DEVELOPMENT OF A GREEN FLUORESCENT PROTEIN (GFP) EXPRESSION VECTOR FOR TRANSFORMATION OF POTATO RING ROT BACTERIUM

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This thesis is dedicated to my parents and my hometown.

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ABSTRACT

Potato bacterial ring rot caused by *Clavibacter michiganensis* subsp. *sepedonicus* (CMS) (formerly *Corynebacterium sepedonicus*), a gram-positive bacterium, is a big concern to seed and processing potato producers in Canada and worldwide. However, many issues, particularly the biology and the pathogenicity of CMS are not fully understood. To facilitate studies on these issues, a plasmid, pHN216 (13.8 kbp), previously modified from two wild type plasmids isolated from *C. michiganensis* subsp. *michiganensis* (CMM) was used as a cloning vector. Two variants of jellyfish (*Aquaria victoria*) green fluorescent protein (GFP) gene (*gfp*) were inserted in plasmid pHN216 in order to express GFP in CMS. GFP has been used as a reporter in various organisms including bacteria. In this study experiments were conducted to transform both CMS and CMM with the recombinant plasmid. Although CMS was the target of primary interest, transformation of CMM was also done since it is taxonomically closely related to CMS but is less fastidious and is the source of the original plasmid. CMM is a pathogen of tomato and does not infect potato. In this study, two recombinant plasmids, pHNG and pHNCG were constructed. CaMV 35S and *LacZ* promoters were cloned into pHNCG and pHNG upstream of the *gfp* gene, respectively. *Escherichia coli*, CMS and CMM cells were then transformed with these plasmids. A new method for the preparation of competent cells and for electroporation was developed in this study for enhancing the efficiency of transformation and all three bacterial species were successfully transformed with either pHNG or pHNCG. Glycine and PEG were used to treat cultured and re-suspended cells after harvesting to enhance their competency. Two electrical pulses (20 seconds interval, 1.5 Kv/cm, 600 Ω) were employed during electroporation. Transformed

bacteria were screened initially by antibiotic selection followed by purification of plasmids, and detection of GFP and target gene sequences, *e.g.* resistance gene for neomycin, promoter(s), and *gfp* gene. The presence of GFP in the transformed cells was monitored by fluorescence microscopy, ELISA and Western blotting. The sequences of both CaMV 35S promoter and *gfp* gene were confirmed by polymerase chain reaction, Southern hybridization, and DNA sequencing. The results of all tests showed that both pHNG and pHNCG were transformed into *E. coli*, CMM6 and CMS R2 cells. The level of GFP expression and green fluorescent signal in *E. coli* cells transformed with pHNCG was much higher than that detected in CMM6 cells. This is the first report to show successful GFP expression in *Clavibacter michiganensis*, a gram-positive bacterium. CaMV 35S promoter may play an important role for driving GFP expression in CMM6 cells since no expression was detected with the *Lac Z* promoter. GFP and fluorescent signal were not detected in CMM6 cells transformed with pHNG. CMS R2 cells transformed with either pHNG or pHNCG also did not express GFP although these bacteria were resistant to neomycin and were confirmed to carry the recombinant plasmid used. The new recombinant plasmid carrying a *gfp* gene under the control of CaMV 35S promoter can be used in CMM as a model system to optimize GFP expression in the gram positive bacterium, *Clavibacter michiganensis*. This may eventually lead to development of a suitable expression plasmid for use in CMS to study the biology and pathogenicity of CMS in potato.

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LIST OF ABBREVIATIONS

Amp	ampicillin
BCIP	5-brome-4-chloro-3-indolyphosphate
bp	base pair
BRR	bacterial ring rot of potato
$CaCl_2$	Calcium chloride
CaMV	cauliflower mosaic virus (*Caulimovirus*)
CIP	calf intestinal alkaline phosphatase
CMM	*Clavibacter michiganensis* subsp.*michiganensis* (formerly *Corynebacterium michiganensis*)
CMS	*Clavibacter michiganensis* subsp. *sepedonicus* (formerly *Corynebacterium sepedonicus*)
Da	dalton
DEPC	Diethyl pyrocarbonate
dH_2O	distilled water
DNase	deoxyribonuclease
dNTP	2'-deoxyribonucleotide triphosphate (*e.g.* dATP, dTTP, dGTP, and dCTP)
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid

EGFP	enhanced green fluorescent protein, the product of modified *gfp* gene
ELISA	enzyme linked immunosorbent assay
EtOH	ethanol
g	gravitational constant (9.8 m/s^2)
GFP	green fluorescent protein, the product of *gfp* gene
Gn	Gentamicin
HCl	hydrochloric acid
hr	hour(s)
IFM	immunofluorescence assay
IPTG	isopropyl-β-D-thiogalactopyranoside
KAc	potassium acetate
Kan	kanamycin
kb	kilobase
kbp	kilobase pair
Klenow	*E. coli* DNA polymerase I, Klenow fragment (or large fragment)
LB	Luria-Bertani (media)
M	molar
mA	milliampere
mbar	millibar (= 10^{-3} bar = 100 newtons per square meter)
MCS	multiple cloning site
µg	microgram

mg	milligram
µl	microliter
µM	micromolar
µm	micrometer
min	minute(s)
mM	millimolar
mm	millimeter
M_r	molecular weight
NaAc	sodium acetate
NaOH	sodium hydroxide
NBT	nitroblue tetrazolium
Neo	Neomycin
ng	nanogram
NH_4Ac	ammonium acetate
$(NH_4)_2SO_4$	ammonium sulfate
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
PEG 8000	polyethylene glycol molecular weight 8000
pmole	picomole
RNase	ribonuclease

rpm	rotation per minute
SDS	sodium dodecyl sulfate
sec	second(s)
SSC	1 ×SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0
TBE	1 ×TBE: 0.1 M Tris, 0.1 M Boric acid and 2 mM EDTA, pH 8.3
TBS	Tris buffered saline
TBY	Tryptone broth with yeast medium
TE	10 mM Tris-HCl, pH 8.0, 1 mM EDTA
Tris	Tris(hydroxymethyl)-aminomethane
UV	ultraviolet
V	volt
v/v	volume for volume
W	watt
w/v	weight for volume
YGM	bacterium culture medium containing yeast extract, glucose, and mineral salts
YT	bacterium culture medium containing yeast extract and tryptone

1. INTRODUCTION

1.1 *General information of potato ring rot disease*

Potatoes (*Solanum tuberosum* L.) are a crop of major significance in human nutrition, ranking fourth in world production, after wheat, corn, and rice. Potatoes are grown on approximately 50 million acres in more than 125 countries, with an annual production of about 250 million tons. Potatoes are grown across North America, with production concentrated in several areas of the northern United States and eastern and western Canada. North American potato production accounts for about 10% of the world total (Rowe, 1993). For the last ten years, potato production has increased at an annual average rate of 4.5%. More remarkable still is that as potato output continues to expand, the growth rate for area planted and production continues to accelerate (Database, International Potato Centre 2001).

The potato has hundreds of recognized pests and pathogens (disease agents, including many weeds, insects, nematodes, fungi, bacteria, and viruses). Because a potato crop is vegetatively propagated from tubers, which easily carry pathogens and pests, many diseases have followed potatoes to wherever they are grown. Bacterial diseases (e.g. bacterial ring rot, bacterial soft rot, blackleg, pink eye, and common scab) are particularly important as tuber borne contamination. Bacterial ring rot (BRR) is one of the many serious diseases of potatoes, and is one of the main reasons for the rejection of seed lots in potato certification programs in North America. This disease is a big concern to seed potato producers as well as commercial processing potato producers due to the highly contagious bacterium carried by the seed tubers,

the significant yield loss at harvest and in storage, and destructive damage to tuber quality (Gudmestad and Secor, 1992).

Bacterial ring rot of potato was originally reported in Germany in 1906 and has since been found in many other areas. This disease was first found in Canada in 1931 and in the United States a year later. By 1940, the disease had been found in most North American potato production areas. It often has caused major potato yield losses in Canada, but since the early 1970's rigid seed inspection procedures and disease control programs have kept outbreaks of this disease to a minimum (Evans and Otrysko, 1994; Manzer and Genereux, 1980). The objective in Canada is to systematically eradicate bacterial ring rot (De Boer and Slack, 1984). A zero tolerance level has been assigned to potato bacterial ring rot for both import and export of seed potatoes in Canada, the United States, and countries in the European Community (Shepard and Claflin, 1975; Munro, 1978; Anon, 1990, and Clayton and Slack 1988). In Canada, there are also restrictions (e.g. seed potato certification, field inspection and post harvest tuber testing) in several provinces including Alberta, British Columbia, New Brunswick and Prince Edward Island.

1.2 *The causal organism of potato ring rot disease*

The causal organism of potato ring rot is *Clavibacter michiganensis* subsp. *sepedonicus* (Davis *et al.*, 1984)(formerly *Corynebacterium sepedonicus* [Spieck & Kott.] Skapt. & Burkh.). It is a Gram-positive, nonmotile bacterium. Cells are 0.4~0.6 × 0.8~1.2 μm and predominantly wedge-shaped, although curved and straight rods are also present.

Single cells are most abundant, but V and Y configurations are often observed. Growth on all media is slow, and colonies rarely exceed 1 mm in diameter after five days on nutrient glucose agar. The colonies on nutrient agar are white, thin, translucent and glistening. Cells are non-acid-fast. Gelatin is not liquefied and nitrates are not reduced. Acid is produced from arabinose, xylose, dextrose, galactose, fructose, sucrose, maltose, cellobiose, manitol and salicin, but not rhamnose. The optimum temperature for growth in culture is 18 to 21°C, with a maximum of 30°C (Schaad *et.al.* 2001).

1.3 *Symptoms of potato ring rot disease*

In nature, the ring rot bacteria are capable of causing disease only in potato, but under experimental conditions they can also infect tomato (*Lycopersicon* spp.), eggplant (*Solanum melongena* L.) and other *Solanum* species. Young eggplant and tomato plants have been used in the bioassay to demonstrate the presence of ring rot bacteria in suspected potato plants. Ring rot bacteria have also been reported to colonize sugar beet (*Beta vulgaris* L.) roots under field conditions but do not cause disease in this crop. The role of sugar beet in the epidemiology of potato ring rot in areas where both potato and sugar beet are grown is unknown (Bugbee *et al.* 1987; Bugbee and Gudmestad 1988).

Symptoms of infected potato plants in the field may vary with cultivar and overall crop condition and can range from latent infection to distinctive symptoms. Leaf symptoms usually do not appear until after flowering, but certain cultivars, such as Russet Burbank, may develop an early season dwarf rosette. More conspicuous foliar symptoms generally appear during

middle to late growing season, and usually begin on middle and lower leaves. Wilted leaflets are slightly rolled at the margins, and light green to pale yellow areas develop in the interveinal spaces. As wilting progresses, the affected leaves become necrotic and symptoms move upward until the entire stem wilts and dies. The most important diagnostic symptoms used in the field are one or more wilted stems in a hill and a milky exudate that can be squeezed from the stem near the point of attachment to the mother tuber. Vascular tissues in those stems may appear brown. Under cool growing conditions or in fields with high levels of soil nitrogen, plants may not develop symptoms but still produce infected tubers. Infected tubers may be symptomless at harvest and can take two or three months to develop ring rot symptoms in storage (Bonde and Covell, 1950; Guthrie, 1959; Stead, 1993).

The characteristic tuber symptom of ring rot is a decay of the tissues of the vascular rings. Vascular discolouration is usually most apparent at the stem end of the tuber, which may exudate a milky or cheesy ooze when squeezed. This decay usually occurs after the appearance of foliar symptoms, but it does not always develop in the tubers of infected plants. Badly affected tubers exhibit ragged skin cracks, reddish-brown discolouration near the eyes, and may collapse into a semi-liquid soup when handled. Secondary invaders, notably soft rot bacteria, can cause a foul-smelling decay that masks the symptoms of ring rot (Kreutzer and McLean, 1943). Symptoms of potato ring rot disease are shown in Fig.1.

1.4 *Disease cycle of potato ring rot disease*

Potato ring rot bacteria normally overwinter in infected tubers, in storage or those that

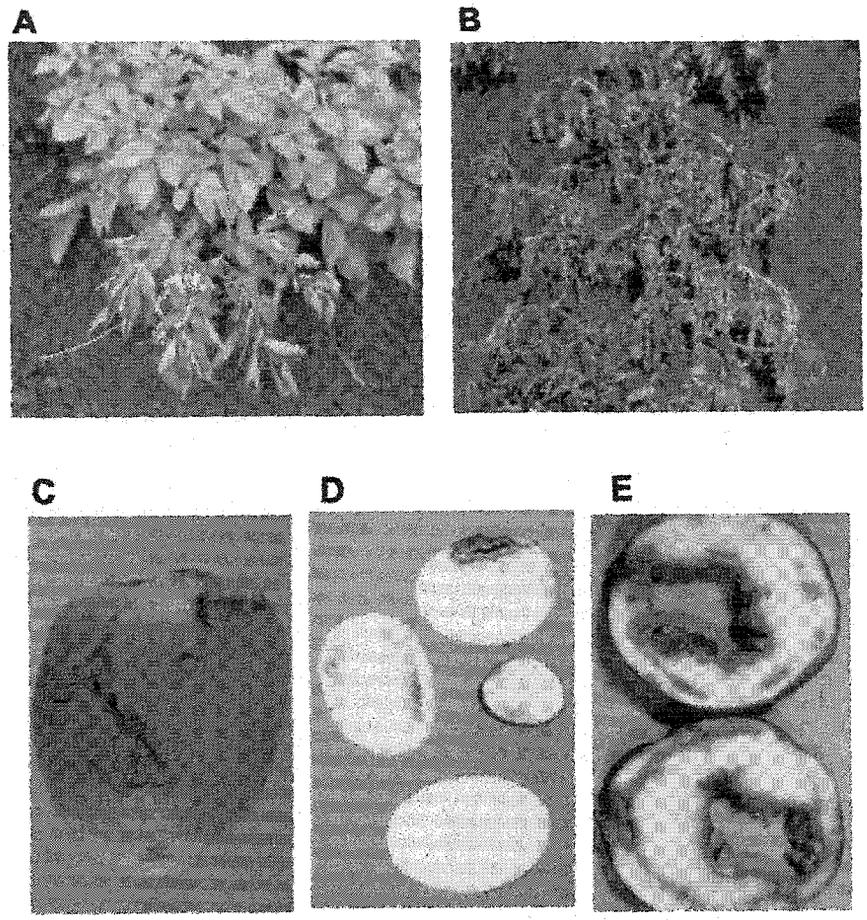


Fig. 1. Symptoms of potato bacterial ring rot disease caused by *Clavibacter michiganensis* subsp. *sepedonicus*. a: wilted stem; b: wilted stems and premature dieback of leaves and plants; c: skin cracking of tuber; d: moderately severe internal rot of tuber; e: severe internal rot of tuber (Evans and Otrysko, 1994).

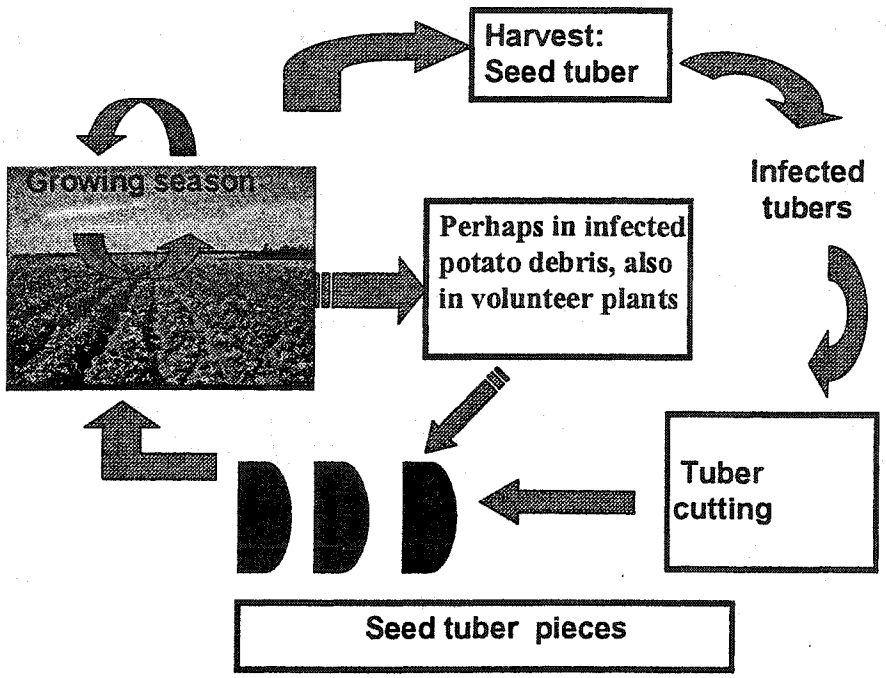


Fig. 2. Life cycle of potato bacterial ring rot disease. After the growing season the seed tubers are harvested. Potato tubers are cut into seed pieces and used to grow new plants in the following season. Bacteria in the infected tubers are thus transferred into the next life cycle through seed tuber cutting. This is belived to be the major way this bacterium moves from region to region. The other possible way is that the bacterium in infected potato debris, also in volunteer plants serves as an inoculum for the next cycle

survive the winter in the field (See Fig.2 for the disease cycle). The bacterium cannot survive in non-sterilized soil, but it may remain viable for many months on dry surfaces such as bags, crates and walls at temperatures below freezing (Nelson, 1980). However, the viability of the organism is rapidly lost in warm and moist soil. Wounds are necessary to enable ring rot bacteria to penetrate seed pieces. Tuber infection generally occurs through wounds from seed-cutting knives, picker planters and harvesters. Conditions for the spread of ring rot are most favourable at planting when tubers are cut into seed pieces. Bacteria from infected tubers are smeared onto freshly cut seed surfaces by cutting knives. Extremely high levels of infection can occur from very few infected tubers. If the infected plants exceed 5% of total plants in a field, there will be a high risk of the entire crop rotting in storage (Nelson, 1982). Reports from the United States suggest that Colorado potato beetle (*Leptinotarsa decemlineata*) and green peach aphid (*Myzus persicae*) can serve as vectors for this pathogen (Christie *et al.*,1991). Disease develops most rapidly at 18-22°C soil temperatures, but higher temperatures decrease infection from seed piece inoculation. In general, warm, dry weather hastens symptom development, but temperatures above optimum delay symptom expression (Bishop and Slack, 1987).

1.5 *Diagnosis of potato ring rot*

The diagnosis of potato ring rot is usually based on the characteristic leaf or tuber symptoms. The bacterium invades the vascular tissue of stems and tubers and can cause severe wilting of the foliage and decay of the tubers. The bacterium can also be present in potato

stems and tubers without manifestation of macroscopic symptoms (Stead, 1993; De Boer *et al.*, 1994). Such symptomless (or latent) infections are of particular concern because the only control for the disease is avoidance of the pathogen (De Boer and Slack, 1984). Because the etiologic agent is mainly borne by tubers used for planting, avoidance is achieved by planting tubers that do not exhibit ring rot symptoms and that were harvested from ostensibly healthy plants.

Visual inspection to determine the presence of ring rot infections is not reliable because many factors can affect the symptom expression and cannot be used to determine whether asymptomatic infections are present. Visual inspection for symptoms also is frequently inadequate because symptoms are generally not expressed until late in the growing season when natural senescence and other diseases are present. Various procedures, such as the examination of tubers under ultraviolet (UV) light and Gram staining (Lachance *et al.*, 1962), have been implemented from time to time in an attempt to determine whether a crop of symptomless potatoes is free of the ring rot pathogen. The Gram stain has been used for many years as an easy and rapid testing method. The appearance of blue-stained cells of the characteristic size and shape in the exudate squeezed from a suspected stem or tuber is regarded as a positive test (De Boer and Copeman, 1974; De Boer *et al.*, 1992).

Serological methods including agglutination test, immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) have been developed and adopted for detecting potato ring rot bacterium. ELISA and IFA based on monoclonal antibodies (MAbs) have proven to be useful for BRR testing in Canada and in some European countries (Claflin and

Shepard, 1977; De Boer *et al.*, 1986; De Boer *et al.*, 1988; De Boer and Copeman, 1980; Dinesen and De Boer, 1995). Pathogenicity tests with eggplant (*Solanum melongena*) are sometimes employed as a confirmatory procedure. In recent years, some progress in diagnosis has been made by using polymerase chain reaction (PCR) for detecting the bacterial ring rot pathogen. PCR is more sensitive and accurate than any other diagnostic methods (Schneider *et al.*, 1993; Rademaker and Jane, 1994; Li and De Boer, 1995; Slack *et al.*, 1996).

1.6 *Questions related to pathogenesis to be answered*

The life cycle and the infection mechanism of this disease and the molecular biology of the pathogen are poorly understood although this pathogen has been known for almost 100 years. Many questions related to pathogenesis of BRR have not yet been answered. Do bacteria introduced on leaves infect the progeny tubers? How do the bacteria move from the seed piece to progeny tubers (*via* the vascular system, *via* the soil, external to the plant)? How does the location of bacteria in seed tubers affect the movement of bacteria into the plant and development of ring rot disease? What environmental factors (*e.g.* soil temperature, saturation, and aeration) before and after planting affect proliferation and movement of the bacteria in the seed piece and sprout or plant? The development of molecular biology in recent years can provide some useful means for studying the biology of *C. michiganensis* subsp. *sepedonicus* and for understanding the mechanism of the pathogenesis of these bacterium.

1.7 *Plasmids found in potato ring rot bacterium*

Previous studies have shown that many isolates of *C. michiganensis* subsp. *sepedonicus* harbour a 51 kb native plasmid whose function is unknown, (Clark and Lawrence, 1986; Mogen and Oleson, 1987; Mogen *et al.*, 1988) and produces extracellular polysaccharides (Gorin *et al.*, 1961; Spencer *et al.*, 1961), a putative toxic glycopeptide (Strobel, 1967, 1968, 1970) and an extracellular cellulase (Goto and Okaba, 1958; Baer and Gudmestadand, 1995). A recombinant plasmid, pHN216 (13.8 kb) modified from the native plasmid, pCM2 of *Clavibacter michiganensis* subp. *michiganensis* was constructed and evaluated for the transformation of *C. michiganensis* subsp. *sepedonicus* (Laine *et al.*, 1996). Transformation of a cellulase-deficient mutant of *C. michiganensis* subsp. *sepedonicus* with pHN216-C8, a construct containing a reporter marker, (β-1,4-endoglucanase-encoding gene), demonstrated that the production of cellulase was restored (Laine *et al.*, 1996).

1.8 *Plasmids associated with Clavibacter michiganensis subp.michiganensis*

Clavibacter michiganensis subp.*michiganensis* (CMM) is the causal agent of tomato (*Lycopersicon esculentum* L.) bacterial canker, a highly contagious and destructive disease in tomato production (Strider, 1969). Wilting of lower leaflets is normally the first observable symptom of this disease. Older leaflets curl upward, progressively die from the margin inward and turn brown (Menzies and Jarvis, 1994). Further symptoms include plant stunting, discolouration of vascular tissue, open stem cankers and plant death (Pitblado and Tartier, 1994). *Clavibacter michiganensis* subp. *michiganensis* is taxonomically related to *Clavibacter michiganensis* subp. *sepedonicus*. Like CMS, CMM is also a Gram-positive

bacterium, measuring 1×0.5 μm. However, in contrast CMM is a motile bacterium. It is non-acid fast, non-spore forming and non-lipolytic. It liquefies gelatin slowly and can oxidise carbohydrates (Menzies and Jarvis, 1994). The optimum temperature for the growth of CMM in culture medium is 24 to 32°C (Hayward and Waterston, 1964).

Plasmids, pCM1 (27.5 kb) and pCM2 (72 kb) were isolated from *Clavibacter michiganensis* subp. *michiganensis* (Meletzus and Eichenlaub, 1991). Based on these CMM native plasmids, recombinant plasmids, pDM3, pDM100, pDM302, pDM302-B1 and pDM306 were developed (Meletzus and Eichenlaub, 1991; Meletzus *et al.*, 1993). Some of these recombinant plasmids (pDM302, pDM302-B1 and pDM306) were used for the transformation of *C. michiganensis* subsp. *michiganensis* cells and further studies allowed researchers to identify genes whose products cause wilting of tomato plants (Meletzus *et al.*, 1993).

1.9 *The discovery and application of green fluorescent protein*

Many researchers have found that in the bioluminescent jellyfish *Aequoria victoria*, light is produced when energy is transferred from the Ca^{2+}-activated photoprotein aequorinn to the green fluorescent protein (GFP) (Shimomura *et al.*, 1962; Morin and Hastings, 1971; Ward *et al.*, 1980). The cloning of the wild-type GFP gene (*gfp*) and its subsequent expression in heterologous systems has established GFP as a novel genetic reporter system (Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Inouye and Tsuji, 1994; Wang and Hazelrigg, 1994). This protein can be expressed in many heterologous systems including plants, animals,

viruses, and bacteria. Illuminated by blue or UV light, GFP yields a bright green fluorescence. Light-stimulated GFP fluorescence is species-independent and does not require any cofactors, substrates, or additional gene products from *A. victoria*. Additionally, detection of GFP and its variants can be performed with living tissues instead of fixed samples (Chalfie *et al.*, 1994; Baulcombe *et al.*, 1995; Galbraith *et al.*, 1995; Haseloff and Amos, 1995; Hu and Cheng, 1995; Oparka, *et al.*,1995; Sit *et al.*, 1998).

Bacteria including *Agrobacterium tumefaciens, Bacillus subtillis, Escherichia coli, Mycobacterium* sp. and *Pseudomonas* sp. have been transformed for GFP expression (Yu and den Engh, 1995; Webb *et al.*, 1995; Lewis and Errington, 1996; Andersen *et al.*, 1998). GFP has been used in some of these bacteria for visualization of cell-specific gene expression and subcellular protein localization (Webb *et al.*, 1995; Lewis and Errington, 1996). However, no successful GFP expression has been reported in the gram-positive bacterium, *Clavibacter michiganensis* to date.

A 3.3 kb recombinant plasmid, pGFP, that carries the complete GFP coding sequence has been constructed by Shimomura *et al.* (1962). Plasmid pGFP and its modified types, enhanced GFP (EGFP) and mutant GFP (mGFP), *etc.* are now commercially available (Clontech Laboratories, Inc.). Plasmid pGFP (or pEGFP) has two multiple cloning sites (MCS's): the 5' MCS lies immediately upstream from the GFP start codon and the 3' MCS lies downstream of the GFP termination codon. The expression of GFP in *E. coli* and plant cells driven by the *Lac-Z* promoter has been demonstrated (Reichel *et al.*, 1996). The *gfp* gene is now broadly used as a reporter gene in many research fields including studies of the biology and

pathogenecity of plant pathogens (Heinlein et al., 1995; Santa Cruz et al., 1996; Sit *et al.*, 1998).

1.10 *Objectives of the study*

The primary objective of this study was to develop a recombinant plasmid containing *gfp* gene and use such a plasmid to transform *C. michiganensis* subsp. *sepedonicus* (CMS) cells. These transformed CMS cells would then be useful for monitoring or visualizing the distribution and movement of transformed CMS cells in the infected plants. Alternatively, if CMS cells could not be transformed or express GFP, then since CMS and *C. michiganensis* subsp. *michiganensis* (CMM) are closely related and plasmid pHN216 was developed as an expression vector based on CMM-derived plasmid (Meletzus and Eichenlaub, 1991; Laine *et al.*, 1996), CMM would be transformed with gfp gene containing recombinant plasmid.

GFP has previously been used for the visualization of cell-specific gene expression and subcellular protein localization in some bacteria (Webb *et al.*, 1995; Lewis and Errington, 1996). However, no successful GFP expression has been reported in *C. michiganensis* subsp. Both CMS and CMM are gram-positive bacteria. The transformation of a *gfp* gene containing plasmid into CMS and/or CMM for a successful expression of *gfp* gene is an apparent challenge in this study. Therefore, various strains of CMS and/or CMM will be evaluated for their potential for transformation and GFP expression. Modifications will be made to the methods for competent cell preparation, electroporation and recovery of transformed cells.

To validate successful transformation of CMS and/or CMM, the presence of specific

sequences, *e.g.* antibiotic resistance gene, promoter(s), and *gfp* gene and the expression of GFP in the transformed cells will be evaluated by various serological and molecular methods. The production of GFP will also be monitored by detection of protein and fluorescent signal.

2. MATERIALS AND METHODS

2.1 *Bacterial isolates*

Isolates of potato ring rot bacterium used in this study were *Clavibacter michiganensis* subsp. *sepedonicus*(CMS) and related bacterium *C. michiganensis* subsp.*michiganensis* (CMM) from the collection at Canadian Food Inspection Agency (CFIA), Centre for Animal and Plant Health (CAPH) Charlottetown PEI. *C. michiganensis* subsp. *michiganensis* isolate harboring plasmid pHN216 (Fig. 3) was kindly provided by Dr. Mary C. Metzler (Department of Biology, Laboratory of Plant Physiology and Molecular Biology, University of Turku, FIN-20520 Turku, Finland). *Clavibacter michiganensis* subsp. *sepedonicus*(CMS) was formerly named as *Corynebacterium sepedonicus* [Spieck & Kott.] Skapt. & Burkh.)(Davis *et al.*, 1984). This gram-positive, nonmotile bacterium (Fig. 4) and CMM were grown in standard YGM (yeast extract, glucose, mineral salts) medium (De Boer and Copeman, 1980) at 26-28°C with shaking at 250 rpm. Also the colonies were cultured on YGM supplement with 1.8% agar under the same conditions.

E. coli DH5α (Invitrogen Life Technologies) was used in this study for general gene cloning and for the propagation and amplification of plasmid or recombinant plasmid. *E. coli* DH5α cells were generally cultured using YT medium (1× YT broth: 0.8% Bacto-tryptone, 0.5% Yeast extract, 0.5% NaCl, pH7.5) or LB(lennox L broth) (1× LB broth: 1.0% Bacto-tryptone, 0.5% yeast extract and 1.0% NaCl, pH 7.5) as described by Sambrook *et al.*, 1989.

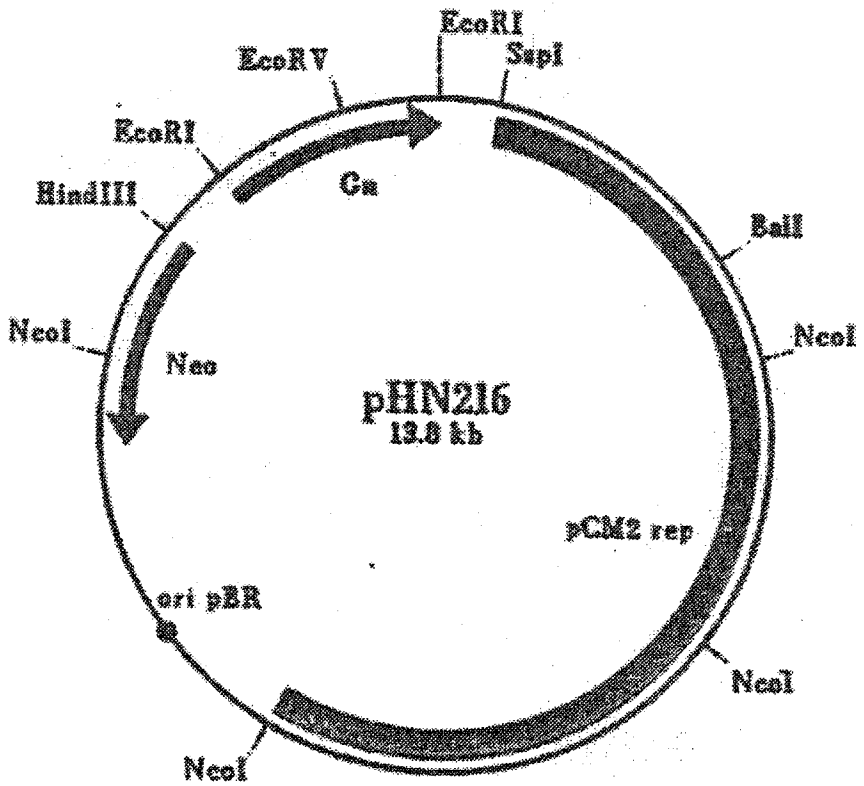


Fig. 3. Plasmid DNA pHN216. This plasmid was modified from native plasmid found in CMM cells and contains a Neomycin (Neo) and a Gentamycin (Gn) resistant gene. This plasmid also contains the origins of replication from pBR and from pCM2, a native plasmid found in CMM cells. (Laine *et al.*, 1996)

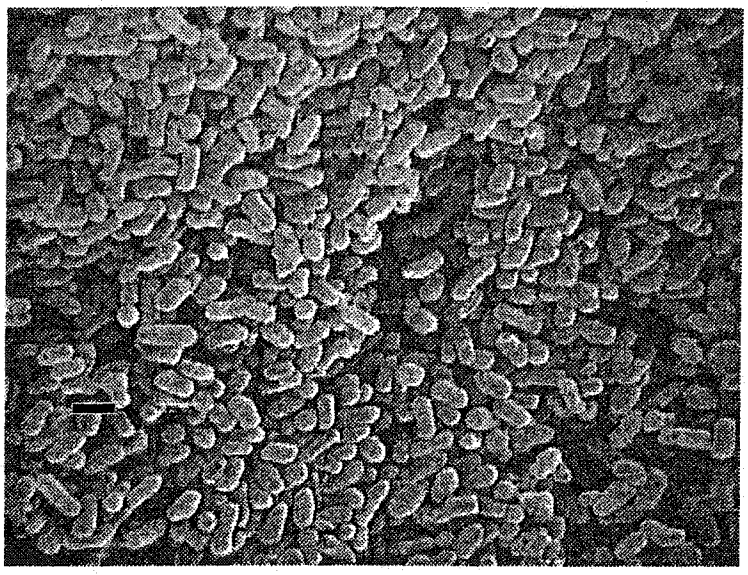


Fig. 4. CMS cells. This scanning electron microscopy image shows the typical cells of *Clavibacter michiganensis* subsp. *sepedonicus*. The cells of *C. michiganensis* subsp. *sepedonicus*, and *C. michiganensis* subsp. *michiganensis* are quite similar. Scale bar in lower left equals 1μm (Courtesy Dr.S.H. De Boer).

2.2 *Preparation of plasmid DNA, pHN216*

2.2.1 Extraction of total DNAs from CMM containing pHN216

A single colony of *C. michiganensis* subsp. *michiganensis* (CMM) cells containing plasmid pHN216, was selected and grown in 2 ml of YGM medium at 26-28°C for two days with constant shaking at 250 rpm. The cells were collected by centrifugation at 12,000 ×*g* for 5 min. The total DNA was then extracted from the cultured cells according to the method described by Ward and De Boer (1990). The cells were resuspended in 1 ml of total DNA extraction buffer (50 mM Tris-HCl, pH 8.0, 25 mM EDTA, 1% SDS and 10 µg/ml of Proteinase K) and the cell suspension mixture was incubated at 55°C for 3 hrs. Five hundred µl of 7.5 M ammonium acetate (NH_4Ac) were added to each tube and mixed by vortexing for 2 - 3 seconds. The mixture was incubated at 4°C (on ice) for 30 min followed by centrifugation at 18,000 ×*g* at 4°C for 10 min. The supernatant was transferred to a fresh tube and 1 volume of ice cold isopropanol was added and mixed by gentle inversion. The sample was stored at -20 or -70°C for 8 - 10 hrs (or overnight). The precipitates were collected by centrifugation at 18,000 ×*g* for 30 min at 4°C. The pellets were washed twice each with 750 µl of ice cold 70 % ethanol (EtOH), drained and dried. Pellets were dissolved in 50 - 100 µl of autoclaved dH_2O at room temperature and the DNA suspension was then stored at -20°C for future use.

2.2.2 Preparation of *E. coli* DH5α competent cells

The competent cells of *E. coli DH5α* were prepared by the cold calcium chloride ($CaCl_2$) method (Sambrook *et al.*, 1989). Two ml of LB broth were inoculated with 10 µl of *E. coli* DH5α glycerol stock solution or a single colony and incubated at 37°C overnight with vigorous and constant shaking (300 rpm). The next day, 0.3 ml of this overnight culture were mixed with 30 ml of LB broth and incubated at 37°C with constant shaking (300 rpm) for about 1.5 to 2 hrs to a density of about 5×10^7 cells/ml. The OD reading was about 0.8 units at 600 nm for *E. coli* DH5α. The cells were cooled on ice for 5 min and collected by centrifugation at 4,000 ×*g* for 5 min at 4°C (4,000 rpm, Sorvall SS34 rotor). The cell pellets were resuspended in half of the original volume of ice-cold calcium chloride (15 ml), kept on ice for 20 min followed by centrifugation as above. The supernatant was discarded and the cells were resuspended in 1/15 of the original culture volume (2 ml) of ice-cold 50 mM $CaCl_2$ and chilled on ice before use.

2.2.3 Transformation of *E. coli* DH5α competent cells with total DNA from CMM

The competent cells of *E. coli* DH5α were transformed with the total DNA extracted from CMM by the heat-shock method (Sambrook *et al.*, 1989). Twenty µl of total DNA were added to a pre-chilled 1.5 ml Eppendorf tube along with 200 µl of competent cells. The mixture was gently vortexed and incubated on ice for 30 min. The mixture was then heat-shocked at 42°C for 2 min. One ml of LB broth was then added into the tube and incubated at 37°C for 40 min to one hour. The cells were centrifuged for 10 to 15 seconds at 12,000 ×*g* in

a micro-centrifuge with most of the supernatant being discarded except for about 200-300 µl which was kept. The pellets were redissolved by vortexing and spread evenly onto the LB agar plate [LB broth with 1.5% (w/v) Bacto-agar] containing 75 µg/ml gentamicin (Gn.). The plates were inverted and incubated at 37°C overnight.

2.2.4 Screening for plasmid DNA, pHN216 in transformed *E. coli* DH5α cell lines

To screen the transformed *E. coli* DH5α cells for the presence of pHN216, plasmid DNA extraction was conducted. Transformed bacterial colonies were selected and inoculated individually into 2 ml of LB broth containing 75 µg/ml Gn. and incubated at 37°C overnight with constant, vigorous shaking (300 rpm). The plasmid DNA was extracted from the bacteria by the rapid alkaline extraction procedure (Birnboim and Doly, 1979), but the volume of each solution I, II and III were scaled up 1.5 times. Overnight cultures were centrifuged in a 1.5 ml Eppendorf tube. The cells were then resuspended with 150 µl of solution I [25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose and lysozyme (3 mg/ml)] and then chilled on ice for 5 min. The cells were lysed completely with 300 µl of freshly prepared solution II (0.2 M NaOH, 1% SDS). After the mixture was incubated on ice for 5 min, 225 µl of solution III [each 100 ml solution III: 60 ml of 5 M sodium acetate (NaAc) or 60 ml of 5 M potassium acetate (KAc), 11.5 ml of glacial acetic acid, pH 4.8)] were added to the mixture to neutralize the lysates and precipitate the chromosomal DNA. At the same time, the protein-SDS complex was precipitated. The mixture was incubated on ice for 15 min. After centrifugation for 10 min at 16,000 ×*g* in an Eppendorf centrifuge, the supernatant was mixed with 675 µl of ice-cold isopropanol

and kept on ice for another 10 min. Plasmid DNA was collected by centrifugation for 12 min at 16,000 ×*g* in an Eppendorf centrifuge, and then dissolved in 100 µl of 0.1× TE (1 ×TE: 10 mM Tris-HCl, 1 mM EDTA, pH8). The preparations were analyzed by agarose gel electrophoresis in1% agarose gel containing Ethidium bromide at a concentration of 1 µg/ml in TBE buffer (0.1 M Tris-HCl, 0.1 M Boric acid and 2 mM EDTA, pH 8.3). For this, 2 µl of the plasmid DNA were mixed with 1 µl of 6 × loading dye [0.25% xylene cyanol FF (w/v), 30% glycerol (v/v)] containing RNase A (0.04 µg/ml), incubated at room temperature for 5 to 10 min, and electrophoresed at 90 volt (V) for 1.5 hr. Lambda DNA doubly digested with *Hin*dIII and *Eco*RI and/or 100 bp DNA ladder (New England Biolabs) were used as molecular weight (*Mr*) markers. DNA bands were visualized under of 304 nm UV light (Sambrook *et al.*, 1989), and the gels were photographed.

2.2.5 Restriction endonuclease analysis of pHN216

To confirm the specific restriction site of pHN216, restriction enonuclease analysis was performed. The plasmid pHN216 was digested with various restriction endonucleases e.g. (*Hind*III and *EcoR*I from New England Biolabs or Invitrogen Life technologies) using the digestion conditions recommended by the manufacturers. For single digestion, approximately 0.5 µg of purified plasmid DNA was digested in a 20 µl reaction volume containing the appropriate buffer and enzyme (1-5 units per 0.1 µg of DNA). The reaction mixture was incubated at 37°C (or appropriate temperature for the enzyme used) for 1.5 to 2 hrs. For double digestion, the enzyme requiring the lowest ionic strength was added first and the reaction

mixture was pre-incubated for about 45 min before increasing the ionic strength to that of the buffer of the second enzyme which was then added and allowed to digest for another 50 min. Eight µl of each restriction digest were mixed with 2 µl of 6 × loading dye and electrophoresed as described in Section 2.2.4.

2.3 *Preparation of plasmids pEGFP and pCAMBIA 1303*

2.3.1 Transformation of *E. coli* DH5α competent cells with pEGFP and pCAMBIA 1303

Plasmids pEGFP (concentration: 1 µg/µl)(Fig. 5) and pCAMBIA 1303 (concentration: 0.5 µg/µl)(Fig. 6) were obtained from Clontech Laboratories, Inc. These plasmids were transformed into *E. coli* DH5α competent cells in this study. The competent cells of *E. coli* DH5α were prepared by the cold $CaCl_2$ method (see section 2.2.2) and the heat shock method was used for the transformation (see section 2.2.3). After transformation the cells were spread on plates of 2 ×YT agar (1.6% Bacto-tryptone, 1% yeast extract, 1% NaCl, 1.5% agar, pH7.5)(Sambrook *et al.*, 1989) containing 75 µg/ml ampicillin (Amp) and the plates were then inverted and incubated at 37°C overnight.

2.3.2 Propagation of pEGFP and pCAMBIA 1303 transformed cells and the purification of these two plasmids

Transformed bacterial colonies were selected and inoculated individually into 2 ml of LB broth containing 75 µg/ml of Amp and incubated at 37°C overnight with constant, vigorous

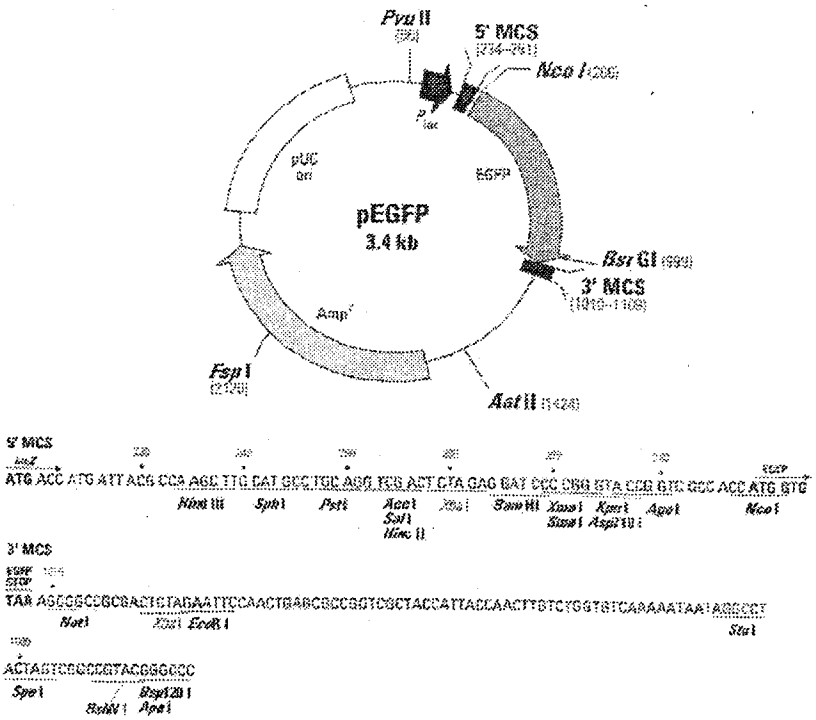


Fig. 5. Plasmid DNA pEGFP. Plasmid pEGFP (3.4 kb) carries enhanced green fluorescent protein gene (*gfp*, approximately 800 bp in length) and a *LacZ* promoter in front of the *gfp* gene. There are two multiple cloning sites (MCS) flanking the EGFP sequence. (Clontech laboratories, Inc.)

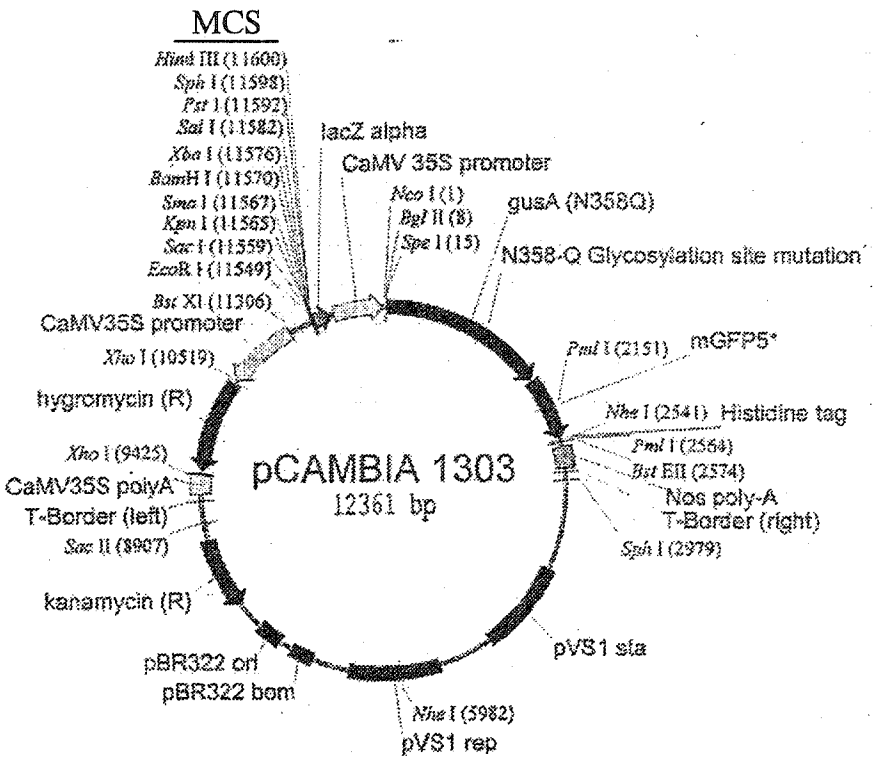


Fig. 6. Plasmid DNA pCAMBIA 1303. This plasmid (12361 bp) carries a mutant *gfp* gene (mGFP) and two copies of CaMV 35S promoter sequence, one located on the upstream of mGFP and one located on the upstream of the hygromycin resistant gene. It also has a kanamycin resistant gene. A multiple cloning site (MCS) is located in between two CaMV 35S promoters(Clontech laboratories, Inc.).

shaking (300 rpm). The plasmid DNA was extracted by the rapid alkaline extraction procedure (Birnboim and Doly, 1979) with modifications (see section 2.2.4). The plasmid DNA extracted from each 2 ml of culture medium was dissolved in 100 µl of 0.1 ×TE and stored at -20°C for future use.

2.3.3 Restriction endonuclease analysis of pEGFP and pCAMBIA 1303

Purified plasmids, pEGFP and pCAMBIA 1303 were evaluated by restriction digestion to confirm the presence of restriction site *Hind*III, *Eco*RI, and *HindIII and SphI* in the plasmids (Figs.5 and 6). The restriction enzymes were purchased either from New England Biolabs or from Invitrogen Life technologies and the digestion conditions were as recommended by the manufacturer (see section 2.2.5).

2.4 *Long-term storage of transformed bacterial cells*

The transformed bacterial cells were treated for long-term storage according to the method described by Sambrook *et al.* (1989). Two ml of overnight cultured transformed bacterial cells in the presence of Amp (75 µg/ml) or Gn (75 µg/ml) were harvested by centrifugation at 16,000 ×*g* for 15 seconds in an Eppendorf centrifuge. Cell pellets were completely suspended in 400 µl of 10 mM $MgSO_4$ and then 400 µl of glycerol were added to the mixture. The solution was mixed thoroughly by vortexing. The treated bacterial cells were then kept at -70°C for long term storage.

2.5 *Preparation of cloning vector and insert DNAs*

2.5.1 Preparation of the DNA fragment of *LacZ* promoter-*egfp* gene

The plasmid pEGFP (Fig. 5) was doubly digested with *Pvu*II and *Eco*RI to release a fragment containing the *LacZ* promoter-*egfp* gene. Firstly, about 5 μg of purified plasmid pEGFP was digested in a 50 μl reaction volume containing 50 mM NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.025% Triton X-100, pH7.5 and 2.5 μl of restriction enzyme *Eco*RI (10 units/μl). The reaction mixture was incubated at 37°C for 1.5 to 2 hrs. The digest was extracted once with one volume of Tris-saturated phenol followed by two extractions each with 2 volumes of chloroform: isoamyl alcohol (24:1). The DNA was then precipitated with 2 volumes of 95% ethanol and 0.25 M potassium acetate (KAc) at -20°C for 20 min followed by centrifugation. The pellets were dissolved in 17 μl of dH_2O and mixed with 1 μl of the second restriction enzyme, *Pvu*II (10 units/μl) and 2 μl of 10 × NEBuffer 2 [1×: 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH7.9)] (New England Biolabs) were added and allowed to digest for another 1.5 to 2 hrs.

Twenty μl of the restriction digest were then loaded in 1% agarose gel for electrophoresis. Low melting temperature agarose and TAE buffer (0.0 4M Tris-HCl, pH8.0, 0.02 M glacial acetic acid, 1 mM EDTA) were used for extracting the DNA fragment of *LacZ* promoter-*egfp* gene from the gel after electrophoresis. The DNA fragment of *LacZ* promoter-*egfp* gene (971 bp in length) was extracted from the gel using a QIAquick Gel Extraction Kit (Qiagen). The procedures used were as those recommended by the kit manufacturer. The

DNA fragment was excised from the agarose gel with a clean, sharp scalpel. Extra agarose was removed to minimize the size of the gel slice. The gel slice was weighed and placed in a 1.7 ml centrifuge tube. Three volumes of the gel slice weight of Buffer QG were added into each tube (100 mg of agarose, 300 µl of buffer). The tube was incubated at 50°C for 10 min, or until the gel slice was completely dissolved. The mixture was vortexed every 2-3 min during the incubation. One gel volume of isopropanol was added to each tube and mixed by inverting the tube several times. The solution was then applied to the MinElute column followed by centrifugation at 16,000 ×g for 1 min. The flow-through solution was collected in a tube attached to the MinElute column and discarded. The DNA binding to the MinElute column was washed once with 500 µl of Buffer QG and then with 750 µl of Buffer PE each for 1 min following the manufacturer's instructions. A fresh, clean 1.5 ml microcentrifuge tube was used to replace the collection tube and 10 µl of Buffer EB (10 mM Tris-HCl, pH 8.5) was added to the center of the membrane of the MinElute column. The column was kept at room temperature for 1 min followed by centrifugation as above for 1 min. The DNA harvested in the 1.5 ml tube was then kept at -20°C for future use.

2.5.2 Preparing the DNA fragment of CaMV 35S promoter-*gfp* gene

The plasmid pCAMBIA 1303 (Fig.6) is 12.361 kbp in size and contains the *gfp* gene, which is controlled by CaMV 35S promoter. The DNA fragment including one of the CaMV 35S promoters *gfp* gene and the 3'-nos terminator is located downstream from the multiple cloning site (MCS). A *Sph*I restriction site is located immediately downstream from the NOS

terminator (Fig. 6). To release the DNA fragment of CaMV 35S promoter-*gfp* gene (3.7 kbp) from pCAMBIA 1303, the plasmid DNA was simultaneously digested with *Hind*III and *Sph*I in a 50 µl reaction volume containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH7.9 and 20 units of each restriction enzyme (New England Biolabs). The 3.7 kbp fragment resulted from the digestion was extracted from low melting temperature agarose gel using a QIAquick Gel Extraction Kit (Qiangen)(see section 2.5.1). The DNA fragment harvested in the 1.5 ml tube was then kept at -20°C for future use.

2.5.3 Linearizing plasmid pHN216

Plasmid pHN216 was linearized by restriction endonuclease *Hind*III (New England Biolabs) using the digestion conditions recommended by the manufacturer. About 5 µg of purified plasmid pHN216 were digested in a 50 µl reaction volume containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH7.9 and 20 units of *Hind*III (New England Biolabs). The reaction mixture was incubated at 37°C for 1 to 1.5 hr.

One volume of TE buffer was added into the digestion mixture and the mixture was extracted with one volume of Tris-saturated phenol followed by two extractions each with two volumes of chloroform: isoamyl alcohol (24:1). Centrifugation was performed after each extraction at 16,000 ×*g* for 5 min. The DNA in the aqueous layer (top) was precipitated with 2.5 volumes of 95% ice-cold ethanol and 0.25 M KAc at -70°C for 20 min. DNA was collected by centrifugation at 16,000 ×*g* for 15 min. The pellets were rinsed with 70% EtOH, vacuum-dried and then resuspended in 32 µl of dH_2O.

2.5.4 Filling in the ends of the linearized pHN216 with Klenow fragment of *E.coli* T4 DNA Polymerase I

The ends of pHN216 linearized by *Hind*III (see section 2.5.3) were filled in with the Klenow fragment of *E. coli* DNA polymerase I in a reaction volume of 50 µl containing 32 µl of linearized plasmid DNA, 5 mM DTT, 7 mM Tris-HCl, pH 7.5, 50 mM NaCl, 7 mM $MgCl_2$, 0.5 mM of each dATP, dTTP, dGTP, and dCTP, and 5 units of *E. coli* DNA polymerase I, Klenow fragment (Invitrogen Life technologies). The reaction was conducted at 37°C for 1 hr and terminated by the addition of 50 µl TE, pH 8.0 and the mixture was passed through a Sephadex G50 spin column. The mixture was extracted once with one volume of Tris-saturated phenol followed by two extractions each with two volumes of chloroform: isoamyl alcohol (24:1). The DNA in the aqueous layer were then precipitated with 0.25 M KAc and 2.5 volumes of ice-cold 95% ethanol at -70°C for 20 min followed by centrifugation at 16,000 ×*g* for 15 min. The pellets were re-dissolved in 46 µl of dH_2O.

2.5.5 Further digestion of pHN216 linearized by *Hind*III and Filling in the ends by Klenow fragment of DNA Polymerase I with *Eco*RI

To generate a compatible end of the vector with the insert for cloning, further digestion of pHN216 linearized by *Hind*III and filling in the ends by Klenow fragment of DNA Polymerase I with *Eco*RI was performed. The plasmid, pHN216 linearized by *Hind*III was filled in (see section 2.5.4) and further digested with *Eco*RI in a 50 µl reaction volume containing 46 µl of DNA (see section 2.5.4) and 2 µl of 10 × *Eco*RI buffer (1 ×: 50 mM

NaCl, 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.025% Triton X-100, pH7.5) and 2 µl of *Eco*RI (10 units/µl). The reaction mixture was incubated at 37°C for 1.5 to 2 hrs. The digest was extracted once with one volume of Tris-saturated phenol followed by two extractions each with 2 volumes of chloroform: isoamyl alcohol (24:1). The DNAs were then precipitated with 2 volumes of 95% ethanol and 0.25 M KAc at -20°C for 20 min and harvested by centrifugation at 16,000 ×*g* for 15 min. The pellets were re-dissolved in 50 µl of dH_2O and stored at -20°C for further use.

2.6 *Construction of recombinant plasmid using linearized pHN216 as a vector and the LacZ promoter-egfp gene as an insert*

The 971 bp DNA fragment of *LacZ* promoter-*egfp* gene (as insert) was ligated to the plasmid pHN216 (as vector) linearized with *Hind*III and end-filled with the Klenow fragment of *E. coli* DNA polymerase I followed by *Eco*RI digestion (see section 2.5.5). The recombinant plasmid was named pHNG (Fig. 7). The ligation reaction mixture (total 50 µl) consisted of 0.5 µg of vector and 0.112 µg of the insert, 10 µl of 5 × ligation buffer [1 ×: 50 mM Tris, pH8.0, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT and 5% polyethylene glycol-8000 (PEG-8000)], and 1 unit of DNA ligase (Invitrogen Life technologies). The molar ratio of insert to the vector was 3:1. All the reagents were combined in a 0.5 ml Eppendorf tube, and the reaction was conducted at 15°C for 12-16 hrs. The ligation mixture was chilled on ice before transformation.

Competent cells of *E. coli* DH5α prepared by the cold $CaCl_2$ method (see section

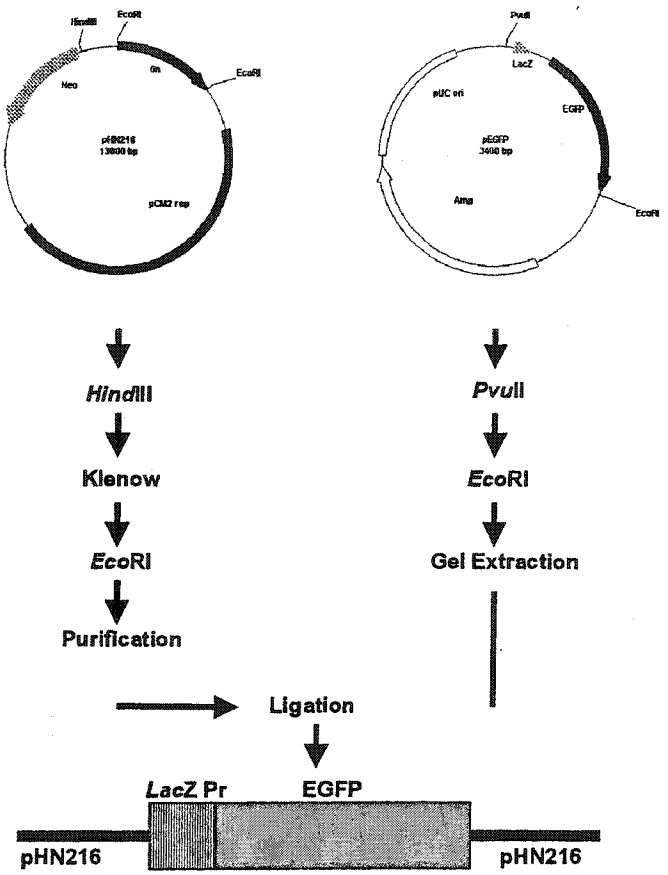


Fig. 7. Construction of recombinant plasmid pHNG. The *LacZ* promoter - enhanced *gfp* gene fragment excised from pEGFP is ligated to the sites of *Hind*III and *Eco*RI of plasmid pHN216. Gentamycin resistant gene *(Hind*III - *Eco*RI - *Eco*RV - *Eco*RI) on pHN216 is removed. The new construct of recombinant plasmid retains the Neomycin resistant gene.

2.2.2) were transformed with the ligation mixture containing recombinant plasmid pHNG. Ten µl of ligation mixture was used for every 200 µl of competent cells in the transformation by the heat-shock method (Sambrook *et.al.* 1989). The cells were then spread evenly onto LB agar plate [LB broth with 1.5% (w/v) Bacto-agar] containing 50 µg/ml of Neomycin (Neo.). The plates were inverted and incubated at 37°C overnight for colony development.

Individual colonies that developed on the LB agar plates were selected and cultured in 2 ×YT broth containing 50 µg/ml of Neo as described previously. The recombinant plasmid pHNG was then purified by the modified alkaline purification method (see section 2.2.4).

2.7 *Construction of recombinant plasmid using linearized pHNG as a vector and the CaMV 35S promoter-gfp gene as an insert*

2.7.1 Linearization of the recombinant plasmid pHNG

The plasmid pHNG was doubly digested with restriction endonucleases *Hind*III and *Sph*I (New England Biolabs) following the manufacturer's recommendations. Approximately 5 µg of purified plasmid DNA was digested in a 50 µl reaction volume containing 5 µl of 10 × NEBuffer 2, 1 µl of *Hind*III (20 units /µl) and 2 µl of *Sph*I (10 units /µl). The reaction was conducted at 37°C for 1 to 1.5 hr and the digested DNA was extracted once with one volume of Tris saturated phenol and twice with two volumes of chloroform: isoamyl alcohol (24:1). The DNA in the aqueous layer (top) was precipitated with 2.5 volumes of 95% ice-cold ethanol and 0.25 M KAc at -70°C for 20 min. DNA was collected by centrifugation at 16,000 ×*g* for

15 min. The pellets were rinsed with 70% EtOH, vacuum-dried and then resuspended in 50 µl of dH_2O for further use.

2.7.2 Ligation of CaMV 35S promoter-*gfp* gene fragment and linearized pHNG

The 3.7 kbp DNA fragment of CaMV 35S promoter-*gfp* gene prepared previously (see section 2.5.2) was used here as an insert and the linearized plasmid pHNG (see section 2.7.1) was used as a cloning vector in this experiment. The insert was ligated into the vector in a 50 µl of reaction volume consisting of 0.2 µg of vector and 0.16 µg of the insert, 10 µl of 5 × ligation buffer and 1 unit of DNA ligase (Invitrogen Life Technologies). The molar ratio of insert to vector was 3:1. All the reagents were combined in a 0.5 ml Eppendorf tube, incubated at 15°C for 12-16 hrs. The new construct of recombinant plasmid DNA was then named as pHNCG (Fig.8). The ligation mixture was chilled on ice before transformation.

2.7.3 Transformation of *E. coli* DH5α competent cells with pHNCG

The competent cells (200 µl) of *E. coli* DH5α prepared by the cold $CaCl_2$ method (see section 2.2.2) were transformed with 10 µl of ligation mixture (from above) by the heat-shock method (Sambrook et al., 1989), and spread evenly onto the LB Agar plate [LB broth with 1.5% (w/v) Bacto-agar] containing 50 µg/ml of Neo. The plates were inverted and incubated at 37°C overnight for colony development.

Individual colonies that developed on the LB agar plates were selected and cultured in 2 ×YT broth containing 50 µg/ml of Neo as described previously. The recombinant plasmid

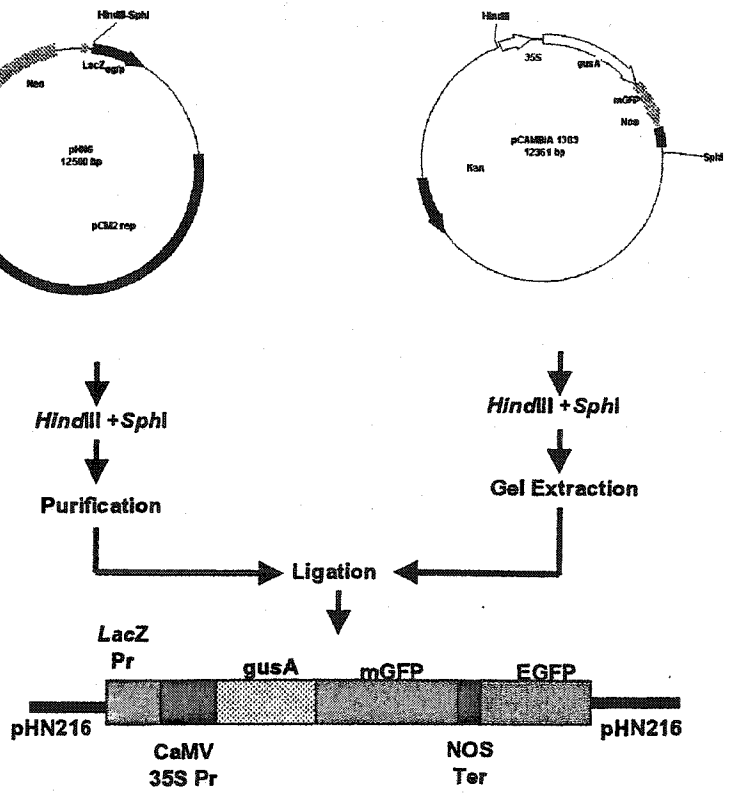


Fig. 8. Construction of recombinant plasmid pHNCG. A DNA fragment (3.7 kbp) of the CaMV 35S promoter - gusA - mGFP - NOS terminator excised from pCAMBIA 1303 is ligated to the sites of *Hind*III and *Sph*I of pHNG, just upstream of the enhanced *gfp* gene.

DNA, pHNCG, was then purified by the modified alkaline plasmid DNA purification method described above from the cultured cells for further use and analysis.

2.8 *Transformation of CMM and CMS competent cells with recombinant plasmid DNA by a modified electroporation method*

2.8.1 Evaluation of various CMM/CMS strains cultured on YGM agar plates

Several CMM and/or CMS strains were obtained from the collection of bacterial isolates at CAPH. The bacterial strains of *C. michiganensis* subsp. *sepedonicus* R2, R9, R12, R14 and *C. michiganensis* subsp. *michiganensis* CMM6 were streaked on YGM agar plates (1.5% agar in YGM). The plates were incubated at 23°C for 5-7 days. Individual colonies were selected and spread on YGM agar plates. The plates were incubated at 23°C for another 3-4 days.

2.8.2 Preparation of CMM/CMS competent cells

2.8.2.1 Method 1

A single colony of CMM or CMS was inoculated into 2.5 ml of YGM broth and cultured at 25°C for 2 days with constant shaking (250-300 rpm). The overnight culture was then diluted in 30ml of YGM broth and grown under the same conditions to an optical density of 1.0 at 580nm (OD_{580}=1.0). The cells were harvested by centrifugation at 2806 ×*g* for 10 min at 4°C . The cells were washed once with ice-cold water followed by centrifugation. The cells were then washed with 10% glycerol and collected by centrifugation. The cells were

suspended in 10% glycerol to an OD_{650} of 0.8 (Laine *et al*, 1996) and then used directly for electroporation.

2.8.2.2 Method 2

To enhance the competency of the cells, a second method was used for the preparation of competent cells. A single colony of CMM or CMS cells was inoculated into 2.5 ml of TBY Broth (1% Tryptone, 0.5% yeast extract, 0.5% NaCl, pH7.5)(Kirchner *et al.*, 2001) and cultured at 25°C overnight with constant shaking (250-300 rpm). The overnight culture was then diluted in TBY Broth to give an OD_{580} of 0.3 and further cultured (approximately 2 hrs) at 25°C with shaking to an OD_{580} of 0.6. Glycine solution (20%) was then added to a final concentration of 2.5%. The mixture was incubated for an additional hour under the same conditions. The cells were harvested by centrifugation at 2806 $\times g$ for 10 min at 4°C . The cells were washed once with ice-cold water followed by centrifugation. The cells were then washed with 10% glycerol and collected by centrifugation. The cells were suspended in 10% glycerol to an OD_{650} of 0.8 (Manzer and Genereux, 1980; Kirchner *et al*, 2001) and used for electroporation.

2.8.3 Electroporation for the transformation of CMM/CMS with recombinant plasmids pHNG and pHNCG

Transformation efficiency using two electrorporation procedures, Methods A and B, developed previously (Laine *et al*, 1996, Kirchner *et al*, 2001) were extremely low and it was

difficult to obtain transformed CMM/CMS cell colonies. Therefore, a modified procedure, Method C, was developed in this study for improving the efficiency of electroporation.

2.8.3.1 Method A

Competent cells of CMM/CMS (20 µl) prepared by Method 1 were mixed with 0.1-5 µg of recombinant plasmid pHNG or pHNCG and 20 µl of ice-cold 40% PEG-8000 in a precooled 0.1 cm Gene Pulser cuvette (Bio-Rad) on ice. The cuvette was then placed in a shocking chamber, and the cells were exposed to two pulses. The time interval between pulses was 20 sec by using a field strength of 15 kV/cm and a resistance of 600 Ω (Manzer and Genereux, 1980). The electroporation conditions for 0.1 ml cuvettes were 2.5 µF, 15 kV/cm and 600 Ω. The cells were treated with two successive pulses with an interval of 20 sec, resulting in a time constant of 12 to 16 milli seconds (ms). Immediately after the pulse, the cells were mixed with 0.4 ml of SB broth (1% Tryptone, 0.5% Yeast extract, 0.4% NaCl, 8.37% Sorbitol, and 20 nM each of $CaCl_2$ and $MgCl_2$)(Yoshihama *et al.*, 1985) and incubated for 3 hrs at 25°C with constant shaking. The cells were then plated on YGM agar plates containing 50 µg/ml of Neo. The plates were incubated at 23°C for two days before the colonies were examined (Laine *et al*, 1996).

2.8.3.2 Method B

Competent cells of CMM/CMS (100 µl) prepared by using Method 2 were mixed with 0.01-5 µg of recombinant plasmid pHNG or pHNCG in a pre-cooled 0.2 cm Gene Pulser

cuvette (Bio-Rad) on ice. The cuvette was then placed in a shocking chamber, and the cells were exposed to 25 μF, 12.5 kV/cm, and 600 Ω, resulting in a time constant of 12 to 16 ms. Immediately after the pulse, the cells were mixed with 0.4 ml of SB broth (1% Tryptone, 0.5% Yeast extract, 0.4% NaCl, 8.37% Sorbitol, and 20 nM each of $CaCl_2$ and $MgCl_2$) (Yoshihama *et al.*, 1985) and incubated for 3 hrs at 25°C with constant shaking. The cells were then plated on SB media agar plates containing 50 μg/ml of Neo. The plates were incubated at 23°C for two days before the colonies were examined (Manzer and Genereux, 1980; Kirchner *et al*, 2001).

2.8.3.3 Method C - modified from methods A and B

Competent cells of CMM/CMS (20 μl) prepared by using Method 1 were mixed with 0.01-5 μg of recombinant plasmid pHNG or pHNCG and 20 μl of ice-cold 40% PEG-8000 in a pre-cooled 0.1 cm Gene Pulser cuvette (Bio-Rad) on ice. The cuvette was then placed in shocking chamber, and the cells were exposed to two pulses. The time interval between pulses was 20 sec by using a field strength of 15 kV/cm and a resistance of 600 Ω (Manzer and Genereux, 1980). The electroporation conditions were 2.5 μF, 15 kV/cm and 600 Ω when the 0.1 cm cuvettes were used. The cells were treated with two successive pulses with an interval of 20 sec, resulting in a time constant of 12 to 16 ms. Immediately after the pulse, the cells were mixed with 0.4 ml of SB broth (1% Tryptone, 0.5% Yeast extract, 0.4% NaCl, 8.37% Sorbitol, and 20 nM each of $CaCl_2$ and $MgCl_2$)(Yoshihama *et al.*, 1985) and incubated for 3 hrs at 25°C with constant shaking. The cells were then plated on TBY agar plates containing

50 μg/ml of Neo. The plates were incubated at 23°C for two days before the colonies were examined.

2.9 *Screening transformed cells of E. coli, CMM, and CMS*

2.9.1 Fluorescence microscopy

The colonies that grew on the agar plate were examined under fluorescence microscopy (FM) (1000 ×, wave length: 450-500 nm). The agar plates were examined on the UV transilluminator and the colonies that produced a fluorescent signal were selected for FM examination. Each selected colony was divided into two parts, one was resuspended in 100 μl 0.01 × PBS solution (1 ×PBS: 137 mM NaCl, 2.68 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2) and the other part was cultured in appropriate media broth containing 50 μg/ml Neo. The PBS cell suspension (40 μl) was loaded on the window of a FM slide and air-dried. A coverslip was mounted on the slide with 5 μl of 90% glycerol in PBS. The slide was examined under oil immersion at 1000 × with a epi-iluminating light source of 450-500 nm.

2.9.2 Colony PCR amplification

To confirm the identity of the *gfp* gene, CMM, and the sequence of CaMV 35S promoter, the colony PCR was preformed. Individual colonies that grew on agar plates were picked up with a sterile Eppendorf pipette tip and inoculated onto replica agar plates. The remaining cell material from the colony was resuspended in 50 μl of sterile water. The cell

suspension was boiled for 5 min followed by centrifugation at 16,000 ×*g* for 10 min in an Eppendorf centrifuge at 4°C. The supernatant was chilled on ice and a 1 µl aliquot was used as template in PCR amplification.

The primer set specific to the *gfp* gene, EGFP-F (5'-ACG AAC TCC AGC AGG ACC ATG-3') and EGFP-R (5'-TGG TCG AGC TGG ACG GCG ACG-3') was purchased from Clontech and the expected size of the PCR product flanked by these two primers was 608 bp. The primer set specific to CMM, CMM-F (5'-CTT GTG GGG TGG GAA CGT-3') and CMM-R (5'-TCT TTG TGA TCC ACC GGA A-3') was based on published sequences (Li and De Boer, 1995) to target the intergenic spacer region between the 16S and 23S rRNA genes of *Clavibacter michiganensis* subspecies and the size of the PCR product expected was 215 bp. The primer set specific to CaMV 35S 35S-S (5'-GCT CCT ACA AAT GCC ATC A-3') and 35S-A (5'-GAT AGT GGG ATT GTG CGT CA-3') was synthesized by Promega based on the sequences provided by Clontech.

One µl of the template was amplified in a total volume of 25 µl containing 1.5 mM $MgCl_2$, 50 µM of each dNTPs, 0.5 µM of each primer and 2.5 units of Taq Gold DNA polymerase (BIO/CAN Scientific) in a buffer supplied with the polymerase. The reaction was carried out in a thermocycler (Thermolyne Amplitron II, Dubuque, IA) as follows: initial denaturation for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at primer optimized temperatures 60°C for 1 min, and extension at 72°C for 1 min. The final extension was performed at 72°C for 5 min. PCR products were separated on a 1.5 % agarose gel stained with ethidium bromide and visualized under UV light.

2.9.3 Modified mini-prep procedure for the purification of recombinant plasmid DNA

Transformed bacterial (*E. coli*) colonies were picked and inoculated individually into 2 ml of 2 ×YT broth containing 75 μg/ml Neo and incubated at 37°C overnight with constant shaking (300 rpm). All selected colonies were screened by the rapid alkaline extraction procedure (Birnboim and Doly, 1979) with some modifications (all solutions were scaled up 1.5 times) for the presence of recombinant plasmid DNA. After final resuspension of plasmid DNA pellets, one volume of 5 M ammonium acetate (NH_4Ac) was added and the mixture was incubated on ice for 20 min followed by centrifugation at 16,000 ×*g* for 12 min at 4°C. The supernatant was transferred into a fresh tube and the plasmid DNA was precipitated with 2 volume of 95% EtOH at -20°C for 20 min (or overnight) followed by centrifugation as above. The DNA pellets were then dissolved in 100 μl of 0.1 × TE and stored at -20°C. The plasmid DNA was analyzed by using 1% agarose gel stained with ethidium bromide (1 μg/ml in TBE) electrophoresis under the conditions described above.

2.9.4 Restriction analysis of recombinant plasmid pHNG

To characterize newly cloned DNA fragment *LacZ* promoter and *egfp* gene in vector of pHN216, the recombinant plasmid pHNG was analyzed by restriction mapping. Purified recombinant plasmid, pHNG containing the *LacZ* promoter and *egfp* gene was digested with restriction endonuclease *Xba*I (Invitrogen Life Technologies) under the conditions recommended by the supplier. About 2 μg of purified plasmid DNA was digested in a 20 μl reaction volume containing 10 mM Tris-HCl, pH 8.3, 2 mM KAc, 5 mM NaCl and 10 units

of *XbaI*. Plasmids pHN216 and pEGFP were used as controls for *Xba*I digestion. The reaction mixture was incubated at 37°C for 1 to 1.5 hr. Eight microliters of each restriction digest were then mixed with 2 µl of 6 × loading dye and examined by agarose gel electrophoresis as previously described (see section 2.2.4).

2.9.5 Western Blotting

To test the expression of GFP fusion proteins, Western Blotting was conducted. Transformed bacteria were collected by centrifugation at 2,806 ×*g* for 10 min at 4°C. The pellets were washed twice with 0.5 ml PBS and then resuspended in 200 µl of lysis solution (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA pH 8.0, and lysozyme 3 mg/ml) and incubated at 37°C for 15 min. Fifty microliters of the lysate were mixed with 10 µl of 6 × SDS sample buffer (62.2 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol and 0.01% of bromophenol blue). The mixture was boiled for 5 min and then loaded on a polyacrylamide gel (20 µl per well plus 10 µl of prestained SDS-PAGE standards in alternate inner wells of the gel). To prepare a mini SDS-PAGE gel, a 12% separating gel monomer solution was mixed with 4 ml of 30% acrylamide/Bis solution, 37.5:1(2.6% C) (Bio-Rad), 3.35 ml of Milli-Q water, 2.5 ml of 1.5 M Tris-HCl and 100 µl of 10% SDS. After degassing in a sidearm flask under vacuum for 15 min, 5 µl of TEMED (N,N,N',N'-teramethy-ethylenediamine) and 50 µl of freshly prepared 10% ammonium persulfate solution (100 mg of ammonium persulfate in 1 ml of milli-Q water) were added in. The mixture was quickly, but carefully poured into the assembled mini-gel sandwich avoiding the generation of bubbles and leaving enough space to

pour the upper stacking gel. Ethanol was overlaid on the separating gel immediately, after the separating gel polymerized (45-60 min), the ethanol was removed followed by a thorough rinse with water. The 4% stacking gel monomer solution was prepared as described for making the separating gel, except the 30% acrylamide solution was reduced from 4 ml to 1.3 ml, and the TEMED solution was increased from 5 µl to 10 µl. After the 4% stacking mixture was quickly poured into the assembled mini-gel sandwich on top of the separating gel taking care not to generate bubbles. The comb was carefully inserted into the stacking gel avoiding production of bubbles and the gel was kept at room temperature for 30 to 45 min for polymerization. The comb was then removed taking care not to break any of the wells, and the wells were rinsed thoroughly with water. Both the upper and lower buffer chambers of the electrophoresis cell were filled with 1 × Tris/Glycine/SDS electrophoresis buffer [25 mM of Tris, 192 mM of Glycine and 0.1% (w/v) SDS, pH 8.3]). The gel was run at 200 volts (constant voltage setting). The current was approximately 60 mA per gel (120 mA for two gels). The total running time was approximately 45 min when the front of the bromphenol blue in the dye reached the bottom of the gel. After the run was complete, one glass plate was removed and a notch was made in the top left hand corner of the gel providing a means of future orientation.

After electrophoresis, the proteins were transferred from the gel to an Immobilon-P membrane using standard techniques. The membrane was cut to the dimension of the gel, and soaked in 100% methanol for 15 sec and then in milli-Q water for 2 min. The membrane was equilibrated for at least 15 min in the transfer buffer. For assembling the transfer stack for a semi-dry system, the anode electrode plate was placed on a level bench top. The two sheets of

filter paper were soaked in the anode Buffer I (0.3 M Tris, 10% methanol, pH 10.4) and placed in the center of the graphite anode electrode plate. A sheet of filter paper was soaked in the anode Buffer II (25 mM Tris, 10% Methanol, pH 10.4), and placed on top of the first two sheets of filter paper. The membrane and gel were placed on top. Three pieces of filter paper were soaked in the cathode buffer (25 mM of Tris, 40 mM of 6-amino-n-caproic acid, 10% methanol, pH 9.4). They were then placed on the top of the gel. Finally the cathode plate was laid on the top of the assembled transfer stack. The black cathode lead (-) was inserted into the cathode plate jack. The red anode lead (+) was inserted into the anode plate jack. The anode lead and cathode lead were connected to their corresponding power supply outputs. The current was set at 1.2 mA/cm^2 and gel was run for 1-2 hrs. When the transfer was completed, the blotted membrane was removed with a pair of forceps.

The membrane was soaked in blocking buffer (5% skim milk powder, 0.2% Tween-20 in PBS, pH 7.4) and incubated at 4°C overnight with constant, gentle shaking. The primary peptide antibody specific to GFP protein (Clontech Laboratories, Inc.) was diluted at 1:1000 in blocking buffer to a concentration of approximately 1 mg/ml. The membrane was incubated in the diluted antibody solution at room temperature for 2 hrs with constant, gentle shaking. The membrane was washed twice with wash buffer (0.2% Tween-20 in PBS, pH7.4) each for 5 min. The secondary antibody - alkaline phosphatase conjugate (AP) (Clontech Laboratories, Inc.) was diluted in blocking buffer at a ratio of 1:3000. The membrane was then incubated with the diluted secondary antibody at room temperature for 1 hr with constant, gentle shaking. The membrane was washed four times with wash buffer each for 10 min with constant, gentle

shaking. After the membrane was equilibrated in AP buffer (10 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 10 to 15 min, the membrane was transferred into the substrate solution (200 µl of NBT/BCIP mix per 10 ml AP buffer) and incubated in the dark for 4-16 hrs at room temperature. The color development was examined periodically. When the reaction was completed, the membrane was transferred into TE buffer to stop the reaction.

2.9.6 Enzyme-linked immunosorbent assay

To test the expression of GFP fusion proteins, ELISA was performed. A 96-well microtiter plate was coated with the primary antibody (full-length A. V. Polyclonal Antibody) specific to GFP protein (Clontech Laboratories, Inc.) diluted in coating buffer (100 mM carbonate buffer, pH 9.6) at a ratio of 1:1000. Two hundred microliters of antibody solution was added to each well. The plate was covered with parafilm and incubated at 4°C overnight. The antibody solution was removed and the plate was washed thoroughly with wash buffer (0.05% Tween-20 in PBS, pH7.4). Blocking buffer (1% BSA in PBS) was then added into each well (200 µl/well) and the plate was incubated at room temperature. After 2 hrs of incubation, the blocking buffer was removed and the plate was washed two times with wash buffer. Samples (transformed bacterial cells treated with bacterial lysis solution, see section 2.9.5) were then added at 200 µl/well. The plate was covered with parafilm and incubated at 4°C overnight. The sample solution was removed and the plate was washed three times with wash buffer. Blocking buffer (1% BSA in PBS) was then added into each well (200 µl/well)

and the plate was incubated at 37°C for 2 hrs. After the incubation, the blocking buffer was removed and the plate was washed two times with wash buffer. A monoclonal antibody (MAb) specific to GFP protein (Clontech Laboratories, Inc.) was diluted at 1:1000 in blocking buffer and the diluted MAb solution was then added into each well (200 µl/well). The plate was incubated at 37°C for 2.5 hrs following by two washes with wash buffer. Alkaline phosphatase (AP) antibody (Goat anti mouse) conjugate (Clontech Laboratories, Inc.) was diluted at 1: 5000 in blocking buffer and 100 µl of the diluted conjugate solution were added into each well. The plate was incubated at 37°C for 2.5 hrs following three washes with wash buffer, and one wash with 1 ×PBS. The substrate solution NBT/BCIP (Roche Molecular Biochemicals), in AP buffer (100 mM Tris-HCl, pH9.5, 100 mM NaCl, 5 mM $MgCl_2$ was then added into each well (100 µl/well) and the plate was incubated at room temperature for 2-4 hrs. The microtiter plate was scanned at a wavelength of 405 nm and the OD readings were recorded.

2.9.7 Southern hybridization

To further confirm the presence of the gfp gene in bacterial cells, Southern hybridization was conducted.

2.9.7.1 Southern transfer

Southern hybridization was used to determinate whether the *gfp* gene was present in the PCR product obtained from amplification of total DNA extracted from transformed bacteria. After electrophoresis, the agarose gel was placed in a container, covered with 0.25 M HCl, and gently agitated for 5-10 min. The solution was removed and replaced with 0.4 M

NaOH, and gently agitated as above. The vacuum blotting apparatus, VacuumGene pump (Pharmacia Biotech) was assembled according to manufacturers instructions. A porous screen was first placed on the bottom half of the blotting unit and overlayed with a piece of filter paper that was completely saturated with 0.4 M NaOH. A piece of positively charged nylon membrane (Roche Molecular Biochemicals) was layered onto the saturated filter paper. The membrane was cut to a size that easily covered the dimensions of the agarose gel. A thin silicone gasket with a cut-out window was layered on the blotter. The window was cut to be slightly (0.1-0.3 cm) smaller than the actual gel dimensions and was centered over the membrane/filter paper combination. The upper part of the blotter was placed and clamped into position. Vacuum tubing was attached to the blotter and the pump was turned on. The vacuum valve was first set to it's minimum setting. NaOH (0.4 M) was pipetted onto the gel and the vacuum was increased to about 60 mbar and held for 2-3 min to seal the gel firmly on the membrane. While maintaining a 60 mbar vacuum, 0.4 M NaOH was added to the surface of the gel (avoiding overflow). The vacuum was maintained for 1 hr. Periodically during this hour 0.4 M NaOH was added to the top of the gel maintain a 0.2 - 0.3 mm depth. After one hour of transfer, the gel was peeled away from the membrane and discarded. The vacuum was released and the pump was turned off. The membrane was air-dried on a piece of Watmann 1 MM filter paper. The air-dried membrane was then exposed to UV light (256 nm) for 1 min and used for subsequent hybridization.

2.9.7.2 Production of digoxigenin-labeled DNA probes by PCR

PCR labeling was performed to produce digoxigenin (DIG)-labeled DNA probes specific to *egfp* or *gfp* sequences in this study. Templates used for PCR labeling were the plasmid DNA, pEGFP and/or pGFP purchased from Clontech Laboratories, Inc. The primers specific to *gfp* gene and PCR conditions were the same as that used for colony PCR (see section 2.9.2) except that PCR-DIG-dNTP mix (Qiagen) was used to replace the normal dNTP used in PCR amplification. The probes generated by PCR amplification were approximately 608 bp.

2.9.7.3 Pre-hybridization and hybridization

The membrane was laid on a piece of nylon mesh spacer. The membrane and the mesh were rolled and inserted into a glass hybridization bottle. Twenty ml of Pre-Hybridization buffer containing 50% formamide, 5 ×SSC, 2% blocking reagent (Roche Molecular Biochemicals), 0.02% SDS, 0.1% N-lauroylsarcosine were added into each small hybridization bottle (15 cm) and incubated at 65°C in a hybridization oven. The pre-hybridization was conducted at this temperature for 2-3 hrs.

About 2 μg of DIG- labeled DNA probes was diluted in 100 μl of diethylpyrocarbonate (DEPC) treated Milli-Q Water (DEPC-dH_2O) and boiled for 5 minutes followed by rapidly chilling on ice for 10 minutes. The diluted probe was mixed with 10 ml pre-hybridization buffer and added into the hybridization bottle to replace the used Pre-Hybridization buffer. The hybridization bottle was incubated at 65°C in the hybridization oven overnight.

The membrane was removed from the hybridization bottle and washed twice at room temperature in 2 × SSC and 1% SDS for 5 min each. The membrane was inserted into a clean hybridization bottle with nylon mesh spacer and washed once in 1 × SSC and 1% SDS at 65°C in the hybridization oven for 15-20 min followed by three washes in 0.1 × SSC and 1% SDS at 65°C in the hybridization oven each for 10 min. The membrane was removed from the hybridization bottle and air dried followed by immunological detection.

2.9.7.4 Detection

The membrane was rinsed in MaS Buffer (0.1 M Maleic Acid, 0.15 M NaCl, pH7.5) for 1 min at room temperature. The membrane was then transferred into blocking buffer [1% Blocking Reagent (Roche Molecular Biochemicals) in MaS Buffer] and incubated with constant gentle shaking for 1 hr at room temperature followed by one wash in MaS buffer at room temperature for 1 min. The membrane was transferred into anti-DIG-AP conjugate solution (diluted at 1:5,000 in blocking buffer) and incubated at room temperature for 1 hr with constant gentle shaking followed by two washes in MaS Buffer at room temperature each for 15-20 min. The membrane was then equilibrated in substrate buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH9.5) at room temperature for 2 min. The membrane was transferred into a plastic hybridization bag filled with substrate solution [45 µl of NBT and 35 µl of BCIP (X-phosphate) in 10 ml of substrate buffer or 200 µl of NBT/BCIP mix per 10 ml of substrate buffer]. All the substrate solutions and hybridization bag were from Roche Molecular Biochemicals. All the air bubbles were removed from the hybridization bag before the bag was

sealed. The hybridization bag containing the membrane and substrate solution was incubated at room temperature in the dark for 4-16 hrs before the membrane was removed and the reaction was terminated by washing the membrane in TE buffer. The membrane was dried under an infrared light and documented by photographing.

2.9.8 Determination of nucleotide sequences of both *gfp* gene and CaMV 35S promoter

Colony PCR was conducted using primer sets specific to *gfp* gene and CaMV 35S promoter sequences. PCR products (see above for details) and the PCR products were purified using a Qiagen PCR purification kit, QIAquick column following the instruction of the supplier. PCR products were applied to the QIAquick centrifugation column and the DNA was retained in the column while other reagents were removed by centrifugation. After several washes, the DNA bound to the column was eluted with 50 µl of 10 mM Tris-Cl (pH 8.5). The eluted DNA was precipitated with 0.25 M KAc and 2 volumes of 95% EtOH at -20°C for 30 min and harvested by centrifugation. The nucleotide sequences of the PCR products were then determined by automated sequencing using dye terminator cycle sequencing method (York University, Toronto, Canada). Sequences were determined from both strands of the PCR products using both reverse and forward primers. These sequences were compared to known sequences of *gfp* gene and CaMV 35S promoter by using Clone Manager v.6.0.

3. RESULTS

3.1 *Transfer plasmid pHN216 from CMM cells to E. coli DH5α cells*

Transformation of competent cells of *E. coli DH5α* with the total DNA extracted from Gentamicin resistant CMM cells yielded more than 50 colonies. The transformation efficiency was relatively consistent with the transformation control, competent cells of *E. coli DH5α* transformed with pBluescript II. Colonies (or cell lines) were selected based on the size of the colonies and their growth in the selective medium for further culture and for plasmid DNA purification. Twelve individual colonies that grew on the selective agar plates were picked and inoculated into 2 ml of 2 ×YT broth containing 50 μg/ml of Gentamicin. The selected colonies all grew in the medium indicating that they were resistant to the antibiotic used.

3.2 *Preparation of plasmid DNA, pHN216*

The plasmid DNA from each of the 12 selected colonies were analysed by agarose gel electrophoresis. The size of the plasmid DNA, pHN216, was estimated by its mobility in 1% agarose gel and by comparing pHN216 to a known plasmid, pBINPLUS (12,396 bp) from Clontech, which was similar to pHN216 in size (van Engelen *et al.*, 1995), loaded on the same agarose gel (Fig.9). Five colonies with plasmids named pHN216a to pHN216e were selected for further analysis.

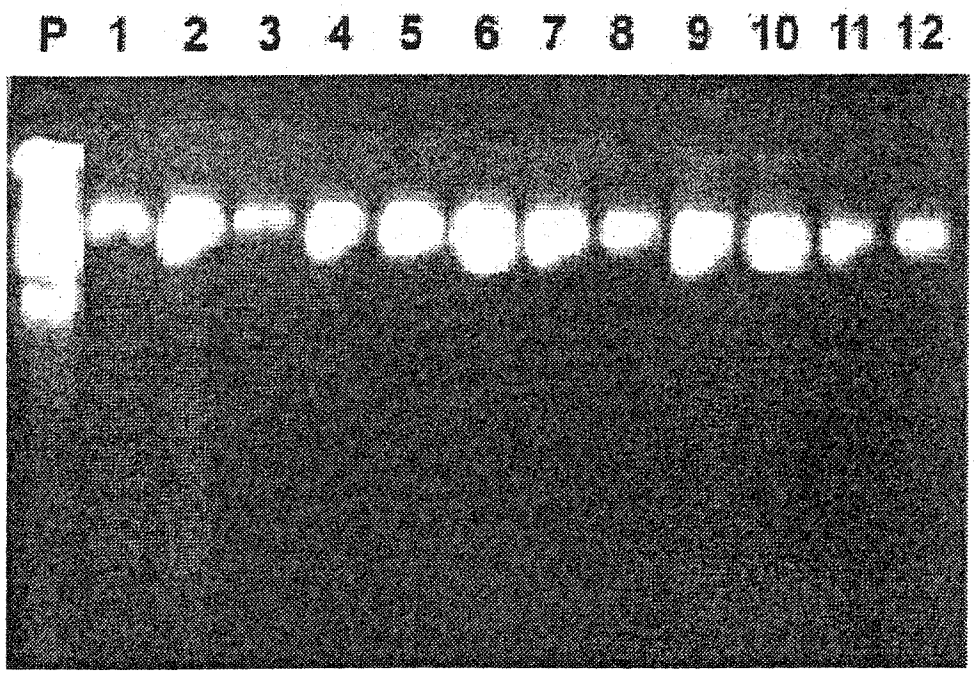


Fig. 9. Mini-prep of plasmid DNA pHN216. Twelve clones of plasmid DNA pHN216 (1-12), purified from *E. coli* DH5α cells were analyzed by agarose gel electrophoresis. P: plasmid pBINPLUS (ClonTech) as a control (for double stranded plasmid DNA). Five clones (lanes 2, 5, 6, 10 and 12) were selected for further analysis and labeled as pHN216a, b, c, d and e, respectively.

3.3 Restriction endonuclease analysis of pHN216

Five selected plasmids pHN216a to pHN216e extracted from five different Gentamycin resistant *E. coli DH5α* cell lines were further evaluated by restriction endonuclease digestion using two restriction endonucleases, *Hind*III and *Eco*RI. Both single digestion with *Hind*III alone and double digestion with *Hind*III and *Eco*RI were conducted. Plasmid pHN216 contains one *Hind*III site, and two *Eco*RI sites based on a previous report (Laine *et al.*, 1996). The restriction digestion yielded the expected restriction sites for all five plasmids, pHN216a to pHN216e (Fig.10). Single digestion of these plasmids with *Hind*III linearized them while double digestion with *Hind*III and *Eco*RI resulted in three fragments, 11,800 bp, 1470 bp and 530 bp, respectively (Fig.10). Non-digested plasmid DNA, pHN216a (no restriction enzyme) was used as a control. The size of the DNA fragments was estimated by comparison with two molecular weight markers, 100 bp DNA ladder (New England Biolabs) and λ DNA digested with *Hind*III and *Eco*RI, loaded on the same gel. The plasmid pHN216a was chosen for subsequent applications.

3.4 *Transfer plasmids, pEGFP and pCAMBIA 1303 into E. coli DH5α cells*

Plasmids, pEGFP (1 μg/μl) and pCAMBIA 1303 (0.5 μg/μl) from Clontech Laboratories, Inc were transformed into *E. coli* DH5α competent cells. Antibiotics (ampicillin for pEGFP, and Kanamycin for pCAMBIA 1303) were included in the medium for the

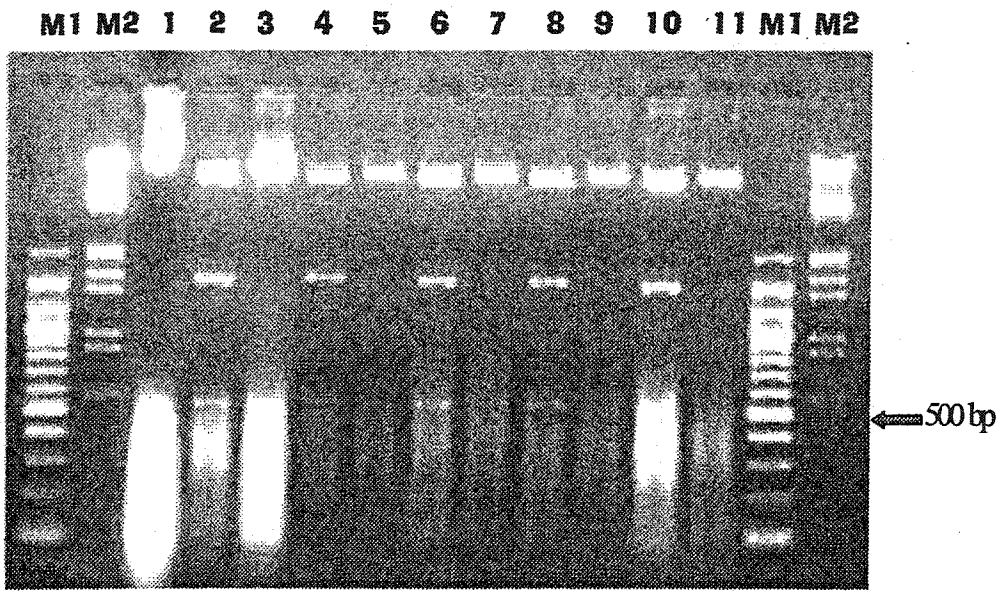


Fig. 10. Restriction endonuclease analysis of pHN216. Plasmid pHN216a - e were either doubly digested with *Hind*III and *Eco*RI (lanes 2, 4, 6, 8, 10) or singly digested with *Hind*III (lanes 3, 5, 7, 9, 11). Non digested pHN216a (lane1) was used as a negative control. M1: 100 bp DNA marker (New England Biolabs); M2: λ DNA double digested with *Hind*III and *Eco*RI.

selection of antibiotic-resistant cells harboring either plasmid. The transformation efficiency was relatively high and approximately 10^5 colonies were obtained from about 50 ng plasmid DNAs used. Several antibiotic- resistant colonies (cell lines) for each plasmid (7 for pEGFP, 8 for pCAMBIA 1303) were picked and inoculated into LB broth individually for the purification of plasmid DNA.

3.5 *Analysis of plasmid DNA, pEGFP and pCAMBIA 1303*

The extracted plasmid DNA was analysed by agarose gel electrophoresis. The size of the plasmids were estimated by their mobility in 1% agarose gel. The original plasmids (purchased from Clontech) were also loaded in the same agarose gel as controls. The plasmids purified from different colonies were named pEGFP-1 to pEGFP-7 (Fig.11a) and pCAMBIA 1303-1 to pCAMBIA 1303-8 (Fig.11b).

3.6 *Restriction digestion of plasmid DNAs, pEGFP and pCAMBIA 1303*

Plasmids, pEGFP and pCAMBIA 1303 were evaluated by restriction endonuclease digestion. Restriction enzymes, *Hind*III and *Eco*RI were used to doubly digest pEGFP-2, pEGFP-4 and pEGFP-6. The results of the restriction digestion confirmed these restriction sites in these plasmids (Figs 5, 12). The double digestion of pEGFP with *Hind*III and *Eco*RI resulted in a fragment of 800 bp (Fig. 12) as expected since pEGFP has a *Hind*III and an *Eco*RI sites in the 5' MCS and 3' MCS, respectively. This fragment consisted of *the egfp* gene sequence (modified *gfp* gene) (Fig. 5) (Clontech Laboratories, Inc.). Enzymes *Hind*III and

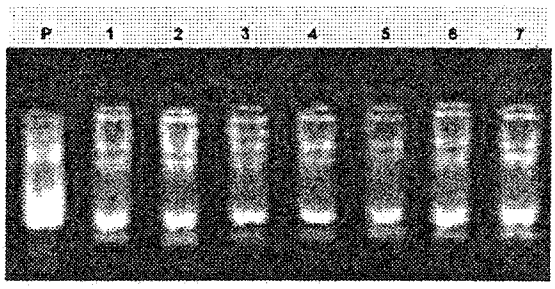


Fig 11a

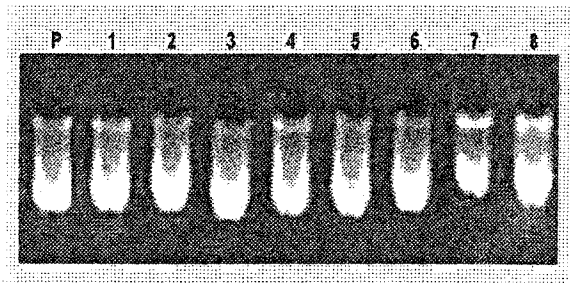


Fig 11b

Fig. 11. Mini-prep of plasmids, pEGFP (a) and pCAMBIA 1303 (b). Different clones of plasmids, pEGFP (1-7) and pCAMBIA 1303 (1-8) purified from *E. coli* DH5α cells were analyzed by agarose gel electrophoresis. Purchased plasmid (P) pEGFP (a) and pCAMBIA 1303 (b) were included as controls. Three pEGFP clones (lanes 2, 4, 6) were selected and labeled as pEGFP-2, -4, and -6. Two pCAMBIA 1303 clones (lanes 2, 8) were selected and labeled as pCAMBIA1303-2, and -5.

*Sph*I were used to doubly digest pCAMBIA 1303-2 and pCAMBIA 1303-8. The double digestion of these plasmids with these two restriction enzymes resulted in a fragment of 3.7 kbp (Fig. 13) as expected since pCAMBIA 1303 contains one site each for *Hind*III and *Sph*I. This fragment consisted of the CaMV 35S promoter sequence and this *gfp* gene (Fig. 6) (Clontech Laboratories, Inc.).

3.7 *Assessment of recombinant plasmid DNAs, pHNG and pHNCG and propagation of these two plasmids in E. coli DH5α cells*

The *Pvu*II-*Eco*RI fragment of *LacZ* promoter-*gfp* gene (971 bp in length) from plasmid pEGFP was inserted into the plasmid pHN216 double digested by *Hind*III and *Eco*RI (the *Hind*III site was filled-in) to generate a new construct - recombinant plasmid pHNG (Fig. 7). This new construct was then transformed into *E. coli* DH5α cells. Neomycin was used in culture medium for the selection of transformed cells and plasmid DNAs were purified from *E. coli* DH5α cells by the modified alkaline plasmid DNA mini-prep procedure (Fig.15a). The purified recombinant plasmids (pHNG clones) were analysed by restriction digestion with *Xba*I (Fig.15b). Recombinant plasmid pHNG (clone 2 or labelled as pHNG-2) was further digested by *Hind*III and *Sph*I followed by phenol/chloroform extraction and KAc/EtOH precipitation and used as another cloning vector. These treatments linearized the plasmid pHNG and removed two base pairs in the 5' MCS just downstream from the *LacZ* promoter.

The *Hind*III-*Sph*I fragment of CaMV 35S promoter-*gfp* gene (3.7 kbp in length) from

plasmid pCAMBIA 1303 was then ligated into the plasmid pHNG doubly digested by *Hind*III and *Sph*I and resulted in a new construct of recombinant plasmid- pHNCG (Fig. 8). This new recombinant plasmid was then transformed into *E. coli* DH5α cells for screening and propagation. Neomycin was used in culture medium for the selection of transformed cells. Plasmids were purified from *E. coli* DH5α cells by the modified alkaline plasmid DNA mini-prep procedure. Seven recombinant plasmids were extracted and examined by gel electrophoresis and by restriction digestion with *Hind*III and *Sph*I (Fig.16a, b). Both pHNCG-4 and -7 contained the expected insert (3.7 kpb fragment) and pHNCG-7 was selected for further use.

E. coli DH5α cells transformed with these two recombinant plasmids, pHNG and pHNCG were also examined by fluorescence microscopy (wavelength: 450-500 nm). Some colonies (cell lines) showed fluorescent signal (Fig.17, 18). These results indicated that the *gfp* gene was expressed in *E. coli* DH5α cells under the control of either *LacZ* promoter (Fig.17a, b) or CaMV 35S promoter (Fig.18a, b). *E. coli* DH5α cells transformed with pHNG and pHNCG showed no obvious difference in their transformation efficiency, intensity of fluorescent signals produced, and growth in liquid culture medium with neomycin (Neo) (see Table 1 for details). The colonies that showed stronger fluorescent signals were selected for further DNA purification and for subsequent experiments.

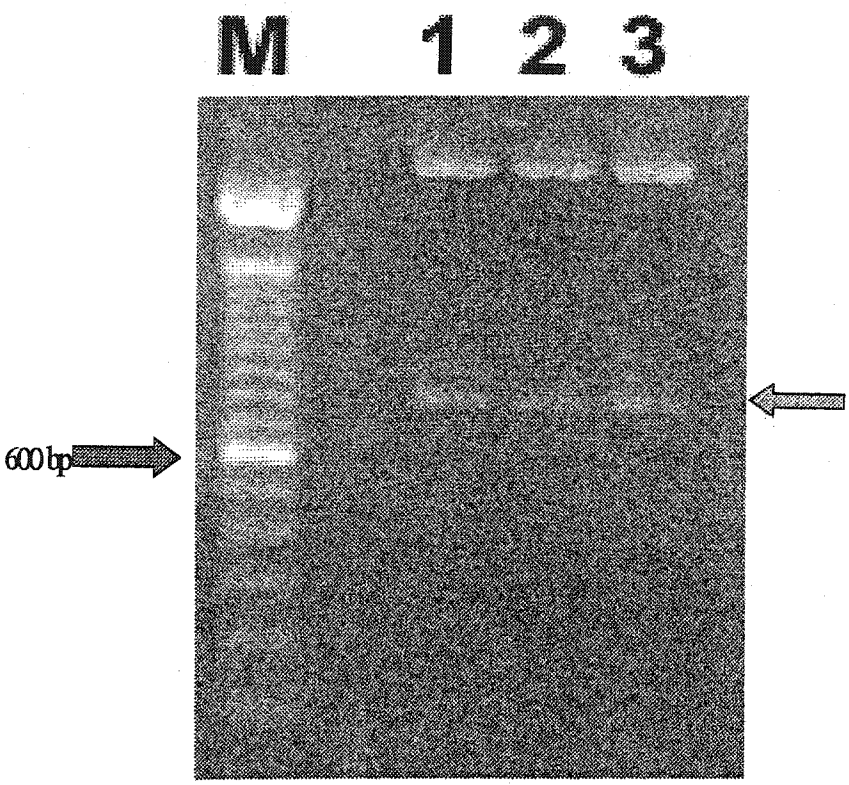


Fig. 12. Restriction endonuclease analysis of pEGFP. Plasmid pEGFP-2, -4, and -6 were double digested with *Hind*III and *Eco*RI and analyzed on 1% agarose gel (lanes 1, 2, 3). A fragment of approximately 800 bp (arrow) was released from each of these clones. M: 100 bp DNA ladder (Invitrogen Canada)(the 600 bp band is stronger).

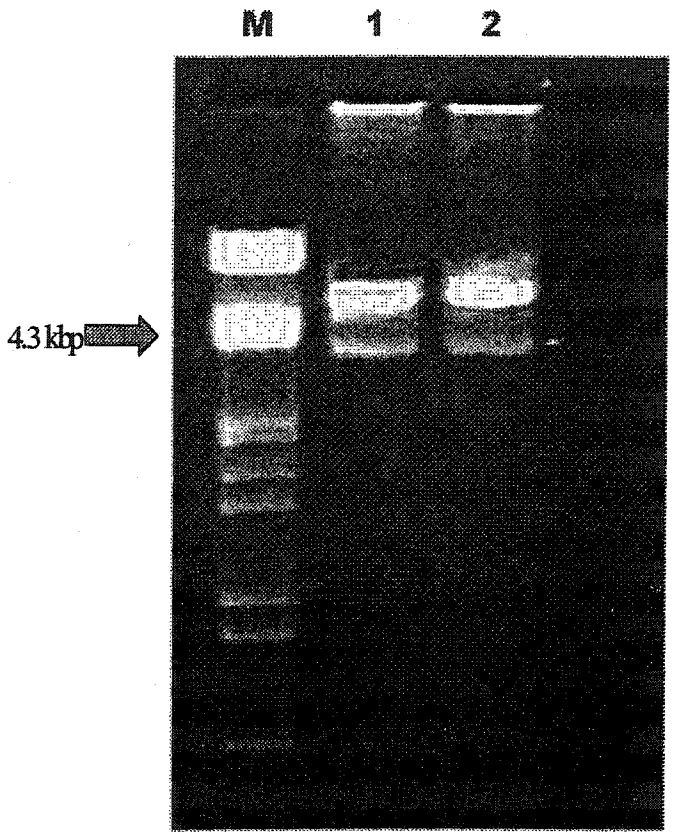


Fig. 13. Restriction endonuclease analysis of pCAMBIA 1303. Plasmid pCAMBIA 1303-2, and -8 were double digested with *Hind*III and *Sph*I and analyzed on 1% agarose gel (lanes 1, 2). The smaller fragment released from each of these clones was approximately 3.7 kbp. M: λ DNA doubly digested with *Hind*III and *Eco*RI.

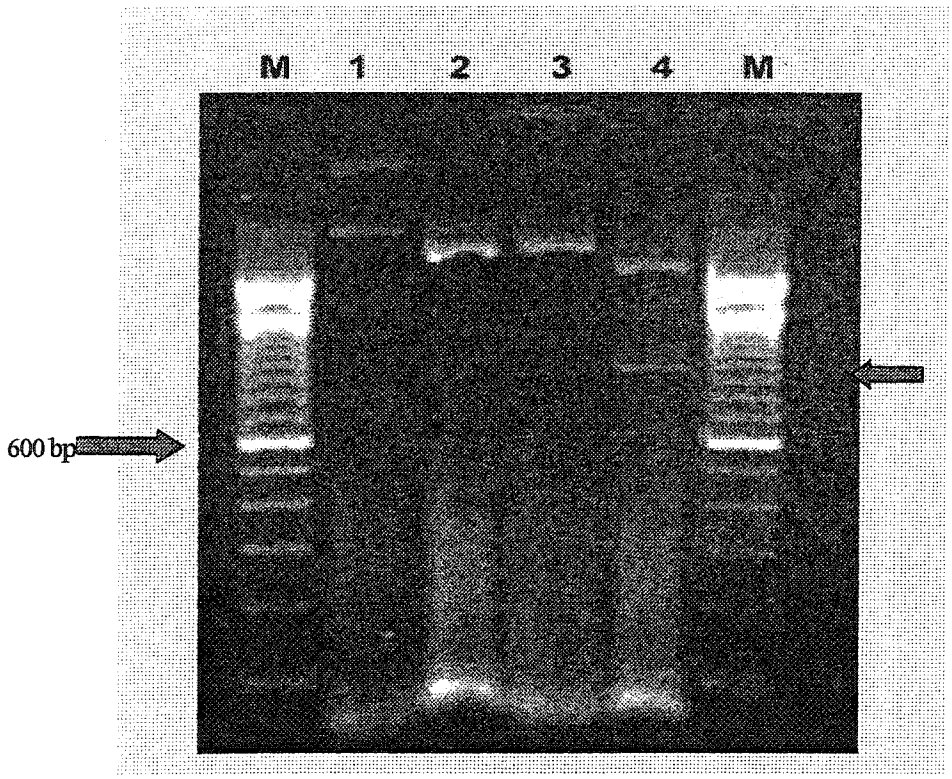


Fig. 14. DNA fragment of *LacZ* promoter-*gfp* gene. Plasmid DNA pEGFP-6 was double digested with *Hind*III and *Pvu*II (lane 4) and resulted in a DNA fragment of 971 bp (arrow). Plasmid DNA (lane 1), pEGFP singly digested with either *Pvu*II (lane 2) or *Hind*III (lane 3) were used to confirm the activity of the enzymes used. M: 1 kbp DNA ladder (Invitrogen Canada).

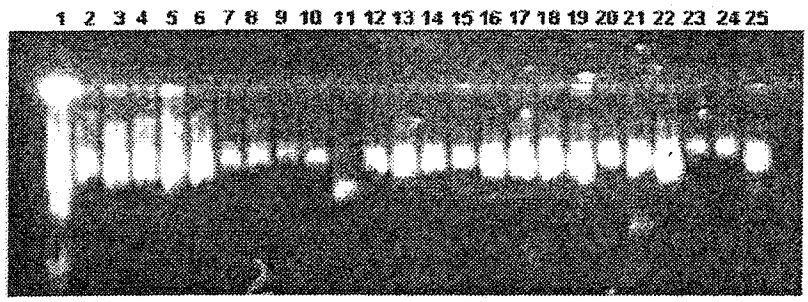


Fig. 15a

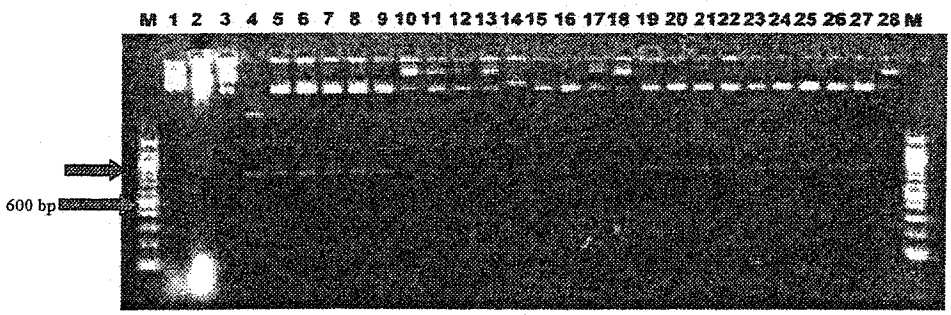


Fig. 15b

Fig. 15. Mini-prep (15a) and restriction analysis (15b) of recombinant plasmid pHNG. 15a) 23 clones of recombinant plasmid DNAs, pHNG purified from *E. coli* DH5α cells were analyzed by agarose gel electrophoresis (lanes2-24). Both original pEGFP (lane1) and pHN216 without insert (lane25) were also loaded on the same gel as controls. 15b) 23 clones of plasmid pHNG were digested with *Xba*I (lanes5-27), M: 100 bp DNA ladder (New England Biolabs); Non digested plasmids, pHN216 (lane1), pEGFP (lane2), pHNG clone 1 (lane3) and clone 23 (lane28) were loaded in the same gel. Plasmid pEGFP cut by *Xba*I (lane4) was used to serve as a positive control. *Xba*I digestion of pEGFP and most of these pHNG clones resulted in a DNA fragment of 800 bp (indicated by an arrow).

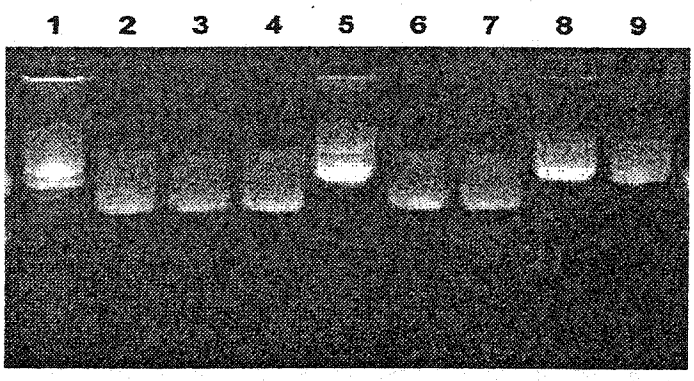


Fig. 16A

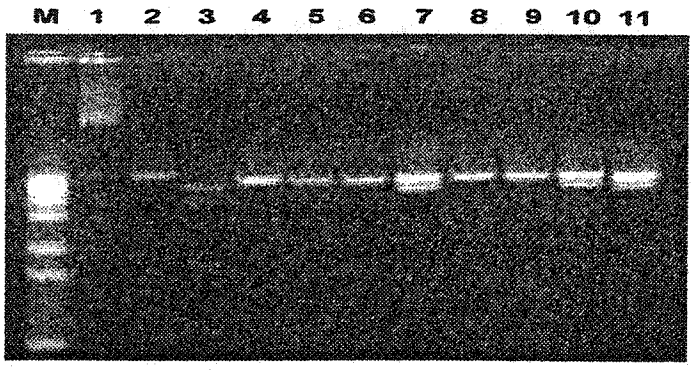
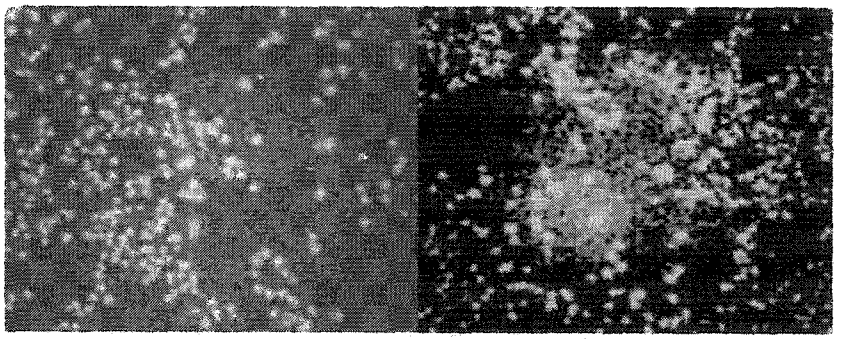


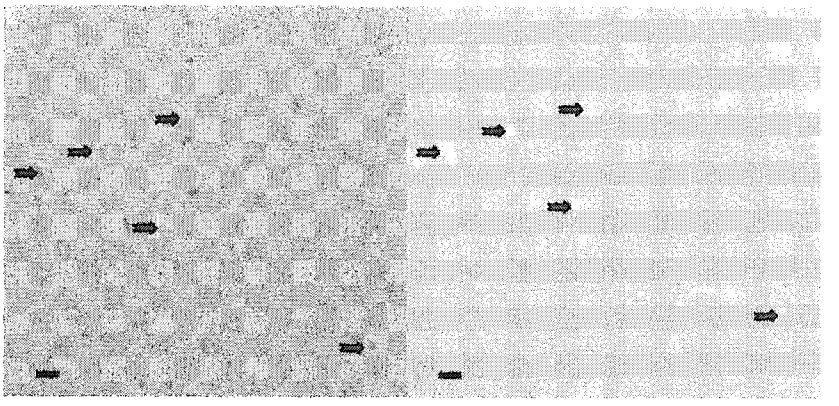
Fig. 16B

Fig. 16. Mini-prep (16a) and restriction analysis (16b) of recombinant plasmid pHNCG. 16a) Seven clones of recombinant plasmid pHNCG purified from *E. coli* DH5α cells were analyzed by agarose gel electrophoresis (lanes2-8). Plasmids, pHNG (lane1) and pCAMBIA 1303 (lane9) were loaded on the same gel as references. 16b) All clones of recombinant plasmid DNA pHNCG were digested with *Hind*III and *Sph*I (lanes4-10). M: 1 kbp DNA ladder (Invitrogen Canada); Lane 1: undigested pHNG; Lane 2: pHNG linearized by *Hind*III; Lane 3: pHNG digested by *Sph*I; Lane11: pCAMBIA 1303 doubly digested with *Hind*III and *Sph*I. Clones pHNCG-4 and pHNCG-7 and pCAMBIA 1303 doubly digested with *Hind*III and *Sph*I each resulted in a fragment of 3.7 kbp



1 2

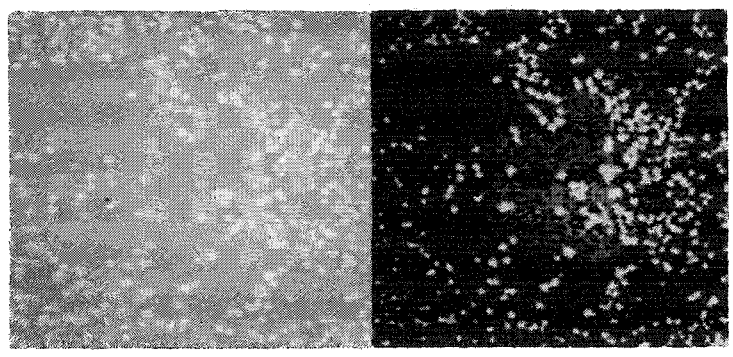
Fig. 17 A



1 2

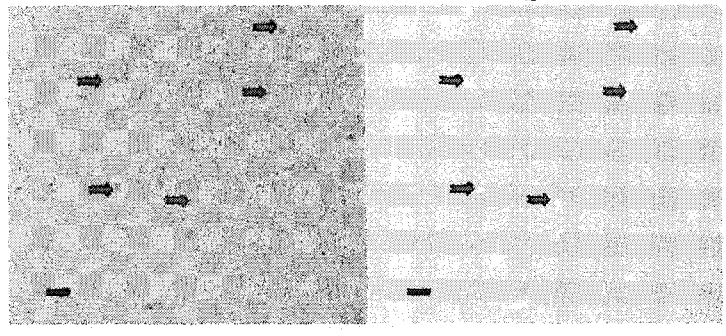
Fig. 17 B

Fig. 17. Fluorescent colonies of *E. coli* DH5α transformed with plasmid pHNG containing the *gfp* gene . The *E. coli* DH5α cells transformed with pHNG were examined under microscopy (wave length: 490 nm). A) colonies on the plates: A1: normal illumination; A2: UV illumination; B) cells from individual colony: B1-phase-contrast illumination, B2-epi-illumination at 450-500 nm showing cells producing fluorescent signals (indicated by arrows). Scale bar in lower left of B(1,2) equals 1μm.



1 2

Fig. 18 A



1 2

Fig. 18 B

Fig. 18. Fluorescent colonies of *E. coli* DH5α transformed with plasmid pHNCG containing the *gfp* gene . The *E. coli* DH5α cells transformed with pHNCG were examined under microscopy (wave length: 490 nm). A) colonies on the plates: A1: normal illumination; A2: UV illumination; B) cells from individual colony: B1-phase-contrast illumination, B2-epi-illumination at 450-500 nm. Showing cells producing fluorescent signals (indicated by arrows). Scale bar in lower left of B(1,2) equals 1μm.

3.8 *Improvement of procedures for the preparation of CMM/CMS competent cells and for electroporation*

3.8.1 Culture of various CMM and CMS strains

CMM strain #6 was cultured at 28°C while CMS strains, R2, R9, R12, R14, and P45 were cultured at 25°C. Two culture media, YGM and TBY were used. The cells were cultured on agar plates of appropriate culture medium for five days before single colonies were picked and inoculated into 2 ml of liquid culture medium, either TBY or YGM broth (see Table 2 for details).

3.8.2 Modified procedures for the preparation of CMM and CMS competent cells

The cell cultures (2 days) were diluted in TBY broth to give an OD_{580} of 0.3. Glycine was then added and the cells were further cultured at 28°C/ 25°C with shaking until the OD_{580} of 0.6 (approximately 2 hrs) was reached. Cells were then harvested and washed for the preparation of competent cells. Another method was to harvest and wash the cells from the 2-day-old culture directly. In this study, glycine solution (20% stock) was added to the cell culture to a final concentration of 2.5% for maintaining a balance of the osmotic pressure. The cells harvested were washed twice in 10% glycerol. The cells were resuspended in 10% glycerol to an optical density at 650 nm of approximately 0.8.

Table 1. Features of *E. coli* DH5α cells transformed with recombinant plasmid DNAs pHNG and pHNCG

Features	pHNG	pHNCG
Transformation efficiency	10^5 colonies/50 ng DNA	10^5 colonies/50 ng DNA
Colonies with fluorescence	more than 90%	more than 90%
Growth rate in medium	Normal*	Normal*
Reaction to Neomycin	Resistant	Resistant

*indicates that the growth rate of these cell lines was similar to that of *E. coli* DH5α cells transformed with plasmid pBluescript.

Table 2. Improvements of procedures for the preparation of CMM and CMS competent cells and electroporation*

Methods	A	B	C
Medium	YGM	TBY	TBY
Cell treatment	No treatment	Treat the cells with glycine for 2 hrs to OD_{580} of 0.6	As method B
Cell mixture	cells: 20 µl 40% PEG: 20 µl Plasmid DNA: 2 µg	Cells:100 µl No PEG Plasmid DNA: 5 µg	As Method A
Electroporation	1.5 kV/cm, 600 Ω 2 pulses (20 sec interval)	1.5 kV/cm, 600 Ω 1 pulse 2 pulses	1.5 kV/cm, 600 Ω (20 sec interval)
Colonies obtained:			
CMM6	92 (5 plates)	84 (5 plates)	≥500 (5 plates)
CMS (P45)	0 (5 plates)	0 (5 plates)	2 (5 plates)
CMS (R2)	0 (5 plates)	0 (5 plates)	1 (5 plates)
CMS (R14)	0 (5 plates)	0 (5 plates)	1 (5 plates)

* Plasmid pHNCG was used for the transformation.

3.8.3 *Modified electroporation parameters for efficient transformation*

Three electroporation methods were evaluated in this study. Method A was based on the procedure described by Laine *et al.* (1996), Method B was based on the electroporation of other organisms (Kirchner *et al.*, 2001) and Method C was a modified procedure based on both methods A and B. The recombinant plasmid DNA was added to the bacterial suspensions (in 10% glycerol) with or without the addition of PEG (Table 3) and the mixtures were used for subsequent electroporation. Method C, used for competent cell preparation and for electroporation, resulted in higher transformation efficiency compared to that of the other two methods (Table 2). The results indicate that the modified electroporation method (C) was more efficient than the other two methods (A, B) as more colonies were obtained by using Method C (500 colonies compared to 84-92 colonies from 5 agar plates of transformed CMM6 cells, Table 2). CMS R2, R14 and P45 cells prepared and transformed by Methods A and B yielded no colonies. CMS R2 and P45 cells prepared and transformed by Method C resulted in 2 and 1 colonies, respectively. Since the transformation efficiency of the modified method (C) was much higher than that of either Method A and B, Method C was used in all subsequent electroporation experiments. Several colonies (cell lines) of transformed CMM and CMS with either pHNG or pHNCG were selected for further evaluations.

3.9 *Fluorescence signals produced by CMM transformed with pHNG and pHNCG*

Individual colonies from agar plates were selected and examined under the fluorescence microscope (FM) at a wave length of 450-500 nm. Fluorescence signals were detected in

some of the colonies (cell lines) of CMM6 cells transformed with pHNCG (Fig. 19). These results indicate that pHNCG was successfully transformed into CMM6 cells and the *gfp* gene was expressed under the control of CaMV 35S promoter to a level detectable by FM. However, colonies of CMM6 and CMS strains transformed with pHNG and colonies of CMS strains transformed with pHNCG developed on the selective agar plates (Table 2) showed no fluorescence signals (Table 3), which indicated that the gene *gfp* either was not expressed or *gfp* products were not detectable by FM, although *E. coli* DH5α cells transformed with either pHNG or pHNCG showed obvious fluorescence signals (Fig.17, 18). Apparently, the neomycin resistance gene (Neo^R) on both recombinant plasmids, pHNG and pHNCG, was expressed in both CMM6 and CMS R2 cells since their growth on the selective agar plates was relatively normal (Table 4). These results indicate that for the recombinant plasmid DNA, either pHNG or pHNCG was successfully delivered into CMM6 and CMS (R2, P45) cells by electroporation and the plasmids replicated normally in the transformed cells. The expression of *gfp*, was dependent on the promoter sequence being upstream. The CaMV 35S promoter (on pHNCG), showed no species-specificity of gene expression, and was better than the *LacZ* promoter (on pHNG) for directing the transcription of *gfp* gene in CMM6. Therefore, CMM6 cell lines transformed with pHNCG no other transformed cell lines were chosen for subsequent evaluations.

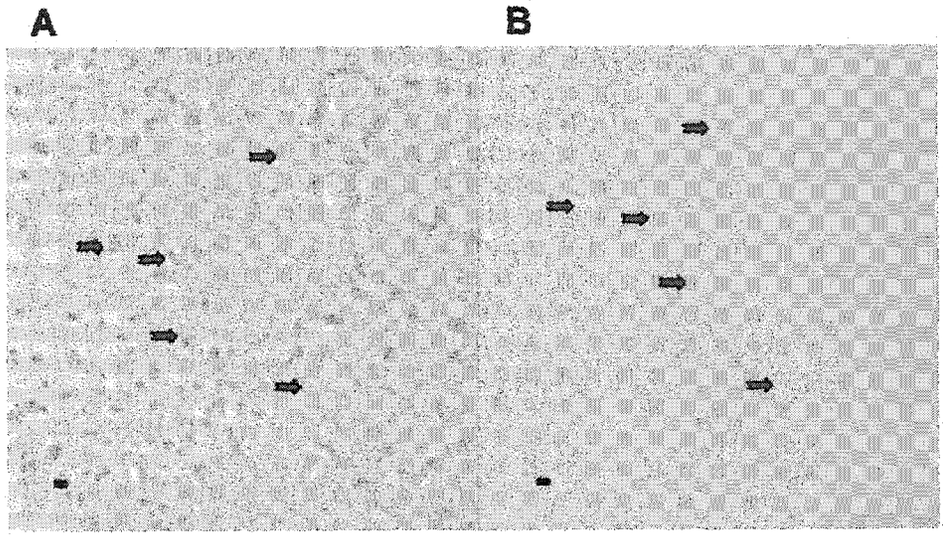


Fig. 19. Fluorescence of CMM6 cells transformed with pHNCG containing the *gfp* gene were examined under microscopy (wave length: 490 nm). This image shows the cells from individual colony. A: under phase-contrast illumination; B: under epi-illumination at 450-500 nm. CMM6 cells producing fluorescent signals are showon (indicated by arrows). Scale bar in lower left of A and B equals 1μm.

Table 3. Detecting fluorescent signal in bacterial cells transformed with pHNG and pHNCG

Bacterial cells	pHNG	pHNCG
E. coli DH5α	++++	++++
CMM6	-	+
CMS R2	-	-
CMS R14	-	-
CMS P45	-	-

++++ : Strong fluorescence signal.

+ : Weak fluorescence signal.

- : No fluorescence signal observed.

Table 4. Growth of CMM6 and CMS cells transformed with recombinant plasmids in selective culture medium

Transformation	Antibiotic in medium		
	Ampicillin	Neomycin	Gentamicin
Non-transformed	-	-	-
Transformed with pEGFP	+	-	-
Transformed with pHN216	-	+	+
Transformed with pHNG	-	+	-
Transformed with pHNCG	-	+	-

- indicates no growth; + indicates growth.

3.10 *Detection of particular DNA sequences in the bacterial cells transformed with gfp gene containing plasmids by PCR*

3.10.1 Specific CMM sequences detected by PCR

PCR amplification was used to confirm the identity of CMM by using primers, CMM-F and CMM-R specifically targeting the intergenic spacer region between the 16S and 23S rRNA genes in both pHNCG transformed and non-transformed CMM6 cells. A PCR product of 226 bp was amplified from DNA of both transformed and non-transformed CMM6 cells (Fig. 20). These results confirmed the CMM identity of both transformed and non-transformed CMM6 cells.

3.10.2 Sequence of gfp gene detected in bacterial cells transformed with recombinant plasmids pHNCG by PCR

PCR amplification was used to confirm the presence of the *gfp* gene in the transformed CMM6 cells. The primer set (GFP-N and GFP-C) specific to the *gfp* gene sequence was used in this experiment. PCR products of 608 bp were amplified from DNA expected from both pHNCG transformed *E. coli* DH5α and CMM6 cells (Fig.21). No PCR product was amplified from total DNAs extracted from non- transformed CMM6 and *E. coli* DH5α cells (Fig.21).

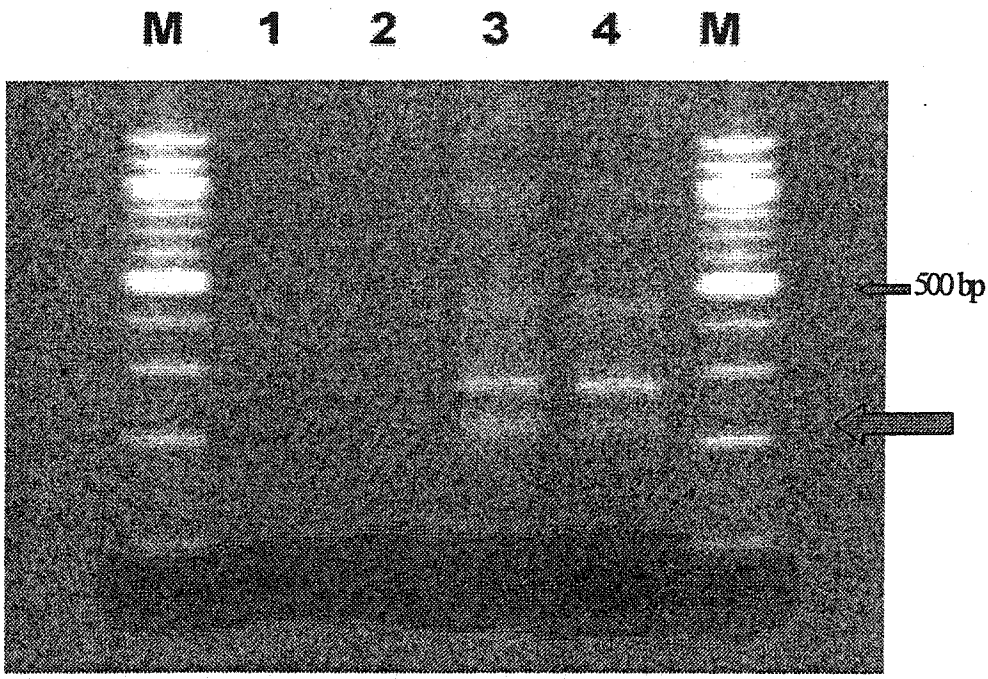


Fig. 20. Confirmation of the identity of CMM cells by colony PCR, using primers specific to CMM 16S gene. M: 100 bp DNA ladder (New England Biolabs); Lane 1: water (no nucleic acid template); Lane 2: *E. coli* DH5α cells transformed with pHNCG; Lane 3: CMM cells transformed with pHNCG; Lane 4: non-transformed CMM cells. Arrow indicates PCR product of 220 bp.

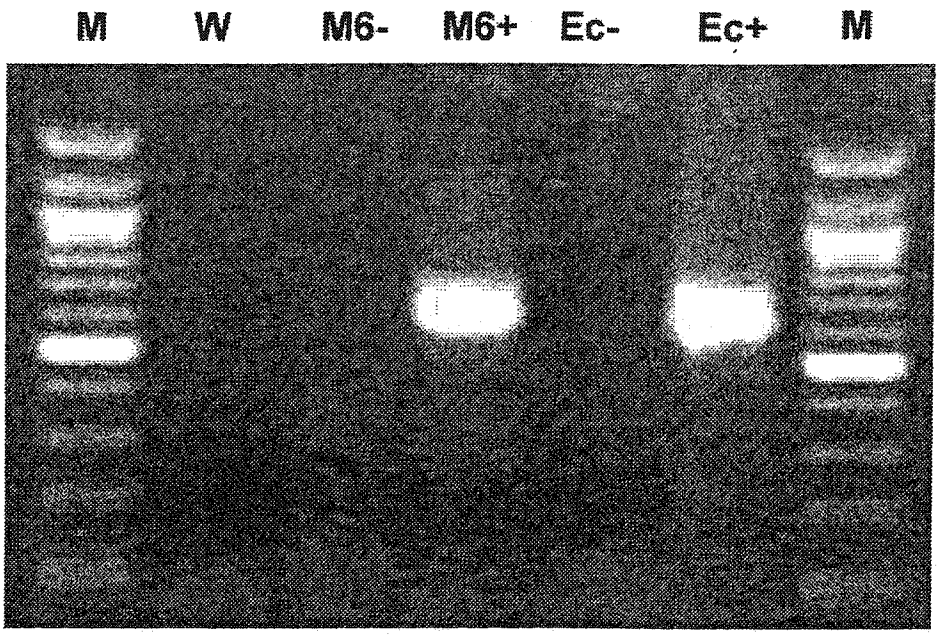


Fig. 21. Detection of *gfp* gene in the bacterial cells transformed with pNHCG. Colony PCR was conducted to screen pHNCG transformed bacterial cells for the presence of *gfp* gene. PCR products were analyzed by gel electrophoresis. M: 100 bp DNA ladder (New England Biolabs); W: water (no nucleic acid template). Transformed CMM6 (M6+) and *E. coli* DH5α (Ec+) cells as well as non transformed CMM6 (M6-) and *E. coli* DH5α (Ec-) cells were screened. PCR products of 608 bp were amplified from both pHNCG transformed CMM6 (M6+) and *E. coli* DH5α (Ec+) cells.

3.10.3 Specific sequence of CaMV 35S promoter detected in bacterial cells transformed with recombinant plasmids, pHNG and pHNCG by PCR

PCR amplification was employed in this study to confirm the presence of the CaMV 35S promoter in the transformed CMM6 cells. The primer set (35S-1 and 35S-2) specific to the 35S promoter sequence was used in this experiment. PCR products of 203 bp were amplified from DNA expected from both pHNCG transformed *E. coli* DH5α and CMM6 cells (Fig.22). A PCR product of the same size was obtained from the original plasmid DNA, pCAMBIA 1303 and no PCR product was amplified from total DNAs extracted from non transformed CMM6 and *E. coli* DH5α cells (Fig.22).

3.11 *Detecting gfp gene products in bacterial cells transformed with gfp gene containing plasmids by ELISA*

Total proteins were extracted from *E. coli* DH5α and CMM6 cells transformed with pHNCG and used for ELISA analysis. The ELISA results indicated that the green fluorescence protein was produced from *gfp* gene to a level detectable in ELISA (Fig.23). The green fluorescence protein was detected in both *E. coli* DH5α and CMM6 cells transformed with pHNCG. However, the concentration of green fluorescence protein accumulated in *E. coli* DH5α was much higher than that accumulated in CMM6 cells (Fig.23). Antibody against the green fluorescence protein did not react with proteins extracted from non-transformed *E. coli* DH5α or CMM6 bacteria, in ELISA. Results obtained from two independent tests were similar (compare test 1 and test 2, Fig.23).

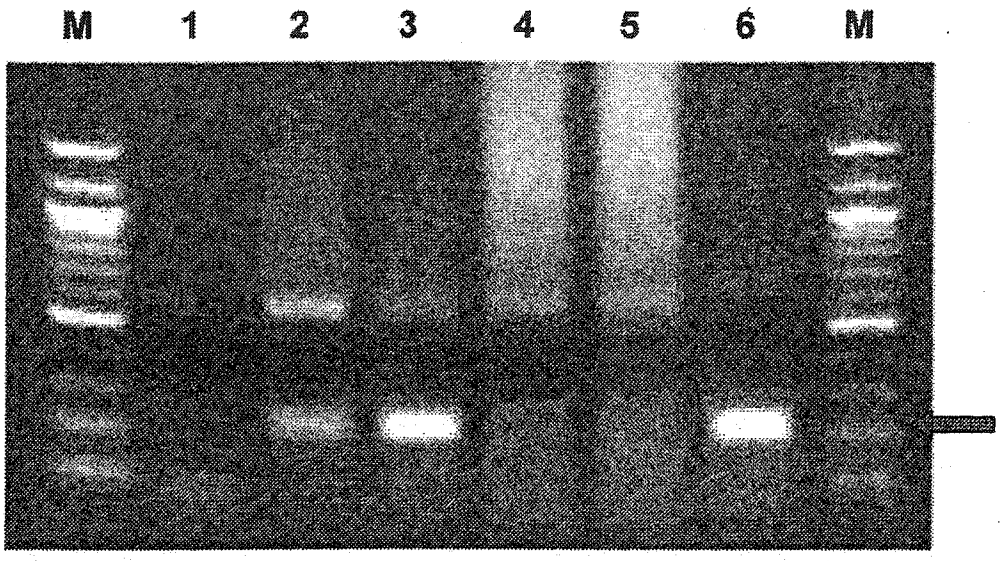


Fig. 22. Detection of CaMV 35S promoter sequence in the bacterial cells transformed with pNHCG. Colony PCR was conducted to screen pHNCG transformed bacterial cells for the presence of CaMV 35S promoter sequence. PCR products were analyzed by gel electrophoresis. M: 100 bp DNA ladder (New England Biolabs); Lane 1: water (no nucleic acid template). Lane 2-3: Transformed CMM6 and *E. coli* DH5α cells; Lane 4-5: Non transformed CMM6 and *E. coli* DH5α cells. PCR products of 210 bp (arrow) were amplified from both pHNCG transformed CMM6 (2) and *E. coli* DH5α (3) cells. Lane 6 positive control a PCR products of 210 bp was amplified from pCAMBIA 1303.

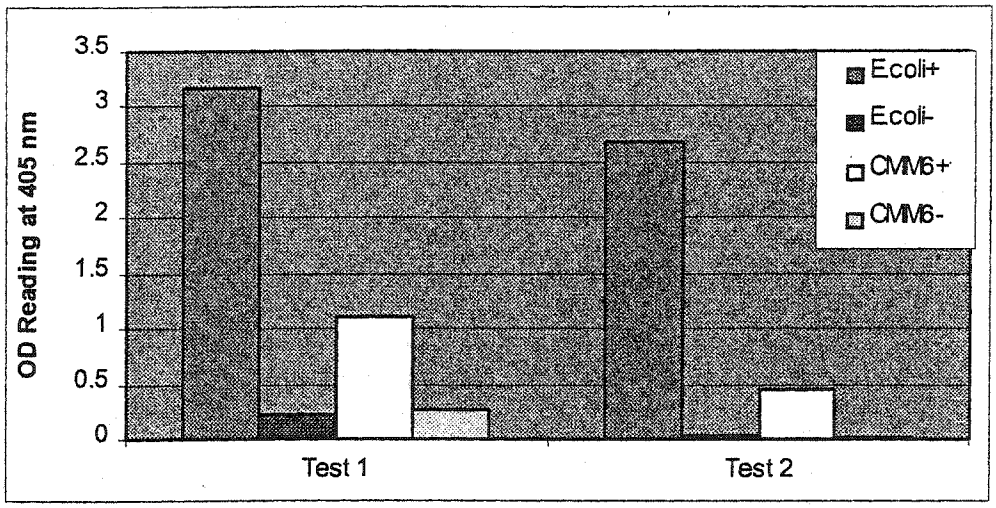


Fig. 23. ELISA detection of green fluorescent protein in bacteria transformed with pHNCG. Total proteins were extracted from both pHNCG transformed and non transformed CMM6 and *E. coli* DH5α cells. The extracted proteins were used in ELISA with antibodies specific to green fluorescence protein. Green fluorescence protein was detected in both CMM6 and *E. coli* DH5α cells transformed (+) with pHNCG, but not in the non transformed (-) cells.

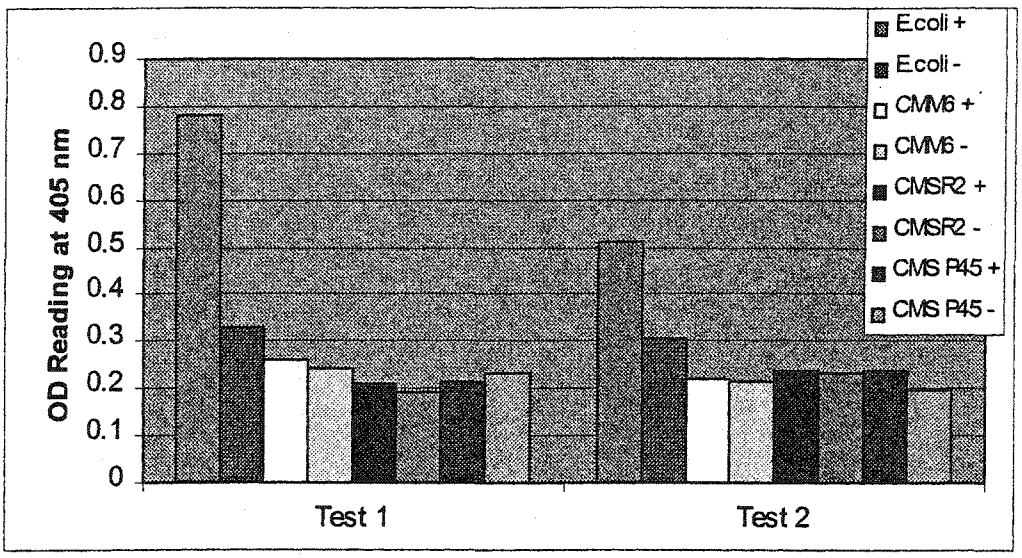


Fig. 24. ELISA detection of green fluorescent protein in bacterial cells transformed with pHNG. Total proteins were extracted from pHNG transformed (+) and non transformed (-) bacterial cells and screened by ELISA using antibodies specific to green fluorescence protein. Bacterial strains were CMM6, CMS R2, CMS P45 and *E. coli* DH5α. Green fluorescence protein was only detected in *E. coli* DH5α cells transformed with pHNG, but not in any other cells including pHNG transformed cells.

Bacterial cells transformed with pHNG were also evaluated in this experiment. ELISA results indicated that *gfp* gene was only expressed in transformed *E. coli* DH5α cells, but not in any other bacterial cells including CMM6, CMS R2 and P45 (Fig. 24).

3.12 *Detecting gfp gene products in bacterial cells transformed with plasmid containing gfp gene by Western blot*

Total proteins extracted from pHNCG transformed *E. coli* DH5α and CMM6 cells were also analysed by Western blot. Proteins separated on SDS-PAGE were blotted onto a membrane and probed with green fluorescence protein-specific antibody. Results showed that the green fluorescence protein of expected size (27 kDa) was produced from the *gfp* gene to a level detectable in Western blot (Fig. 25). The green fluorescence proteins were detected in both *E. coli* DH5α and CMM6 cells transformed with pHNCG. Again, the concentration of green fluorescence protein accumulated in *E. coli* DH5α was much higher than that accumulated in CMM6 cells (Fig.25). Antibodies against green fluorescence protein did not react to proteins extracted from non-transformed bacteria, neither *E. coli* DH5α nor CMM6 in Western hybridization. Bacterial cells transformed with pHNG were also evaluated by Western blot. The results of Western blot showed that *gfp* gene was only expressed in transformed *E. coli* DH5α cells, but not in any other bacterial cells including CMM6, CMSR2, CMSR12, and CMSP45 (Fig. 26).

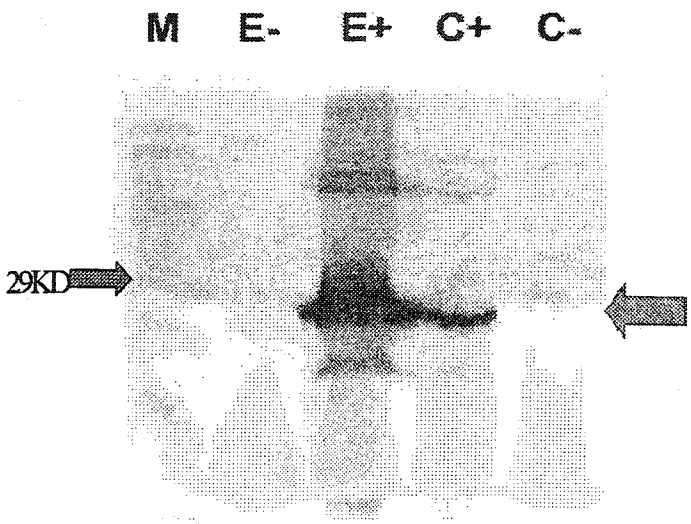


Fig. 25. Western blot of bacterial cells transformed with pHNCG. Total proteins were extracted from both pHNCG transformed (+) and non transformed (-) CMM6 (C) and *E. coli* DH5α. (E) cells and screened by Western Blot using antibodies specific to green fluorescence protein. M: low molecular weight protein marker (Bio-Rad). Green fluorescence proteins were detected in both pHNCG transformed CMM6 and *E. coli* DH5α cells, but not in non transformed cells. The specific protein of 27 kDa is indicated by an arrow.

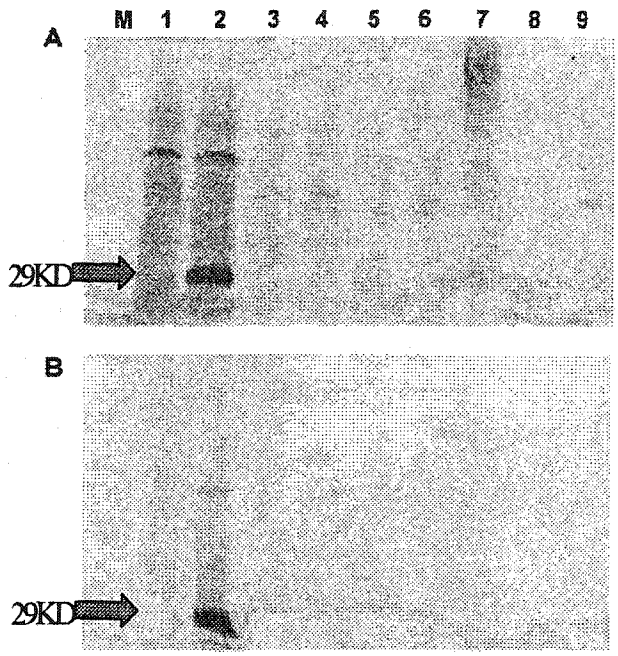


Fig. 26. Western blot of bacterial cells transformed with pHNG. Total proteins were extracted from both pHNG transformed and non transformed bacterial cells and screened by Western Blot using antibodies specific to green fluorescence protein. A: using labeled polyclonal antibody and B: using labeled monoclonal antibody. M: low molecular weight protein marker (Bio-Rad). Green fluorescence proteins were only detected in pHNG transformed *E. coli* DH5α cells (2), but not in any other transformed and non transformed cells including transformed CMM6 (4), CMS R2 (6), CMS R9 (7), CMS R12 (8), CMS P45 (9) and non transformed *E. coli* DH5α cells (1), CMM6 (3), and CMS R2 (5). GFP protein is 27 kDa.

3.13 *Sequence of gfp gene detected in transformed bacterial cells by Southern hybridization*

PCR products amplified from the total DNA extracted from pHNG transformed *E. coli* DH5α, CMS strains and CMM6 cells by using primers specific to *gfp* gene were analysed by agarose gel electrophoresis followed by Southern transfer to blot DNAs onto a nylon membrane (Fig.27a). DNA on the membrane was then probed by DIG- DNA probes generated by PCR labelling using original pEGFP as a template followed by immunological detection. The results confirmed the presence of the *LacZ* promoter in *E. coli* DH5α, CMS strains and CMM6 cells transformed with pHNG (Fig.27b). Southern hybridization further confirmed that there was no cross amplification (Fig.27a, b).

3.14 *Nucleotide sequences of CaMV 35S promoter in transformed E. coli DH5α and CMM6 cells*

The CaMV 35S promoter were amplified by PCR and the PCR products were extracted by using a PCR purification kit (Qiagen). Nucleotide sequences of these PCR products were then determined by automated dye terminator cycle sequencing (ABI Prism 377, York University). The sequences were determined from both orientations of the dsDNA. The sequences obtained were the same as those published previously which confirmed that they were the same as the known CaMV 35S promoter sequence (Fig.28).

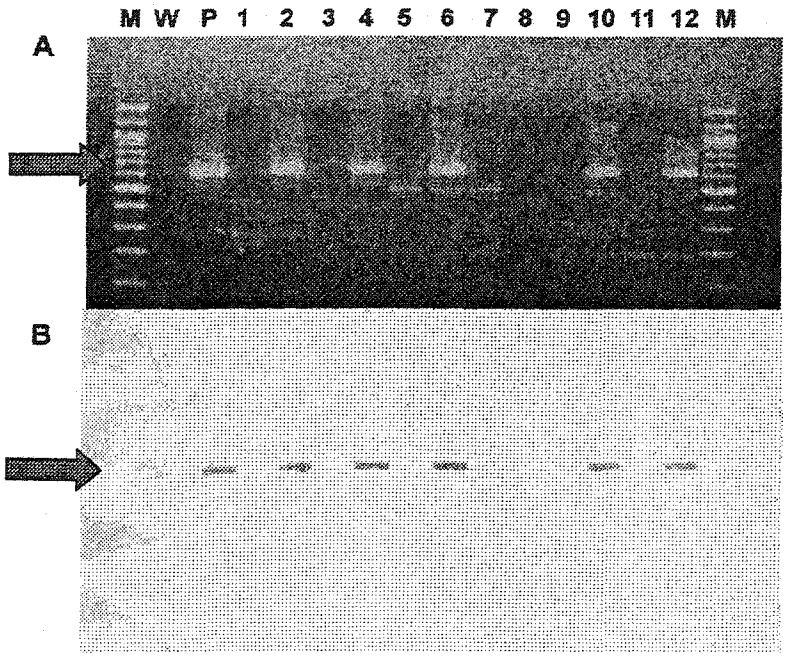


Fig. 27. Detection the *gfp* gene sequence by Southern hybridization in bacterial cells transformed with pHNG. Total nucleic acids extracted from both transformed and non transformed cells were used in PCR. PCR products were analyzed by agarose gel electrophoresis (A) followed by Southern hybridization (B). M: 100 bp DNA ladder (New England Biolabs); W: water (no nucleic acid template); P: plasmid DNA, pEGFP as a positive control. Both transformed (lanes 2, 4, 6, 8, 10, 12) and non transformed (lanes 1, 3, 5, 7, 9, 11) cells were screened. Bacterial strains were *E. coli* DH5α (1, 2), CMM6 (3, 4), CMS R2 (5, 6), CMS R9 (7, 8), CMS R12 (9, 10), CMS P45 (11, 12). A PCR product of 608 bp was amplified from transformed cells of *E. coli* DH5α (2), CMM6 (4), CMS R2 (6), CMS R12 (10), and CMS P45 (12) and confirmed by Southern hybridization as *gfp* gene sequences.

```
nown-primer 1     -CAATTGCGGTGAAACGCGAAGAGGGTGGGCAGGTATCCAACTGGTTGCA
lone 1-primer 1   TTAATTGCGGTGAAACGCGAAGAGGGTGGGCAGGTATCCAACTGGTTGCA
lone 2-primer 1   TTAATTGCGGTGAAACGCGAAGAGGGTGGGCAGGTATCCAACTGGTTGCA

nown-primer 1     CAATCACGCCAATGTTGGCGATGTCGTGAAACTGGTCGCTCCGGCAGGTG
lone 1-primer 1   CAATCACGCCAATGTTGGCGATGTCGTGAAACTGGTCGCTCCGGCAGGTG
lone 2-primer 1   CAATCACGCCAATGTTGGCGATGTCGTGAAACTGGTCGCTCCGGCAGGTG

nown-primer 1     ATTTCTTTATGGCTGTCGCAGATGACACACCAGTGACGTTAATCTCTGCC
lone 1-primer 1   ATTTCTTTATGGCTGTCGCAGATGACACACCAGTGACGTTAATCTCTGCC
lone 2-primer 1   ATTTCTTTATGGCTGTCGCAGATGACACACCAGTGACGTTAATCTCTGCC

nown-primer 1     GGTGTTGGTCAAACGCCAATGCTGGCAATGCTCGACACGCTGAAATCACC
lone 1-primer 1   GGTGTTGGTCAAACGCCAATGCTGGCAATGCTCGACACGCTGAAATCACC
lone 2-primer 1   GGTGTTGGTCAAACGCCAATGCTGGCAATGCTCGACACGCTGAAATCACC

nown-primer 1     AGTCTA-
lone 1-primer 1   AGTCTAA
lone 2-primer 1   AGTCTA-


nown-primer2      AGAAAGGTCAAAGGTTGGATCGTTTGACCCACACCGGTAGAGATTAACGT
lone 1-primer2    AGAAAGGTCAAAGGTTGGAGCGTTTGACCAACACCGGCAGAGATTAACGT
lone 2-primer2    AGAAAGGTCCGCGGGCGGGTAACTTGACCAACACCGGCAGATATTAACGC

nown-primer2      CACTGGTGTGTCATCTGCGACAGCCATAAAGAAATCACCTGCCGGAGCGA
lone 1-primer2    CACTGGTGTGTCATCTGCGACAGCCATAAAGAAATCACCTGCCGGAGCGA
lone 2-primer2    TACTGGTGTGTCATCTGCGACAGCCATAAAGAAATCACCTGCCGGAGCGA

nown-primer2      CCAGTTTCACGACATCGCCAACATTGGCGTGATTGTGCAACCAGTTGGAT
lone 1-primer2    CCAGTTTCACGACATCGCCAACATTGGCGTGATTGTGCAACCAGTTGGAT
lone 2-primer2    CCAGTTTCACGACATCGCCAACATTGGCGTGATTGTGCAACCAGTTGGAT

nown-primer2      ACCTGCCCACCCTCTTCGCGTTTCACCGCAATGCGATAGCCTTTCCTTTA
lone 1-primer2    ACCTGCCCACCCTCTTCGCGTTTCACCGCAATGCGATAGCCTTTCCTTTA
lone 2-primer2    ACCTGCCCACCCTCTTCGCGTTTCACCGCAATGCGATAGCCTTTCCTTTA

nown-primer2      TCGCAA
lone 1-primer2    TCGCAA
lone 2-primer2    TCGCA-
```

Fig. 28. Nucleotide sequence of CaMV 35S promoter amplified from the bacterial cells transformed with pHNCG. Total nucleic acids extracted from both transformed and non transformed cells were used in PCR amplification. PCR products were then purified and their nucleotide sequences were determined by automated dye terminator cycle sequencing from both directions using primers 35S-1 (Primer 1) and 35S-2 (Primer 2). Since the sequences of all 6 PCR samples were the same, only one is shown and compared to the sequence of CaMV 35S promoter in the plasmid pCAMBIA 1303 (ClonTech).

4. DISCUSSION

Potato bacterial ring rot (BRR) disease caused by *Clavibacter michiganensis* subsp. *sepedonicus* (Davis *et al.*, 1984) (CMS) is a significant concern to seed and processing potato producers in Canada and worldwide. Since the first report of this disease in 1906, great achievements have been made worldwide for the isolation and characterization of the causal agent, for understanding the life cycle of the disease, for developing sensitive, specific and efficient methods for the diagnosis and detection of the pathogen, and for the development of strategies for the control and management of the disease. However, many issues, particularly those related to the biology of the bacteria causing potato ring rot, are not fully understood. Of these issues, the movement of ring rot bacterium from the seed tuber to progeny tubers, the distribution and location of the bacterium in potato tubers and sprouts, and the possibility of the ring rot bacterium entering the plant *via* stems and/or leaves have been puzzling researchers for many years and reliable answers or explanations have not been given.

In this study, experiments were conducted to introduce the green fluorescent protein (GFP) gene into plasmid pHN216 (13.8 kbp) which was modified from two wild plasmids, pCM1 (27.5 kb) and pCM2 (72 kb) isolated from *C. michiganensis* subsp. *michiganensis* (CMM)(Meletzus and Eichenlaub, 1991), and then used to transform potato ring rot bacterium CMS. It was considered that the transformed CMS cells could be used for studying the pathogenesis of CMS infection in potato.

Previous studies have shown the successful transformation of both CMM and CMS

with the plasmid pHN216 (Meletzus and Eichenlaub, 1991; Laine *et al.*, 1996). A reporter marker gene encoding β-1,4-endoglucanase inserted into the *Hind*III site of pHN216 was successfully expressed in transformed cells of a cellulase-deficient mutant of CMS (Laine *et al.*, 1996).

In the present study, the gentamicin resistance gene (Gn) in the plasmid pHN216 (*Hind*III - *Eco*RI - *Eco*RV - *Eco*RI fragment) was replaced with a DNA fragment containing *gfp* gene and an appropriate promoter, without damaging the neomycin resistance gene (Neo). Therefore neomycin was used in experiments as the selection marker. The gentamicin resistance gene was previously reported to give a rather poor selection in screening transformants of CMS (Laine *et al.*, 1996). The removal of the gentamicin resistance gene resulted in a smaller plasmid vector, which made it easier to manipulate the vector in cloning and analysis and probably would increase the replication efficiency or copy number of the plasmid in any transformed cells.

It was found that the replication efficiency (or copy number) of pHN216 in either CMM or CMS cells was extremely low, probably due to the size and the origin of the plasmid and it was difficult to obtain a reasonable amount of plasmid DNA from CMM cells for restriction analysis and for subsequent cloning experiments. Therefore, total DNAs were extracted from CMM cells containing pHN216 and used for the transformation of *E. coli* DH5α competent cells. The plasmid was then purified from transformed *E. coli* DH5α cells by a modified mini-prep procedure for plasmid DNA purification. This approach resulted in a relatively high yield of plasmid DNA with adequate quality for any downstream analysis and

application.

The bioluminescent jellyfish (*A. victoria*) produces light when energy is transferred from the Ca^{++}-activated photoprotein aequorin to the green fluorescent protein (GFP) (Shimomura *et al.*, 1962; Morin and Hastings, 1971; Ward *et al.*, 1980). The gene (*gfp*) for producing GFP has been identified and is available commercially. Many modifications have been made for efficient translation and for expression of this gene in various heterologous systems (Prasher *et al.*, 1992; Chalfie *et al.*, 1994; Inouye and Tsuji, 1994a; Wang and Hazelrigg, 1994). When expressed in either eukaryotic or prokaryotic cells and illuminated by blue or UV light, GFP yields a bright green fluorescence. Light-stimulated GFP fluorescence is species-independent and does not require any cofactors, substrates, or additional gene products from *A. victoria*. Additionally, detection of GFP and its variants can be performed in living cells and tissues as well as fixed samples.

To date, several species of bacteria have been transformed for GFP expression (Yu and den Engh, 1995; Webb *et al.*, 1995; Lewis and Errington, 1996; Andersen *et al.*, 1998) and no successful GFP expression has yet been reported in Gram-positive bacterium. Both CMS and CMM are gram-positive bacteria. The transformation of these two bacteria for the expression of *gfp* gene will extend the application of GFP to the clavibacter or coryneform group of bacteria.

The approach of using GFP as a reporter molecule was taken to develop a plasmid vector containing *gfp* gene and deliver this plasmid into CMS. Two plasmids, pEGFP (enhance *gfp* or EGFP) and pCAMBIA 1303 (mutant *gfp* or mGFP) containing the cloned *gfp*

gene with modifications were obtained from Clontech and used as the source of *gfp* gene in this study. These GFP variants are modified from the wild type GFP by converting the 5' end of the gene to a Kozak consensus translation initiation site (Kozak, 1987), removing some potentially inhibitory sequences and introducing some silent mutations. All of these changes increased the translation efficiency of the mRNA and, consequently, the expression of the GFP variants in various systems. The expression of *gfp* in the plasmid pEGFP is under the control of *LacZ* promoter, a widely used protein expression promoter in various species of bacteria. The *gfp* expression in the plasmid pCAMBIA 1303, however, is controlled by CaMV 35S promoter, a widely used gene expression promoter in various species of plants as well as bacteria.

To develop a recombinant plasmid containing *gfp* gene, three approaches were taken in this study. The first attempt was to obtain a dsDNA copy of the *gfp* gene by PCR amplification using a pair of primers flanking the region from the initiation codon to the termination codon of the target gene. Sequences of restriction enzyme *Hind*III were incorporated in each of these two primers. After digestion with *Hind*III, the PCR amplified dsDNA was then inserted into the *Hind*III site of pHN216. In this case, the expression of *gfp* was completely dependent on the promoter sequence right in front of the *Hind*III site in pHN216 as described by Laine *et al.* (1996). The second approach was to excise a fragment of *LacZ* promoter-*gfp* gene from the plasmid DNA pEGFP and this fragment of DNA was then inserted into pHN216 without the gentamycin resistant gene. This approach resulted in a recombinant plasmid pHNG containing *gfp* gene. The third approach was to excise a fragment of DNA containing the CaMV 35S promoter-*gfp* gene from the plasmid DNA pCAMBIA 1303. The CaMV 35S promoter-*gfp*

gene was then inserted into the *Hind*III-*Sph*I sites of pHNG to generate another recombinant plasmid, pHNCG.

To deliver the recombinant plasmids into CMM and CMS cells, the conventional methods using $CaCl_2$ for the preparation of competent cells and heat-shock for the transformation were employed with no success. This might be due to the fact that the conditions used in the conventional methods for competent cells and for cell transformation were optimized for *E. coli* cells (*e.g.* strains HB101, JM101, DH5α), but were not optimal for either CMM or CMS cells, particularly because large plasmids were used. To transform CMM and CMS cells with large recombinant plasmids, pHNG and pHNCG, transformation efficiency must be high. Procedures described previously (Laine *et al.* 1996; Kirchner *et al.*, 2001) for the preparation of CMM or CMS competent cells and for electroporation were used initially in this study, but the transformation efficiency was not high enough to obtain *gfp* expressing cell lines. Modifications were made in this study to treat the cultured cells for the preparation of competent cells of CMM or CMS and for electroporation as described by Laine *et al.* (1996) and Kirchner *et al.* (2001). Cultured CMM or CMS cells were treated with glycine and the harvested cells were then maintained in PEG before electroporation. Electroporation at 15 kV/cm, 600 Ω, 2 pulses (20 sec interval) was used for the transformation. This combination resulted in a higher efficiency of transformation for both CMM and CMS.

An attempt was made to transform several bacterial species and strains with constructs

of recombinant plasmids, pHNG and pHNCG. These included *C. michiganensis* subsp. *michganense* Strain 6 (CMM6), CMS strains R2, R9, R12, R14 and P45, and *E. coli* DH5α. Colonies were obtained from CMM6, CMS R2 and P45 and *E. coli* DH5α cells transformed with both pHNG and pHNCG, although the transformation efficiency of CMS cells was significantly lower than for CMM. Conditions used in this study for cell treatment and for electroporation were probably optimal for CMM6 and *E. coli* DH5α cells, but not optimized for CMS cells. Furthermore, the transformation failed to introduce either recombinant plasmid into CMS R9, R12 and R14. Plasmid pHNCG transformed CMS R2 and P45 cell lines did not survive in subsequent culture in the medium containing antibiotic, which indicated that these cell lines were not stable transformants.

Although the *gfp* gene was successfully inserted into the desired site of pHN216 as determined by restriction analysis, PCR amplification and antibiotic selection, the *gfp* gene was not expressed in either *E. coli* DH5α and CMM6 cells since neither fluorescence signal nor GFP was detected in any transformed cells by IMF, respectively (data not shown). The reason was probably that the promoter sequence in pHN216 for the expression of gentamicin (right in front of *Hind*III) was not able to initiate the expression of *gfp* gene. Therefore the focus of this study was shifted on the second and third approaches to employ a better promoter for *gfp* gene expression in CMM cells.

In the recombinant plasmid pHNG, the expression of *gfp* gene was dependent on *LacZ* promoter sequence. In *E. coli* cells transformed with pHNG, GFP was successfully expressed as determined by fluorescence microscopy (FM), ELISA and Western blot.

Although the *gfp* gene sequence was detected in pHNG transformed cells of CMM6 and CMS R2, R14, and P45 based on the results of PCR and Southern hybridization (Fig. 27), the green fluorescence protein, or a translational product of the gene *gfp*, was not detectable by either ELISA or Western blot (Fig. 24, 26). The *LacZ* promoter (22 bp), originated from *E. coli*, a gram-negative bacterium and has been used in many gene cloning systems for the expression of *LacZ* gene to produce a α-complementation unit for β-galactosidase since 1977 (Messing *et al.*, 1977). This promoter was sufficient in *E. coli*, DH5α cells, but might not be suitable in the cells of CMM and CMS, two gram-positive bacteria, for the expression of *gfp* gene cloned in pHNG, even in the presence of isopropythio-β-D-galactoside (IPTG), a *LacZ* operon inducer.

Cauliflower mosaic virus (CaMV) 35S promoter appears to show no species-specificity for expression (Odell *et al.*, 1985; Benfey and Chua, 1990) and is used in various gene delivery systems such as for the development of transgenic species including plants, *e.g.* tobacco and potato, and bacteria, *e.g. Agrobacterium tumefaciens* (Bevan, 1984; Dalta *et al.*, 1992). Therefore, experiments were conducted to transfer the CaMV 35S promoter sequence from the plasmid pCAMBIA1303 upstream of the *gfp* gene in the recombinant plasmid pHNG to develop a new recombinant plasmid construct, pHNCG. The DNA fragment of 3.74 kbp transferred from pCAMBIA 1303 into pHNG contained CaMV 35S promoter-*gfp* gene-3' nos terminator flanked by two restriction sites, *Hind*III and *Sph*I. This construct of recombinant plasmid pHNCG was used with the improved methods for preparation of competent cells and for electroporation. Some CMM6 cell lines with antibiotic resistance were obtained and the CMM identity of all these cell lines were confirmed by PCR

amplification using primers specific to CMM (Fig.20). *E. coli* DH5α cell lines containing pHNCG were obtained by using either these improved methods or conventional $CaCl_2$ and heat-shock methods. All *gfp* expressing bacteria were resistant to neomycin in the culture media.

Further examination of all *gfp* expressing bacteria (both from CMM6 and *E. coli* DH5α) confirmed the presence of the recombinant plasmid, pHNCG since both *gfp* gene and CaMV 35S promoter sequences were detected by PCR and nucleotide sequencing (Fig. 21, 22, 28). The *gfp* gene product, green fluorescence protein, was detected by both ELISA (Fig.23) and Western blot (Fig.25) and the fluorescent signal was observed by fluorescence microscopy (at a wave length of 450-500 nm)(Fig.19). The improved methods for the preparation of CMM competent cells and for their transformation by electroporation enhanced the transformation efficiency and resulted in more transformed cells. The 35S promoter sequence is expected to contribute to the efficient expression of *gfp* in the transformed cells. Several domains of CaMV 35S promoter have been reported to greatly increase the level of promoter activity and the level of transcription of a gene cloned downstream from the 35S promoter (Odell *et al.*, 1985; Benfey and Chua, 1990).

The accumulation level of *gfp* gene products or the intensity of green fluoresce signal in pHNCG transformed CMM6 cells were clearly lower than that detected in pHNCG transformed *E. coli* DH5α cells (Fig. 23). This result can be due to the fact that the concentration of GFP in CMM6 cells is much lower than that accumulated in *E. coli* DH5α cells, or the expression efficiency of *gfp* in CMM6 cells is lower than that in *E. coli* DH5α cells.

Furthermore, the GFP chromophore consists of a cyclic tripeptide derived from Ser-Tyr-Gly in the primary protein sequence (Cody *et al.*, 1993) and is only fluorescent when embedded within the complete GFP protein. The GFP structure can provide a proper environment for the chromophore to fluorescence by excluding solvent and oxygen (Ormö *et al.*, 1996; Yang *et al.*, 1996). Any mutation that alters this tripeptide or change the proper GFP structure will possibly alter or abolish the production of a fluorescent compound. The solubility of GFP produced in different bacterial cells might be significantly different. Both EGFP from pEGFP and mGFP from pCAMBIA 1303 are expressed in *E. coli* cells mainly as a soluble and fluorescent protein even under conditions in which the wild type GFP is expressed in a nonfluorescent form in inclusion bodies (Crameri *et al.*, 1996). These GFP variants also appear to have a low toxicity to *E. coli* cells (Crameri *et al.*, 1996). However, the solubility of GFP expressed in CMM6 cells might be significantly lower than that in *E. coli* cells and thus resulted in a low OD reading in ELISA, Western blotting, and a weak fluorescent signal (Fig.23, 25, 19). Alternatively the accumulation of GFP expressed in CMM6, a gram-positive bacterium, might be toxic to the host resulting in a low rate of cell growth and a low level of GFP accumulation. GFP toxicity might be a factor that contributed to the death of transformed CMS cells. This possibility should be further studied. The differences in cell wall components, and cell biology between gram-positive and negative bacteria might be important factors for different efficiency of cell transformation and GFP expression and accumulation. This might be a major reason why there are no reports in the scientific literature to indicate successful GFP expression in gram-positive bacteria.

In the *gfp* expressing cells, the fluorescent signal observed in *E. coli* DH5α was much stronger than that found in CMM6 under the same conditions for preparation of slides and observation by fluorescence microscopy. Any extreme pH values (lower than pH5.5 or higher than pH11) or possible reducing agents, such as β-mercaptoethanol and dithiothreitol (DTT) were avoided in the experiments to maintain an oxidizing environment condition for fluorescence production (Patterson *et al.*, 1997). However, it is not clear if the low intensity of fluorescent signal in CMM6 cells is solely due to the low concentration or accumulation of soluble GFP, and/or if the cell contents of CMM6 contain reducing agents that may convert GFP into a nonfluorescent form to result in a low fluorescent signal.

Different plasmid constructs with the same origin of replication might be quite different in their stability in any given cell (Laine *et al.*, 1996). Furthermore, the stability of the same recombinant plasmid construct may vary in different cell systems, such as *E. coli* DH5α and CMM6 and the stability of plasmid DNA in different transformed cells might be another factor causing a low yield of green fluorescent protein and a poor fluorescent signal as observed in this study. However, molecular detection, such as PCR and Southern hybridization employed in this study did not reveal the removal or deletion of the recombinant plasmid from the transformed cells examined.

5. FUTURE STUDY

A recombinant plasmid carrying a *gfp* gene and an appropriate promoter has been developed. Both CMM and *E. coli* DH5α cells have been successfully transformed with this recombinant plasmid and *gfp* gene products have been detected in both transformed species of bacteria. However, the stability of the construct of recombinant plasmid, pHNCG in either CMM6 or *E. coli* DH5α cells should be evaluated in the future. This evaluation will be a long process, which cannot be completed in this study. Furthermore, the copy numbers of recombinant plasmid and the accumulation of RNA transcripts of *gfp* gene also should be examined in the future.

The solubility of GFP expressed in CMM6 cells may be lower than that in *E. coli* DH5α cells. The GFPs expressed in CMM6 cells may be associated with some other unknown molecules or compounds, which make it extremely difficult to obtain a high amount of GFP for the tests of ELISA and/or Western hybridization, or make it impossible to produce fluorescent signal after illumination by UV light. Further work may be necessary to study various factors in culture medium that may reduce GFP stability and solubility and prevent the formation of GFP chromophore of GFP expressed in CMM6 cells. Appropriate approaches may be taken to enhance the accumulation of soluble GFP and increase the production of fluorescent signal in the cells.

The GFP variants used in this study appear to have no toxicity to *E. coli* cells, but the possible toxicity of GFP accumulation to CMM6 cells should be evaluated in the future. The

cell death of transformed CMS, a gram-positive bacterium, may be caused by the toxicity of GFP. Further studies may be able to improve the stability of transformed cells and enhance the accumulation of GFP and increase the intensity of fluorescent signal by using different GFP variants and/or change the ingredients of cell culture medium as described in another GFP expression system (Crameri *et al.*, 1996).

The nucleotide sequence of recombinant plasmids, pHNG and pHNCG developed in this study should be determined in the future, which can indicate a need for codon-adjusting of the gfp gene for expression in CMS.

Studies should also be conducted in the future to address the inhibitory effects of reducing agents (Inouye and Tsuji, 1994) in CMM6 cells and cell culture conditions, *e.g.* various incubation temperatures on the production of fluorescent signal. It has been reported that the formation of GFP chromophore is temperature sensitive and a low temperature (15°C) may enhance the strength of fluorescence in plant and yeast systems (Lim *et al.*, 1995; Chiu *et al.*, 1996).

Since the CMM6 cells transformed with pHNCG have been confirmed to carry the *gfp* gene, to accumulate GFP and to generate fluorescent signal. The transformed CMM6 cell lines can be used as a model system in future studies to optimize the efficiency of *gfp* gene transcription and GFP accumulation and the production of fluorescent signal. Eventually, an appropriate plasmid carrying a suitable GFP variant can be introduced into CMS (*e.g.* R2 or P45) cells for a stable expression of *gfp* gene and production of fluorescence. The transformed CMS cells can then be used to inoculate potato and indicator plants, *e.g.* eggplant for the

studies of CMS biology and pathogenicity in the host plants.

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