experiment Station in Kainaliu, Hawaii. Four diseased corms were selected for isolation of fungi. Yellowish, brownish and severely rotten lesions were observed on these four diseased corms.

5.2.1.2 Isolation of pathogenic fungi

Eight samples for isolation of fungi were excised and placed on two plates containing water agar (4 sections per plate). Excess soil was brushed from the corms. Loose and decayed plant tissues around the outside of the corms were removed by rinsing and cutting. The margins of the brownish lesions in the corms were selected as sources for isolation of fungi and cut into sections (approximately 3 cm^2). These sections were surface-sterilized in 20% sodium hypochlorite (Clorox bleach) solution for times ranging from 30 seconds to 2 minutes, depending on the appearance of cleanliness in the sample material. These samples were cut into smaller sections (approximately 0.5 cm^2) and placed in 5% bleach solution to sterilize for approximately 30 seconds and rinsed in sterile water for approximately 30 seconds. After blotting with sterile tissue papers to remove the surface water of the samples, the samples were placed on 1.5% water agar medium (BACTO, Kansas City) for fungal isolation. Five

samples were placed in each plate maintained at 25 °C with a 8h photoperiod. Fungal

germination and mycelial growth around the corm samples were observed daily. When

mycelia were visible with the naked eye, a single mycelial tip was selected and excised using

a sterilized scalpel under the dissecting microscope. The agar containing a single mycelial tip

was placed on the center of a plate containing 10% V8 agar (VA) medium (Miller, 1955) for

purification and better growth at 25 °C with a 16h photoperiod. The isolated fungal cultures

were either maintained on the10% VA agar medium plates or transferred to potato dextrose

agar (PDA) medium plates for multiplication for use in further experiments.

5.2.1.3 Identification of the fungi

Characteristics of the isolated fungal cultures on water agar, 10% VA, and PDA

media were observed with both the naked eye and under the dissecting microscope.

For identification of the fungal pathogen, *Sclerotium rolfsii*, the sclerotial formation, structure,

and color were observed, as well as the mycelial color and structure. Characteristics of

Sclerotium rolfsii that were used in identification include: a) the sclerotia produced an

abundant white, aerial mycelium approximately 7 days after inoculation on PDA plates; b)

the immature sclerotia were roundish and white; c) mature sclerotia were round, brown or

black in color when cultured on PDA medium; and d) under the microscope, the mycelial

clamp connection was evident (Barnett and Hunter, 1998a).

For identification of the fungal pathogen, *Fusarium oxysporum*, the following

characteristics observed under the microscope were used: a) formation of chlamydospores

(survival structures); b) formation of microconidia and macroconidia on phialides; and c)

microconidia have false heads but no chains. For the identification of the fungus, *Rhizoctonia*

solani, abundant rapidly growing mycelia were observed either on water agar, 10% VA, or

PDA medium plates. Under the microscope, cross walls were observed on these mycelia. The

mycelia were stained with safranin stain for observation of nuclei. The presence of four nuclei per cell indicated that the fungus was the pathogenic *Rhizoctonia solani* (Barnett and Hunter, 1998b).

5.2.2 Results

5.2.2.1 Isolation and identification of *Sclerotium rolfsii*

White aerial mycelia around the diseased samples were observed after 5 days of culture on water agar. Under the microscope, the single mycelial tips were separately excised and placed on 10% VA plates. After 7 days of culture on 10% VA, approximately 20 small, round, white sclerotia were formed. One sclerotium was transferred to a PDA plate for better growth. After 7 days of further culture, approximately 50 small, round, light yellowish sclerotia formed (Fig. 5.1). After 14 days of further culture, these sclerotia turned brownish or black in color (Fig. 5.2). Under the microscope, the mycelial clamp connections were observed. More than 80 mature sclerotia were obtained after 20 days of culture on PDA medium.

5.2.2.2 Isolation and identification of *Fusarium oxysporum*

After two days of culture on 10% VA medium, many microconidia, macroconidia, monophialides, and chlamydospores were observed. In addition, microconidia exhibited false heads. Based on these characteristics, 14 cultures were identified as *Fusarium oxysporum*.

5.2.2.3 Isolation and identification of the fungus *Rhizoctonia solani*

Mycelial growth around several samples on the water agar plates was observed after one night. After 24 h culture on 10% VA medium, many mycelial cross walls were observed on several fungal cultures. In most cultures, two nuclei were observed per cell under the microscope, indicating the presence of binucleate *Rhizoctonia solani*-like non-pathogens. Two cultures were observed having 4 nuclei per cell, indicating the presence of the pathogenic *Rhizoctonia solani*.
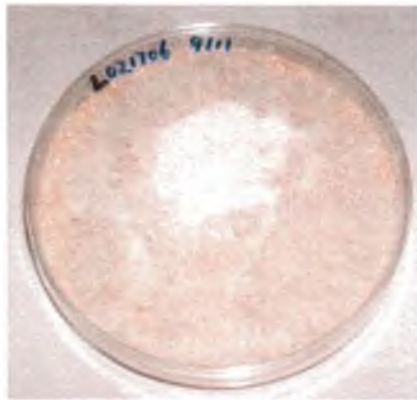
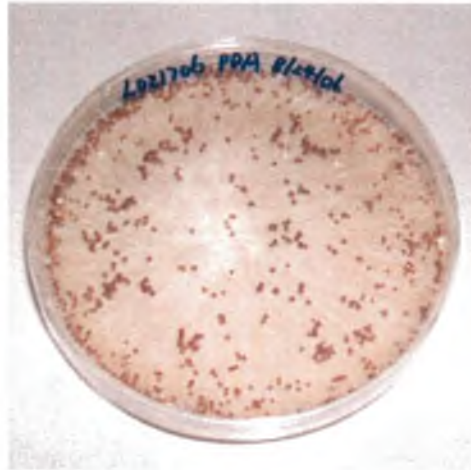Fig. 5.1 Immature sclerotia cultured on the PDA medium

Fig. 5.2 Mature sclerotia cultured on the PDA medium

5.3 Pathogenesis test

5.3.1 Materials and methods

5.3.1.1 Materials

The taro cv. Bun Long hulis (vegetative propagating materials composed of the upper 0.5 cm of corm and lower 30 cm of petioles) were obtained from the Maui Branch Station. The hulis were rinsed, outer petioles were peeled away, and the outer surfaces of the corm were removed. The remaining portion of the huli were placed in 10% Clorox bleach solution for 1 minute and then planted in 5 cm pots. The hulis were grown in the greenhouse for one month to produce sufficient roots and leaves for inoculation with fungal pathogens. Three isolated fungal pathogens, *Sclerotium rolfsii*, *Fusarium oxysporum* and *Rhizoctonia solani*, were tested for their pathogenicity on taro.

5.3.1.2 Inoculation of pathogens

To inoculate the plant roots, two squares (approximate 2 cm^2) of 10% VA agar containing a *Sclerotium rolfsii* culture with dark brown, mature sclerotia (5 sclerotia / agar square) were placed, sclerotia-side-down, and pressed firmly with a scraper on the roots of one healthy taro plant. The roots of plants inoculated with *Sclerotium rolfsii* were observed without magnification on the third and fourteenth days after inoculation. Two squares (approximate 2 cm^2) of 10% VA agar without any fungi were pressed firmly on the roots of one healthy taro plant as a negative control.

Two squares (approximately 2 cm^2) of 10% VA agar containing a *Rhizoctonia solani* culture with germinating spores and mycelia were placed spore-side-down, and pressed firmly with a scraper on the roots of one healthy taro plant. Two squares (approximate 2 cm^2) of 10% VA agar without any fungi were pressed firmly on the roots of one healthy taro plant as a negative control. At 3 and 14 days after inoculation with *Rhizoctonia solani*, the roots of plants were observed with the naked eye.

The spores of *Fusarium oxysporum* were counted using a hemacytometer. To inoculate plant roots, 30 ml (1 x 10^6 spores / ml) of the *Fusarium oxysporum* water solution were poured into the root areas of one healthy taro plant. As a negative control, 30 ml of sterile distilled water was poured into the root areas of another healthy taro plant. At 3 and 14 days after inoculation with *Fusarium oxysporum*, the roots of plants were observed with the naked eye.

5.3.2 Results

At day 3 after inoculation with *Sclerotium rolfsii*, several brown and rotten roots were observed near the inoculation point. At 14 days after inoculation, it was estimated that approximately 80% of all roots had become brownish, soft, and rotten (Fig. 5.3).

At 3 days after inoculation with *Rhizoctonia solani*, one brownish and soft primary root, as well as several small brown secondary roots were observed near the site of inoculation. At 14 days after inoculation with *Rhizoctonia solani*, it was estimated that approximately 1% of the roots had become brownish, soft, rotten, and had died (Fig. 5.4). No brown lesions were observed on roots of the control plant (Fig. 5.3; Fig. 5.4).

At 3 days after inoculation with *Fusarium oxysporum*, two brownish and soft primary roots were observed near the inoculation point. At 14 days after inoculation, approximately 1% of the roots was brownish, soft, rotten, and had died (Fig. 5.5). No brownish lesions were observed on roots of the control plant inoculated with 30 ml sterile distilled water (Fig. 5.5).

5.3.3 Discussion

Based on these pathogenicity test results, *Sclerotium rolfsii* appeared to be the most severe taro cv. Bun Long pathogen. In two weeks, *Sclerotium rolfsii* caused severe root rot disease of taro. In contrast, the fungal pathogens *Rhizoctonia solani* and *Fusarium oxysporum* showed low pathogenicity to taro cv. Bun Long. The fungal pathogen *Sclerotium rolfsii* was

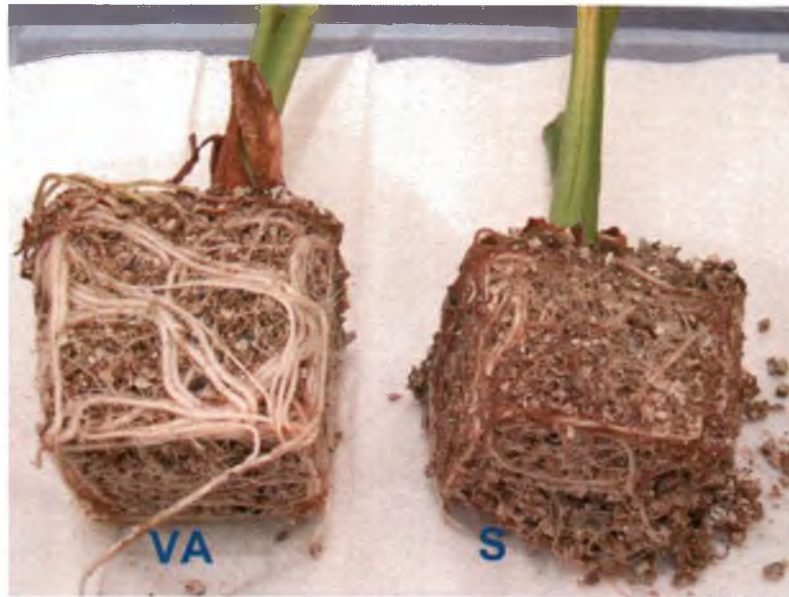selected to conduct the preliminary pathogen resistance bioassay of transgenic taro cv. Bun Long plants.

Fig. 5.3 Pathogenicity test of *Sclerotium rolfsii* on taro cv. Bun Long

VA: Control plant inoculated with 10% VA agar

At 14 days after inoculation, No brown lesions were observed on the roots

S: Test plant inoculated with *Sclerotium rolfsii* in 10% VA agar

At 14 days after inoculation, approximately 80% of all roots had become brownish, soft, and rotten
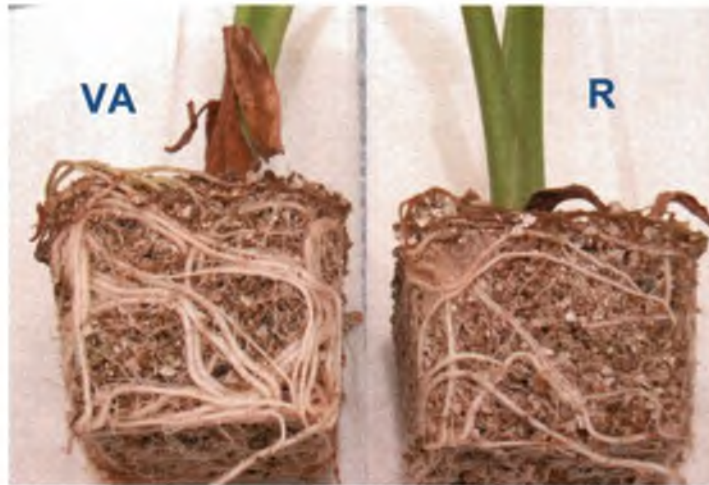
Fig. 5.4 Pathogenicity test of *Rhizoctonia solani* on taro cv. Bun Long

VA: Control plant inoculated with the clean 10% VA agar

At 14 days after inoculation, No brown lesions were observed on the roots

R: Test plant inoculated with *Rhizoctonia solani* in 10% VA agar

At 14 days after inoculation, approximately 1% of all roots had become brownish,

soft, and rotten

Fig. 5.5 Pathogenicity test of *Fusarium oxysporum* on taro cv. Bun Long

W: Control plant inoculated with sterile distilled water

At 14 days after inoculation, No brown lesions were observed on the roots

F: Test plant inoculated with *Fusarium oxysporum* water solution

At 14 days after inoculation, approximately 1% of all roots had become brownish,

soft, and rotten

5.4 Preliminary pathogen resistance bioassay of transgenic plants

5.4.1 Transgenic plants with the rice chitinase gene *RICCHI11* via particle bombardment transformation

5.4.1.1 Materials and methods

In a preliminary test for disease resistance, one positive transgenic line with a chitinase gene obtained via particle bombardment transformation was rapidly propagated by transferring to Magenta boxes (PhytoTechnologies Laboratories, Shawnee Mission, KS, USA) that contained liquid M15 multiplication medium. After the emergence of roots, transgenic and non-transgenic seedlings with three to five leaves were transferred from Magenta boxes to 5 cm pots placed in an environmentally controlled chamber maintained at 25 °C with a 16 h photoperiod. After approximately 40 days of growth, they were transferred to 10 cm pots. An additional 30 days were required before plants were ready for *Phytophthora colocasiae* inoculation.

This test was performed based on the method of Brooks (2000). *Phytophthora colocasiae* was cultured on a 10% VA agar medium (Miller, 1955). A period of one or two weeks was required to produce active sporangia. A plug 4 mm in diameter containing *P. colocasiae* active spores was placed onto the upper surface of a leaf. Disease severity was scored using the diameter of the infected leaf area at 2 and 4 days after inoculation.

5.4.1.2 Results

Transgenic plant growth and morphology appeared normal and similar to non-transformed controls. In total, 50 transgenic plants from the one transformed line and 20 non-transformed plants were successfully inoculated with *P. colocasiae*. Disease severity was scored using the diameters of the infected leaf area after 2 days and 4 days after inoculation. At 2 days after inoculation, on average, the diameter of the infected leaf area was 2.75 cm in the 50 transgenic plants and 2.70 cm in the 20 non-transformed plants. At 4 days after

inoculation, on average, the diameter of the infected leaf area was 5.09 cm in the 50 transgenic plants and 5.13 cm in the 20 non-transformed plants. Obviously, no significant differences in disease resistance were found between transgenic plants and non-transformed plant controls.

5.4.2 Transgenic plants with the rice chitinase gene *RICCHI11* via *Agrobacterium*-mediated transformtion

5.4.2.1 Materials and methods

Transgenic plantlets multiplied from the six independent lines were used in the bioassay with the fungal pathogen *Sclerotium rolfsii*. One mature, brownish black sclerotium was placed on the cut shoot base of one plantlet. One inoculated plantlet was placed on moistened filter paper in a Petri dish sealed to maintain 100% humidity. The Petri dishes were incubated at room temperature with a 10 h photoperiod. Three non-transformed plantlets and three transgenic plantlets per transgenic line (six lines total) were inoculated for each trial and each trial was repeated three times. After inoculation, the inoculated plantlets were observed for lesion initiation and extension daily after for three days. The lengths of the necrotic lesions on the shoots of plantlets were measured on the third day after inoculation and data was analyzed using the general linear model (GLM) program of SAS software (Statistical Analysis System, Cary, N.C.).

5.4.2.2 Results

Necrotic lesions of non-transformed plantlets were visible on day 1 after inoculation with the fungal pathogen *Sclerotium rolfsii*. In contrast, necrotic lesions on all six transgenic plantlet lines were visible only on day 2 or 3 after inoculation. The lengths of lesions measured at 3 days after inoculation were significantly shorter ($P < 0.05$) in all six transgenic lines with the chitinase gene *RICCHI11* compared to those of the non-transformed plantlet controls (Fig. 5.6; Fig. 5.7). On average, the lesions of transgenic lines were approximately

42-63% shorter compared to those of the controls (Fig. 5.6; Fig. 5.7). The non-transformed plantlets were dead, on average, 3.5 days after inoculation. In contrast, the transgenic plantlets died, on average, 5 days after inoculation.

5.4.3 Transgenic plantlets with the wheat oxalate oxidase gene *gf2.8* via *Agrobacterium-mediated transformtion*

5.4.3.1 Materials and methods

In the evaluation of disease response to *Sclerotium rolfsii*, transgenic plantlets multiplied from the independent line P5 with the *gf2.8* gene were used as the bioassay plant materials, and the fungal pathogen *Sclerotium rolfsii* was used as the pathogen material. One mature brown and black sclerotium was placed on the shoot base side of each plantlet. Each inoculated plantlet was placed on a water-soaked wet filter paper in a sealed Petri dish (100% humidity). Three non-transformed plantlets and three transgenic plantlets of the line P5 were treated for one trial. Ttrials were repeated three times. The inoculated plantlets were observed for lesion initiation and extension every day after inoculation. The lengths of the necrotic lesions on shoots of non-transformed plantlets and transgenic plantlets were measured at the third day after inoculation and data was analyzed using the general linear model (GLM) program of SAS software (Statistical Analysis System, Cary, N.C.).
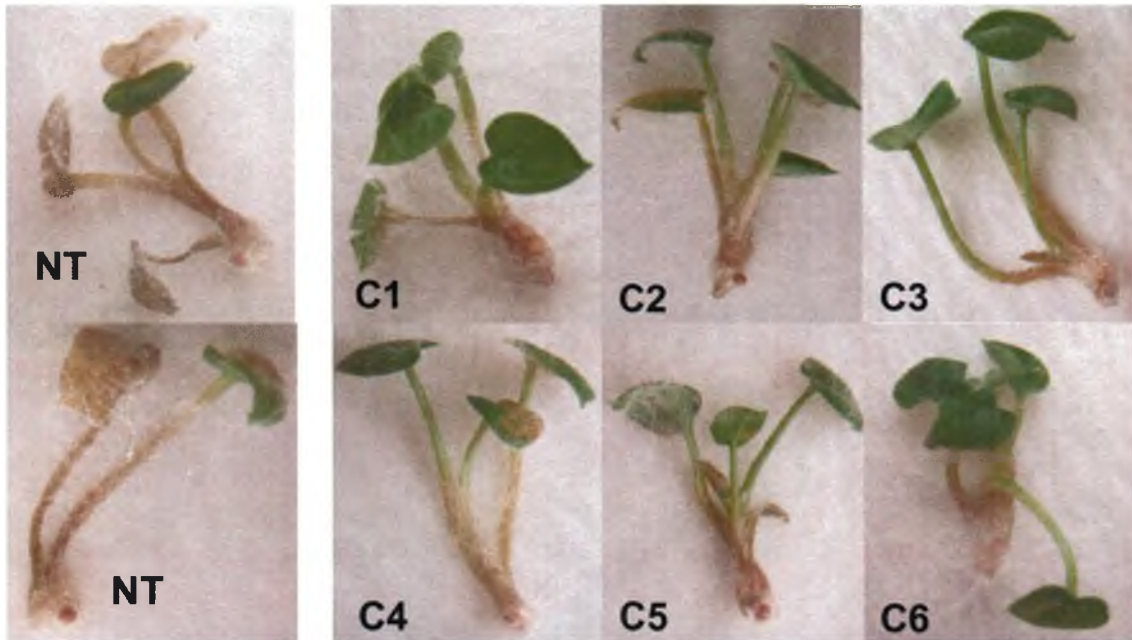
Fig. 5.6 Preliminary bioassay of transgenic taro cv. Bun Long plantlets (six transgenic lines C1-C6) with EHA105:pBI121/*RICCHI11* containing the rice chitinase gene *RICCHI11* and two non-transformed plantlets (NT) challeged with *Sclerotium rolfsii*, 3 days after inoculation. One mature, brownish black sclerotium was placed on the cut shoot base of each plantlet. Necrotic brown lesions of NT were visible and extended throughout whole plantlet at day 3 after inoculation. Necrotic brown lesions of C1-C6 were significantly shorter than NT at day 3 after inoculation.
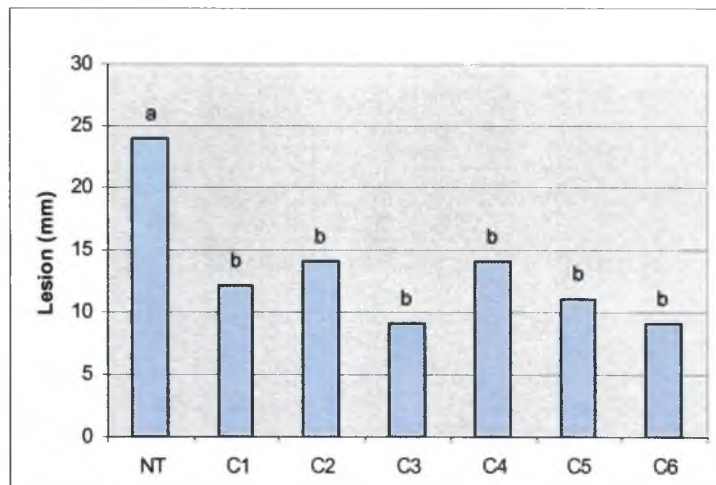
Fig. 5.7 Lesion length (mm) on *Sclerotium rolfsii*-inoculated shoots of transgenic taro cv. Bun Long lines C1-C6 with the rice chitinase gene *RICCHI11* and non-transformed control (NT) measured 3 days after inoculation. Different letters (a, b) indicate significant differences ($P < 0.05$) by the least significant difference (LSD) test of SAS (Statistical Analysis System, Cary, N.C.)

In the evaluation of disease response to *Phytophthora colocasiae*, transgenic plantlets multiplied from the independent line g5 with *gf2.8* gene were used as the bioassay plant materials, and the oomycete pathogen *P. colocasiae* was used as the pathogen. One plug of VA agar bearing active spores (approximate 0.2 cm^3) was placed spore-side down on the cut shoot base of each plantlet. Each inoculated plantlet was placed on moistened filter paper and sealed in a Petri dish to maintain 100% humidity. Three non-transformed plantlets and three transgenic plantlets of the line g5 were used in one trial. This trial was repeated three times. After inoculation, the inoculated plantlets were observed daily for lesion initiation and extension for 30 days.

5.4.3.2 Results

In the bioassay of response to *Sclerotium rolfsii*, necrotic lesions were visible in non-transformed plantlets on day 1 after inoculation. In contrast, necrotic lesions in transgenic plantlets of the line g5 were not visible until 2 or 3 days after inoculation. The lengths of lesions measured 3 days after inoculation were significantly shorter ($P < 0.05$) in transgenic plantlets of line g5 with the wheat oxalate oxidase gene *gf2.8* compared to those of the non-transformed plantlet (Fig. 5.8; Fig. 5.9). On average, the lesions of transgenic plantlets of line P5 were approximately 71% shorter in length compared to the controls (Fig. 5.8; Fig. 5.9). The non-transformed plantlets died at an average of 3.5 days after inoculation, whereas, the transgenic plantlets died at an average of 6 days after inoculation.

In the bioassay of response to *Phytophthora colocasiae*, soft, rotten lesions of non-transformed plantlets were visible 3 days after inoculation, and these lesions extended in size over time (Fig. 5.10). The non-transformed plantlets were dead, on average, 8 days after inoculation. In each transgenic plantlet of the line g5, hypersensitive response-like lesions were observed (Fig. 5.10). These small reddish brown lesions occurred at the inoculation area within three days after inoculation, and they did not extend much further even 30 days after

inoculation (Fig. 5.10). All transgenic plantlets of the line g5 were alive and appeared healthy (green leaves and shoots) 30 days after inoculation (Fig. 5.10). The invasion of the pathogen *Phytophthora colocasiae* was almost completely inhibited in transgenic plantlets of line g5.

5.4.4 Discussion

In conclusion, preliminary pathogen resistance analysis indicated that the single plant line transformed with the chitinase gene *RICCHI11* via particle bombardment transformation did not exhibit increased resistance to *Phytophthora colocasiae*. This result was not unexpected because this taro pathogen is a water mold and does not contain chitin. In the preliminary bioassay of response to the true fungal pathogen *Sclerotium rolfsii* all six transgenic lines transformed with the chitinase gene *RICCHI11* via *Agrobacterium* showed tolerance to the fungal pathogen *Sclerotium rolfsii*.

The transgenic plantlets with the wheat oxalate oxidase gene *gf2.8* also exhibited tolerance to the fungal pathogen *Sclerotium rolfsii*. Moreover, the transgenic plantlets with the wheat oxalate oxidase gene *gf2.8* exhibited hypersensitive response-like cell death when challenged by oomycete pathogen *Phytophthora colocasiae*. The invasion of this oomycete pathogen was largely inhibited in transgenic plantlets. Genetic transformation of taro with the chitinase gene *RICCHI11* or the wheat oxalate oxidase gene *gf2.8* is an encouraging and promising strategy to increase disease resistance against the major fungal and oomycete pathogens of taro.

5.5 Future work

All transgenic lines with either the rice chitinase gene or the oxalate oxidase gene are multiplied for further bioassay. The one line of transgenic taro with the rice chitinase gene via particle bombardment, the six lines transformed with the rice chitinase gene via *Agrobacterium*, and the two lines transformed with the wheat oxalate oxidase gene via *Agrobacterium* will be tested for increased resistance to the fungal pathogen *Sclerotium*

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rolfsii in the green house. In addition, the two transgenic lines with the wheat oxalate oxidase

gene will be tesed for increased resistance to the oomycete pathogen *Phytophthora*

colocasiae in the greenhouse. If increased disease resistance is found, then field tests of

transgenic taro will be planned. Molecular analysis of transgene integration in taro genome

will be conducted. For example, FISH (Fluorescence in situ hybridization) analysis will be

conducted to localize the transgene in taro chromosomes. Flanking sequencing will be

conducted to know more molecular information of transgene insertions and analyze whether

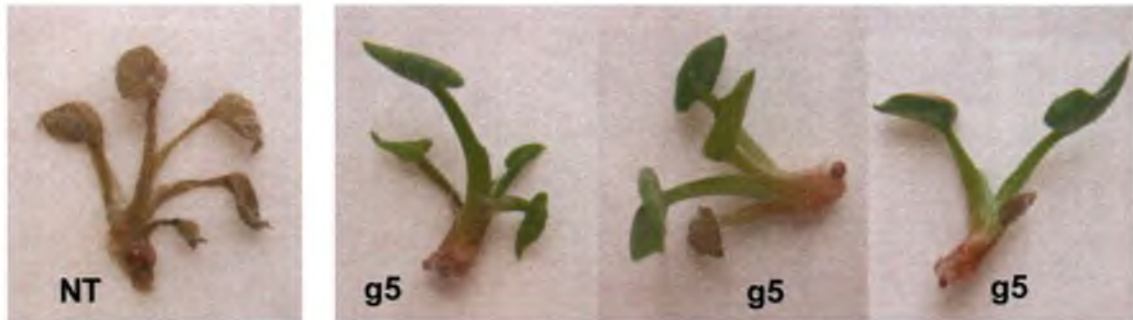transgene insertions affect other genes in taro genome.

Fig. 5.8 Preliminary bioassay of three replicates of transgenic plantlet line 5 (g5) with EHA105:pBI121/*gf2.8* containing the wheat oxalate oxidase gene, *gf2.8* and one non-transformed plantlet control (NT) challenged with *Sclerotium rolfsii* 3 days after inoculation. One mature, brownish black sclerotium was placed on the shoot base of each plantlet. NT showed brown, soft lesion from shoot base extending to whole plantlet. Three replicates of g5 exhibited green, turgid plantlets with infection restricted to brown base.
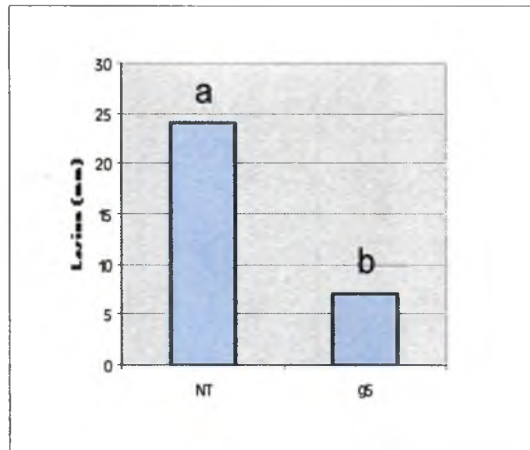
Fig. 5.9 Lesion length (mm) of *Sclerotium rolfsii*-inoculated shoots of transgenic taro cv. Bun Long line g5 with the wheat oxalate oxidase gene *gf2.8* and non-transformed control (NT) measured 3 days after inoculation. Different letters (a, b) indicate significant differences (*P*< 0.05) by the least significant difference (LSD) test of SAS (Statistical Analysis System, Cary, N.C.)

Fig. 5.10 Preliminary bioassay of one plantlet of transgenic taro cv. Bun Long line 5 (g5) with EHA105:pBI121/*gf2.8* containing the wheat oxalate oxidase gene, *gf2.8* and one non-transformed plantlet control (NT) challenged by *Phytophthora colocasiae* at 12 days after inoculation. NT showed soft, rotten lesion from inoculated shoot base to top, and leaves appeared yellow, soft. The g5 appeared green and healthy with hypersensitive-like response lesion (the small reddish brown lesion at inoculated shoot base) and they did not extend further

* arrow indicates hypersensitive-like response lesion

5.6 Literature cited

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