Faculty of Resource Science and Technology

Transformation of Fungal Endophyte, *Ceratocystis paradoxa*

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Declaration

I hereby declare that this thesis entitled "Transformation of endophyte fungi, *Ceratocystis paradoxa*" is my own work and all sources have been quoted and referred to have been acknowledge by means of complete references. It has been submitted and shall not be submitted to other university or institute of higher learning.

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(NIK ZALIKHA BINTI NIK MUSTAFFA)
Date:

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List of Abbreviations

AGE	agarose gel electrophoresis
BAR	*Phosphinothricin acetyl transferase gene*
bp	base pair
Cm	chloramphenicol
GUS	*β- glucuronidase gene*
LA	Luria agar
LB	Luria broth
PDA	potato dextrose agar
PBA	potato dextrose broth
µM	micromolar
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
psi	pounds per square inch
rpm	rotation per minutes
T-DNA	Transfer DNA
Ti-plasmid	*Tumour-inducing plasmid*
vir	*virulence* gene

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ABSTRACT

Advancement in modern biotechnology, especially in medicine, food, waste utilization and bioremediation are resulted from exploitation of fungi. Endophyte fungi have a wide range of relationship with host varies from symbiosis, mutualism and pathogenic. The endophyte fungi, *Ceratocystis paradoxa* is habitually living within wide plant species these includes coconut, pineapple, sugarcane and banana. *C. paradoxa* was among the amylolytic fungus isolated from sago palm pith. In this research, the *Agrobacterium*-mediated transformation protocol was used to transform the endophyte fungi, *C. paradoxa* to carry a binary vector, pGSA1131. The expression of *BAR* selectable marker genes was shown by the fungi that were able to grow through the top agar supplemented with Basta®. The integration of T-DNA region was determined via polymerase chain reaction (PCR) using GUS primer.

Keywords: *Agrobacterium*-mediated transformation, endophyte fungi, Basta® selection, *GUS* gene, polymerase chain reaction, *Ceratocystis paradoxa*

ABSTRAK

Kemajuan bioteknologi terutamanya dalam bidang perubatan; makanan, penggunaan sisa buangan dan bioremediasi adalah disebabkan oleh pengeksploitasian famili fungus. Kulat endofit mempunyai hubungan yang luas dengan tanaman penumpangnya, berbeza dari simbiosis, mutualis dan patogenik. Kulat endofit, *Ceratocystis paradoxa* hidup di dalam bahagian dalam pokok tumpangannya ini termasuklah pokok kelapa, nenas, tebu, dan pisang. *C. paradoxa* adalah antara kulat amilolitik yang diambil daripada empulur sagu. Dalam kajian ini, transformasi perantara-*Agrobacterium* telah digunakan bagi mentransform kulat endofit, *C. paradoxa* untuk membawa vektor binari, pGSA1131. Ekspresi gen *BAR* dapat dilihat melalui kemampuan kulat yang berjaya tumbuh di atas media agar yang dilengkapi dengan Basta®. Bahagian T-DNA yang di integrasi dapat dikesan melalui reaksi rantaian polimeras (PCR) menggunakan GUS primer.

Kata kunci: transformasi perantara-*Agrobacterium*, kulat endofit, pemilihan Basta®, *GUS* gen, reaksi rantaian polimeras, *Ceratocystis paradoxa*

1.0 Introduction

Genetic modification has led to both potential benefits to improve human nutrition, health and unknown risk for human population and environment. Due to increase in human population, there is an increase in the need for food products and nutrition especially in developed and developing countries. Thus, genetic engineering is one or the effective way to increase food production, improve agriculture crop to alleviate starvation in poor countries as stated by Guha et al. (2013).

Advancement in modern biotechnology, especially in medicine; food, waste utilization and bioremediation are resulted from exploitation of fungi (Pointing & Hyde, 2001). Fungi utilization for human will become an indispensable in the future. The evolution of molecular and cellular biology leads to the genetic transformation to introduce the exogenous genes into cells and tissues (Jenes et al., 1993).

The endophyte fungi live within the host plant to get nutrients for growth. Thus, endophyte fungi are a suitable model for plant transformation and subsequently the characteristic of its host also can be study, an example of this would be *Ceratocystis paradoxa*. Endophyte can have a wide range of relationship with host varies from symbiosis, mutualism and pathogenic. *Ceratocystis paradoxa* is habitually living within wide plant species these includes coconut, pineapple, sugarcane and banana and associated with bole rot disease (Jonathan et al., 2012). In this research, *C. paradoxa* is fungal endophytes derived from sago pith is used as the transformation model. Industrial applications such as high fructose syrup production, paper making, food processing and ethanol production was there result of exploitation of diverse fungal amylolytic enzymes, including α-amylase, gluocoamylase and α-glucosidase (Chen et al., 2012).

The focus of this research is to transform endophyte fungi to carry a desired foreign DNA by using *Agrobacterium tumefaciens*-mediated transformation protocols. The foreign DNA is carried by binary vector, pGSA1131 that has *BAR* selectable markers and *GUS* reporter gene. The selection of the transformed endophyte fungi is analyzed by the resistance to growth in Basta® medium that contains glufosinate and phosphinothricin (Chaure et al., 2000). According to Ngieng et al. (2013) the transformed endophyte will be observed by PCR using GUS primer. The PCR product will be analyzed using agarose gel electrophoresis.

Problem Statements

I. Can the endophyte fungi *C. paradoxa* genetically transformed via *Agrobacterium tumefaciens*-mediated transformation?
II. Can *BAR* gene of transformed endophyte fungi be expressed in a transformed *C. paradoxa*?

Objectives

i. To assess the capability of endophyte fungi, *C. paradoxa* to be transformed with a vector carrying foreign DNA, pGSA1131 via *Agrobacterium tumefaciens*-mediated transformation.
ii. To determine the transformed fungi and observe the expression of *BAR* gene of the transformed *C. paradoxa*.

2.0 Literature Review

2.1. The endophyte fungi, *Ceratocystis paradoxa*

Currently, there are approximately 80 000 to 120 000 species of fungi exist, whilst the estimated number of around 1.5 million species (Hawksworth, 2001). Endophytic fungi have a relationship with plants; it reside entirely within plant tissues and may grow within the roots, stems and leaves, emerging from the plant host to sporulate (Stone et al., 2004). Endophyte can has a wide range of relationship with host that varies from symbiosis, mutualism and pathogenic.

This work uses the endophyte *Ceratocystis. paradoxa,* that was isolated from sago pith and obtained from Molecular Genetic Laboratory (Ngui Sing Ngieng, pers comm.). *Ceratocystis paradoxa* is among the best amylolytic fungus isolated from sago pith industrial waste as stated by Sallehin et al. (2009), although it has pathogenic effect against some crop variety which is coconut, pineapple, sugarcane and banana. Nevertheless, it has been studied for its starch degrading and enzyme properties and production. The diverse fungal amylolytic enzymes including α-amylase, gluocoamylase and α-glucosidase, have wide industrial applications such as high fructose syrup production, paper making, food processing and ethanol production (Chen et al., 2012).

C. paradoxa lies in the domain Ascomycota, habitually living within a wide plant species that includes coconut, pineapple, sugarcane and banana, and associated with bole rot disease (Jonathan et al., 2012). In North Africa, Bayoud disease caused by the parasitic fungus *Ceratocystis paradoxa,* is a common threat to date palms (Juhany, 2010). Post-

harvest diseases of coconut reported at Jiouru, Changjr, Neipu in Pintung county, showed a fruit rot disease caused by *C. paradoxa* (Tzeng & Sun, 2009). Principal symptoms of this disease are blackening of exocarp, mesocarp and then endocarp of coconut fruit. The affected fruit usually emitted fruit-type fragrant.

The chlamydospores and conidia of the asexual are the vital inoculum origin. Both spore forms persist in the soil as stated by MacFarlane, (2015). The dark, thick-walled chlamydospore helps in long-term survival of *C. paradoxa*. The distribution is through rain-splash and airborne dispersal, and by transport of infested soil. As the symptoms on infected trees, a soft, yellow rot can be seen and the affected areas of the trunk show a dark discoloration. The trunk decay of *C. paradoxa* tissues, results in a reddish brown liquid that bleed from the point of infection, producing a sap flow that extends down the trunk and blackens as it dries. The level of pathogenicity of *C. paradoxa* may cause a heart rot, resulting in reduced growth, necrosis of lower leaf and ultimately defoliation and death of infected palms (MacFarlane, 2015).

2.2. Genetic engineering

Genetic engineering is the process of adding foreign DNA to an organism. The goal is to add desired traits that are beneficial. Currently, the genetically modified organisms that are in the market include those to increase productivity of crops, high nutritional value crops, plants with resistance to some insects and tolerate herbicides, and crops with modified oil content (n.d, 2005). Genetic engineering is also applied in medical area such as liver cancer studies. Hepatocyte gene therapy is being developed as an alternative to liver transplantation, which is the only effective therapy for many liver diseases (Guha et al., 2013). It has shown to have high survival rates because the liver has unique features that make it attractive for *in vivo* gene transfer. Thus, genetic engineering is one or the

effective way to increase food production, improve agriculture crop to prevent starvation in poor countries and help cures disease.

In addition, there are discussions about the development of GMOs and the safety and efficacy of the new products. This includes public concerns about gene technology in issues of ethical concerns, socio-economic issues, effects on the environment and food safety and human health.

2.3. *Agrobacterium*-mediated transformation

The evolution of molecular and cellular biology leads to the genetic transformation with the aim to introduce exogenous genes into cells and tissues (Jenes et al., 1993). Crown gall disease is disease that affects most plants and caused by *Agrobacterium tumefaciens,* a gram negative bacteria (Slater et al., 2003). *A. tumefaciens* has the ability to transfer bacterial genes into the plant genome, resulting in tumorous tissues growth. The features of *A. tumefaciens* are rod-shaped, motile bacterium that lives in rhizophere and get nutrients from the roots. When the plant roots are damaged, *A. tumefaciens* can infect the wound area, causing several symptoms as described by Slater et al. (2003).

Due to the characteristics of infection, *A. tumefaciens* have be manipulated to carry foreign genes, used to infect plants and be applied to agricultural sector. Thus, the modification of plants to then carry desirable traits can be achieved. In genetic transformation, the *Agrobacterium* oncogenic gene is eliminated to produce disarmed helper tumour-inducing plasmid that still has the ability to transfer foreign DNA. According to Chawla (2003), the Ti-plasmid for fungi transformation is extensively modified so that most of the features are eliminated, with only the left and right border sequences being used to transfer the foreign DNA, to be integrated into the host genome, and genetically produces modified host

genome. The stable gene transferred by *A. tumefaciens*-mediated transformation is achieved as stated by Chawla (2009).

In the previous research, *Agrobacterium*-mediated transformation of the endophytic fungus *Acremonium implicatum* associated with *Brachiaria* grasses, have been reported by Abello et al. (2007). In the study, it is showed that the transformation was possible and the efficiency doubled for both mycelia and conidia transformation with increase in co-cultivation period for *A. tumefaciens* and *A. implicatum* from 48 to 72 h. The concentrations of acetosyringone is high in the *Agrobacterium* culture and co-cultivation medium proved to be indispensable for successful transformation (Sarahan et al., 2004).

2.4. Binary vector, pGSA1131

The practices of molecular biology technique enable creation of the binary vector for fungi transformation. The binary vector system consist of two autonomously replicating plasmid within *Agrobacterium*, a shuttle vector that contain gene of interest between T-DNA border and a helper Ti plasmid that provide *vir* genes product to facilitate transfer into host cells (Chawla, 2003). The small size of pGSA1131 which is 9394 bp, made the plasmid easily to be manipulated. The desirable features of a binary vector are that they are small in size, easy to manipulate and less liable to damage (Slater et al., 2003). To ensure the replication of high copy number in *E. coli*, the binary vector contained an origin of replication (Slater et al., 2003). To determine for transformed fungi, the selectable marker (*BAR* gene) and reporter gene (*GUS* gene) in the binary vector can be analyzed.

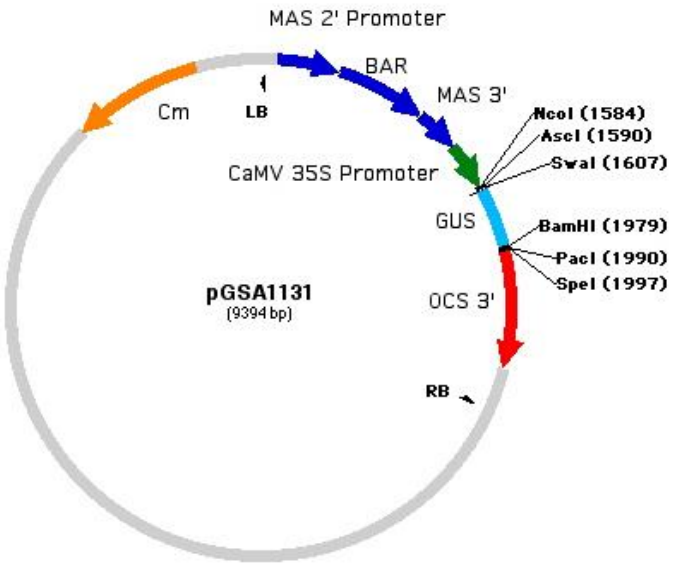

Figure 1. The map of binary vector, pGSA1131

2.5. *GUS* reporter gene

The reporter gene that contained within the T-DNA region of the binary vector acts as an indicator of the transformation. *β-glucuronidase* (*GUS*) originated from *E. coli* strain RAJ201 (Slater et al., 2003). The hydrolyzation of β-glucuronides to glucuronic acid can be performed by the product of *GUS* gene as stated by Hames & Rickwood (1993). The gene encodes a polypeptide of 602 amino acids. *GUS* is a versatile reporter gene system as it can be detected via spectrophotometric, fluorometric and histochemical assay for enzyme activity (Jefferson, 1987). The use of *GUS* enables for both qualitative and quantitative monitoring of fungal expression (White et al., 2005).

2.6. BAR Selectable Marker

Fungi transformation is a very low frequency event. Therefore, it is important to select the transformed endophyte fungi and utilizing the selectable marker that was incorporated into the fungus genome. In the transformed fungus filaments, the expression of the selectable

marker makes it resistant to the toxic materials (Slater et al., 2003). The *BAR* gene contains a bialaphos resistance cassette is an inhibitor of glutamine synthetase (Chaure et al., 2000), which is a herbicide with the active component phosphinothricin, commercially available as Basta®. The positive selectable marker for bacteria, fungi, and plants can be driven from the *BAR* gene that is isolated from the bialaphos, encodes a phosphinothricin acetyl transferase that inactivates the herbicide (Chaure et al., 2000). The growth and sporulation of the *M. cladophyllus* in the bialaphos medium indicates the successful of transformation.

3.0 Materials and Methods

absolute ethanol (Scharlau)
acetosyringone (PhytoTechnology Lab)
calcium chloride (J. T. Baker)
carbenicillin
chloramphenicol (Naqalai Tesque)
potato dextrose agar
CIA 24:1 (Amresco)
EDTA (HmbG)
ethanol
formic acid (Sigma)
glucose (R&M Chemical)
glycerol stocks (HmbG)
isopropyl alcohol (Amresco)
induction media
Luria agar and broth (Himedia)
magnesium chloride (Lab Reagent)
NaOH (MERCK)
NaCl (UNI CHEM)
potasium acetate (MERCK)
potato dextrose broth (MERCK)
SDS (MERCK)
sodium chloride (UNI-CHEM)
Tris-HCl (Amresco)
plasmid miniprep kit (Bio-Rad)

3.1. Preparation of reagent

3.1.1. Preparation of Luria broth

Approximately 6.25 g of Luria broth medium was dissolved in 250 ml bottle contained distilled water and autoclaved. The bottles was cooled and stored in 4^{o}C freezer.

3.1.2. Preparation of Luria agar

Approximately 8.78 g of Luria agar was dissolved in 250 ml bottle contained distilled water and autoclave. The bottles was cooled to 55^{o}C and poured into petri dish. The agar was settled to harden. Then, the plate was inverted, sealed with parafilm and stored in 4^{o}C freezer.

3.1.3. Preparation of potato dextrose agar

Approximately 9.75 g of potato dextrose agar was dissolved in 250 ml bottle contained distilled water and the mixture was autoclaved. The bottles was cooled and stored in 4^{o}C freezer.

3.1.4. Preparation of potato dextrose broth

Approximately 2.4 g of potato dextrose broth was dissolved in 100 ml bottle contained distilled water and autoclaved. The bottles was cooled and stored in 4^{o}C freezer.

3.2. The preparation of competent cells

3.2.1. Culture *Escherichia coli*

This work is done in laminar flows cabinet where 70% alcohol was used to surface sterilized the work surfaces and UV light was switched on for 15 minutes. About 10 ml of LA was poured into each petri dish. Approximately 5 µL, 10 µ and 20 µL of XL-1 blue strain of *E.coli* was pipette and added into two LA plates as disused by Sim et al. (2011). A sterile inoculation loop was drag through streak and spread the *E. coli* over a section of the plate. The plates were incubated in 37^{o}C for 16 h.

3.2.2. Growing bacterial culture

The next day, a single colony of *E. coli* was picked and transferred into 10 ml LB medium. The culture will be incubated in 3500 rpm shaker for 16 h at 37˚C.

3.2.3. Harvest cell and storage of competent cell

The next day, 100 µL of overnight culture was poured into 13 ml of LB. The OD_{600} was monitored, until reading was at 0.55. The culture vessel was transferred to ice box for 10 min as discussed by Sim et al. (2011). Then, the cell was centrifuged at 3500 rpm for 5 min in 4^{o}C. The supernatant was discarded and the tube was air dried. Then, the cell pellet was washed gently by resuspend in 5 ml of ice cold 100 mM calcium chloride solution. The cell suspension was kept on ice for 10 min and centrifuged again 3500 rpm for 2 min. After that, the supernatant was discarded and the tube was air dried. The cell pellet was resuspend in 2.5 ml of cold 100 mM $CaCl_2$ solution. Then, cell suspension was incubated

on ice for 1 h. About 20% glycerol stocks was added and snap freeze the competent cell in liquid nitrogen and stored at -80^{o}C.

3.2.4. Transformation of competent cells

Approximately, 10 ml of pGSA1131 was added to the 50 ml competent cell suspension and placed on ice for 20 min. The tubes were transferred to a 42℃ water bath. Then, the tubes were placed on ice for 2 min. About 950 µL of LB medium without chloramphenicol antibiotic was added and the tube was placed on a shaker for 1 h 30 min at 150 rpm at 37℃. Then, the 100 µL of Cm antibiotic will be plated only on the top of the LA plate only. The cells were spread evenly. The medium was left overnight at 37℃ or until the colonies of *E. coli* contained the pGSA1131 appeared (Chawla, 2003).

3.2.5. Mini Preparation for Extraction of Double Stranded Plasmid DNA in *Escherichia coli*

GeneMATRIX plasmid miniprep DNA purification kit was used for extraction of pGSA1131 in *E. coli*. About 40 µl of activation buffer PL was applied onto spin column and kept at temperature until transferred lysate to spin-column. The overnight culture is poured into 1.5 ml tubes and spin in 13 500 rpm for 2 min in microcentrifuge machine. The supernatant was poured off and the tube was air-dried. About 250 µl of cell R buffer was added and resuspended completely. About 350 µl of neutralization and binding buffer neutral B was added and mixed by inverting the tube a few time and until the blue colored disappeared. Then, the tubes was spun in 13 500 rpm for 7 min in a microcentrifuge. The supernatant was poured off into spin column placed in receiver tube and spin down for 12 000 rpm for 1 min. The spin column was removed; the supernatant was poured off and placed back in receiver tube. Approximately, 500 µl wash PLX1 buffer and spin down at 12 000 rpm for 1 min. The spin column was removed, supernatant was poured off and spin column was replaced back. Then, about 650 µl of wash PLX2 buffer was added and spin down at 12

000 rpm for 1 min. The spin column was removed, supernatant was poured off and spin column was replaced back. spin down again at 12 000 rpm for 2 min to remove any traces of wash PLX2 buffer. The spin column was placed into new 1.5 ml receiver tube and 50 µl of elution buffer to elute bound DNA. The tubes was incubated at for 2 min at room temperature and spin down at 12 000 rpm for 2 min. The spin column was removed and capped the receiver tube. Plasmid DNA was ready for AGE and PCR for analysis.

Table 1. GUS primer sequences

Primer name	Primer sequence	Annealing temperature
GUS-eF	5' CCC CAG ATG AAC ATG GCA TC 3'	62.4^{o}C
GUS-eR	5' GAT CCC CAT CAA AGA GAT CGC 3'	62.7^{o}C

Table 2. PCR profiling for *GUS* gene

Segment	temperature	duration	No. of cycle
Initial denaturation	95	3 min	1
Denaturation	95	30 sec	35
Annealing	57.5	30 sec	35
Extension	72	45 sec	35
Final extension	72	5 min	1
Final hold	20	∞	1

Table 3. PCR ingredients for 25 µl reaction mixture for GUS primer

Reagent	Volume
10x taq buffer	2.5 µl
dNTP mix	0.5 µl
GUS-eF	1 µl
GUS-Er	1 µl
25 mM $MgCl_2$	1.5 µl
Template DNA	1 µl
Taq DNA polymerase	1 µl
Ultrapure distilled water	16.5 µl
Total volume	25 µl

3.3. *Agrobacterium*–mediated transformation

3.3.1. Preparation of *Agrobacterium* culture

Approximately 5 µL and 10 µL of *Agrobacterium* LBA4404 stock was pipetted and added into two petri dishes containing 10 ml LA with 3 µl chloramphenicol and 20 µl rifampicin. A sterile loop was used to streak the *Agrobacterium* and the plates were incubated in dark, at room temperature for 2 days.

3.3.2. Growing *Agrobacterium*

After two days of incubation, a single colony of *Agrobacterium* was selected and transferred into 10 ml LB medium with 3 µl chloramphenicol and 20 µl rifampicin. The culture in was incubated in a shaker incubator at 1800 rpm for 2 days at room temperature in the dark.

3.3.3. Harvesting and storage of *Agrobacterium* cells

The next day, 200 µl of overnight culture was poured into 15 ml of LB supplemented with 30 µl rifampicin and 4.5 µl chloramphenicol. The OD_{600} was monitored, until reading was at 0.6. The culture vessel was transferred to ice box for 10 min (Sim et al., 2011). Then, the cell was centrifuged at 3500 rpm for 5 min in 4^{o}C. The supernatant was discarded and the tube was air dried. Then, the cell pellet was washed gently by resuspend in 5 ml of ice cold 100 mM calcium chloride solution. The cell suspension was kept on ice for 10 min and recentrifuged again 3500 rpm for 2 min. After that, the supernatant was discarded and the tube was air dried. The cell pellet was resuspended in 2.5 ml of cold 100 mM $CaCl_2$ solution. Then, cell suspension was incubted on ice for 1 h. About 20% glycerol stocks (HmbG) was added, and snap freeze the competent cell in liquid nitrogen and stored at -80^{o}C.

3.3.4. Freeze thaw transformation of *Agrobacterium* with pGSA1131

Approximately 10 µl plasmid DNA was added to 100 µl of competent cells and mixed by gently by pipetting up and down 2 to 3 times. Incubate reaction on ice for 20 min. Then, the tube was placed in liquid nitrogen for 5 min (Sim et al., 2011). The mixture was then heat shocked in 37^{o}C water bath for 5 min and returned to ice for 5 min. One milliliter of LB was added to the tube, incubated on a 28^{o}C in 180 rpm rotating shaker for 4 hrs and overnight. Then, 100 µl of culture was spread on 7 ml LA plate containing a selection agent, 2.1 µl chloramphenicol and 14 µl rifampicin. Remaining culture was kept at 4^{o}C for up to 14 days. The plate was allowed to grow for 2 d at 28^{o}C (room temperature) and proceed to DNA plasmid extraction and colony PCR to confirm for transformants.

3.3.5. Mini preparation for extraction of double stranded plasmid DNA in *Agrobacterium* cells

The same GeneMATRIX plasmid miniprep DNA purification kit was used for extraction of pGSA1131 in transformed *Agrobacterium.* Plasmid DNA was ready for AGE and PCR for analysis.

3.4. The endophyte fungi, *Ceratocystis paradoxa*

3.4.1. Sub-culture of *Ceratocystis paradoxa* from pure culture

The pure culture of *C. paradoxa* was obtained from Molecular Genetic Laboratory. A single colony of endophyte fungi strains was sub-cultured on PDA and incubated at room temperature for 3 days. After incubation, white coloured colonies were observed on the plates. After a further 2 days of incubation, the colonies colour blackened and produce a fruity-like odour. A pure culture of colony type on each plate was obtained and maintained. The maintenance was done by sub-culturing each of the colonies onto the PDA plates and incubated at room temperature again for 3 days.

3.4.2. Preparation and harvest *Ceratocystis paradoxa* cells

The conidia of *C. paradoxa* were used for transformation. The fungus was grown on PDA for 3 d and incubated at 28℃. Conidia were washed in a 10 ml solution of 0.15 M NaCl physiological salt. The conidia were collected by pipette 1 ml solution in 1.5 ml tube.

3.4.3. Select the working concentration of Basta® to inhibit growth of *Ceratocystis paradoxa*

C. paradoxa strains were sub-cultured in three PDA plate at room temperature for 3 d. The concentration of Basta® used was 0, 50 and 100 µg/ml. Fungi growth was detected at 0 and 50 µg/ml of Basta®. At 100 µg/ml concentration, no growth of fungi was detected, therefore,this concentration was used for selection of transformed fungi.

3.4.4. Transformation of *Ceratocystis paradoxa* with pGSA1131

In the transformation of fast growing fungi, a high concentration of carbenicillin (100 µg/ml) was used. According to Abello et al. (2008), carbenicillin (Cm) act as inhibitor for *A. tumefaciens* growth. Thus, high concentration of carbenicillin was adequate to inhibit growth in *A. tumefaciens* while allowing *Ceratocystis paradoxa* putative transformants to grow on selection media as described by Mullins et al. (2001). *C. paradoxa* strains and LBA4404 was transformed with vector pGSA1131, using the methods described by Park (2001).

About 200 µl of transformed *Agrobacterium* contained pGSA1131 was grown in 20 ml LB medium supplemented with 6 µl Cm, 40 µl rifampicin and incubated at 28℃ rotating shaker in the dark for 2 d to an OD_{600} of 0.66. Bacterial cells in 2 ml and 4 ml aliquot were harvested and washed with induction medium, and then resuspended with 5 ml of infiltration medium (IM) in the presence and absence of 200 µM acetosyringone (AS). Suspension cells of *A. tumefaciens* of approximately 100 ml, were mixed with 100 ml of *C.*

paradoxa conidia. Subsequently, each 200 ml of these mixtures was placed on PDA in the presence or absence of 200 µM AS. The plates were incubated at 28℃ for 3 days.

3.4.5. Selection of transformed endophyte fungi, *BAR* selection

Selection for *BAR* genes in *C. paradoxa* transformants was performed by overlaying the top plate of *C. paradoxa* with Basta®. About 25 ml of PDA overlay agar containing 100 µg/ml of Basta® was used to select for transformed fungi and 100 µg/ml of carbenicillin was used to inhibit the growth of the *Agrobacterium* was included in top agar medium. The medium was incubated for 7 d at 28℃ and transformed *C. paradoxa* growing on the top agar were sub-cultured onto a new PDA medium containing 100 µg/ml of Basta® for mitotic stability verification.

3.4.6. Analysis of transformed *Ceratocystis paradoxa*

The extraction of fungal DNA started with pre-warming the extraction buffer in 70°C water bath (Cubero et al., 1999). At the same time fungal mycelium was filtered with filter paper to remove the potato dextrose broth and dried. Liquid nitrogen was poured onto the mycelium and grinded until fungal mycelium was turned into powder. Fungi powder was transferred to a 1.5 ml eppendorf tube. About 500 µl of pre-warmed extraction buffer was added into these tubes and incubated in 70°C water bath for 30 minutes. Then, an equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and mixed by inverting the tubes 10 times. The tubes were spun down for 5 min at 10,000 rpm at room temperature. The upper layer aqueous phase was collected and transferred into a new eppendorf tube. About two volumes of precipitation buffer was added to the supernatant and the resulting mixture in tubes was inverted for 2 min to mix. The tubes were spun down for 10 min at 13,000 rpm at room temperature and the supernatant was discarded. The pellet was resuspend with 350 µl of 1.2 M NaCl, an equal volume of chloroform:isoamyl alcohol (24:1 v/v) was added and mixed vigorously. Then, the tubes were spun down for 5 min at