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# VECTOR CONSTRUCTION FOR SOD GENE OVEREXPRESSION AS AN ATTEMPT TO DEVELOP PHYTOREMEDIATION PLANTS

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## Abstract

*We construct a new binary plasmid vector designated pCHISOD, harboring* SOD *gene. A 564-bp NCO II /Best EIII fragment containing the intact SOD gene and its flanking regions was initially inserted into pCHIE. The purpose of this research was to construct binary plasmid which will introduce the SOD gene into Mel stoma plants in order to create transgenic plants of rphytoremediation of metals. Transgenic plants of M. malabathricum carrying the SOD gene have been developed via Agro bacterium tumefactions-mediated transformation. This method*

*involved the use of leaf explants co-cultivated with disarmed Agro bacterium strain LBA4404, which on the T-DNA region carries SOD genes. The presence of the T-DNA in the regenerated shoots will confirmed by amplification of the transgene using polymerase chain reaction.*

**Keywords**

Melastomamalabathricum, SOD phytoremediation

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## 1. Introduction

*Melastomamalabathricum* L. Is one of the most common weeds that grow abundantly in waste grounds and open fields? This woody species is highly tolerant to Al stress, but little is known about their ability to grow in heavy metal-contaminated soils. Elevated concentrations of both essential and non-essential heavy metals in the soil can lead to toxicity symptoms and growth inhibition in most plants (Li et al., 2008). Plants vary in their ability to absorb and accumulate minerals from the soil solution. *M. malabathricum*accumulates more than 10 mg Al $kg^{-1}$ in leaves and roots. Generally, plants are classified as accumulators if they accumulate at least 1000 mg kg-1 in their leaves (Hutchinson & Wollack, 1943).Our previous analysis in plant tissue showedthat *M. malabathricum* accumulate more Pb than Cd and Cu, and only small amount were transported to stem and leaves, so *M. malabathricum* is suitable as agent of phytoremediation.

The objective of this work is intended as a protocol for producing gene expression constructs, starting with isolated clone, for use in plants that are transformable by *Agro bacterium*. In this construct we use a strong promoter and adjoining upstream sequences to drive high level and constitutive transcription of a SOD gene's coding sequence.

Superoxide dismutase's (SODs) are metal-containing enzymes that catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide. Thus, they are an important antioxidant defense in nearly all cells exposed to oxygen. The enzyme has been found in all aerobic organisms examined where it plays a major role in the defense against toxic- reduced oxygen species, which are generated as by-products of many biological oxidations. The generation of oxygen radicals can be further exacerbated during environmental adversity and consequently SOD has been proposed to be important for plant stress tolerance. It acts to

scavenge molecular oxygen and its univalent reductants, thereby protecting the cell from the harmful effects of these reactive oxygen species (ROS).

We performed an *Agro bacterium*-mediated transformation system of *Melastoma*leaf explants by using hygromycin selection. Regenerated *Melastoma*plants with hygromycin-resistance will be subjected to heavy metal stress. Molecular characterization by polymerase chain reaction (PCR), analysis will reveal how many percent of the hygromycin-resistant regenerated *Melastoma*plants was successfully transformed using this method.

## 2. Materials and methods

### Materials

Seeds of *Melastomamalabathricum*L. Collected from Jasinga, West Java Province was used as plant materials. Plasmid pCHIE (PT. BISI Tbk collection) were used as an expression vector in this study (Figure 1). *Escherichia coli* strain DH5α was used as recipient hosts for recombinant vector. The *Agro bacterium tumefaciens* strain LBA4404 was used for the transformation of the *M. malabathricum*L. All *in vitro* manipulation and cloning was conducted using standard techniques.

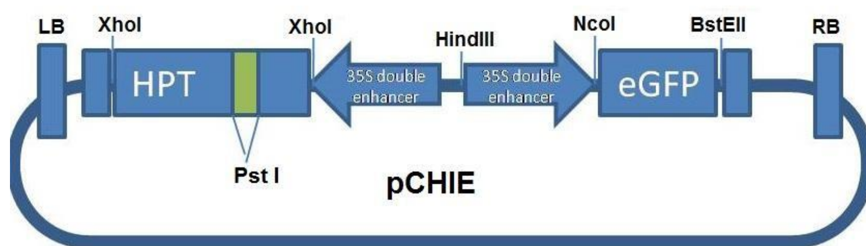


**Figure 1:** *pCHIE original plasmid which carried the eGFP coding region*

### Methods

### 2.2.1 pCHIE-SOD expression vector construction

We were using the p CHIE binary vector, which modified from p Cambia includes a pC1302origin of replication, the HPT gene conferring hygromycin resistance in bacteria, both the right and left T-DNA borders, and a multiple cloning site (MCS), pVS1 replica for high stability in *Agro bacterium.* The e GFP gene was removed and replaced with SOD gene to generate p CHISOD binary plasmid.

Binary plasmids were constructed using standard recombinant techniques. The binary Trans formation vector p CHIE was constructed as follows. A 564-bp fragment of the SOD gene was synthesized by PCR using the specific primers 5'-CCGGGACCATGGTGAAGGCTGAGGTT-3'and

5′GAGCTCGGTCACCTTAACCCTGGAGA-3'. Underlined sequence in primer shows introduced restriction enzyme site: *Nco*I (at forward primer) and *Best EII* (at reverse primer). The resultant insert *NcoI*I /*Best EII*I-sod was then introduced downstream of the 35S Ca MV promoter in *NCOs* /*Best EII*-digested p CHIE, yielding p CHISOD.

One microfiber template plasmid DNA was mixed in a reaction cocktail containing 1xTaq buffer, 4 moment mix, 10 pmol primer *forward*, 10 pmol primer *reverse,* and 2UTaqpolymerase (Ferments) and ddH$_2$O for final volume of 20 µl. PCR was performed as follows: 95$^o$C for 2 minutes, followed by 35cycles of 94$^o$C for 30 seconds, 56oC for 30 seconds; extensión 72oC for

1.5 minutes, and a final extensionista of 72$^o$C for 5 minutes.

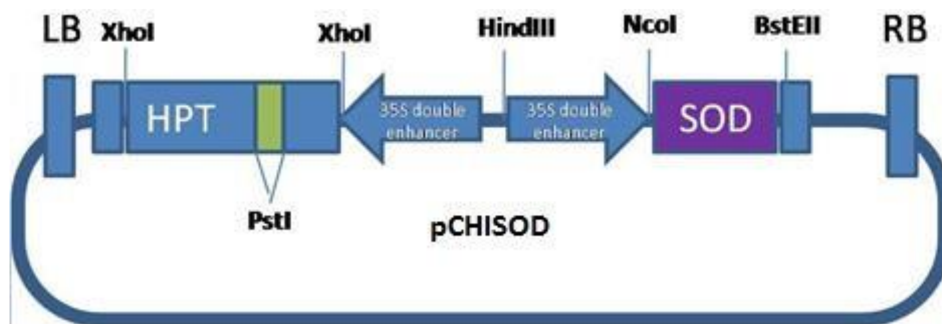


**Figure 2:** *TheT-DNA region of p CHISOD. HPT =hygromycinphosphotransferase II gene, SOD = Super Oxide Dismutase gene, LB = Left Border, RB = Right Border*

The T-DNA of p CHISOD binary vector carried a hygromycin phosphor transfer se II (*hpt*) resistance gene. The *SOD* gene was driven by the CaMV35S promoter (Figure 2). The promoter CaMV35S is a strong, constitutive promoter mainly used in transgenic expression in plants. SOD gene were lye next to the right T-DNA border

The procedure for vector preparación the p CHISOD were double digested with *Nco*I and

*Best EII*. The 200-bp *NcoI/ Best EII* fragment was legated into the MS between left and right border of T-DNA of the binary vector p CHIE. The ligation reaction mixtures contained 6 µl (60ng) insert, 2µl (20 ng) p CHIE, 1 µl (40 U) T4 DNA ligase and 1 µl 10x buffer ligation. The reaction was incubated at 4°C overnight and subsequently introduced into DH5α strain of *E. coli* using heat shock method as described by Shearson (2002).

Mini-prep plasmid DNA from the Trans formant clones was performed to identify successful sub clones. Isolation of plasmid DNA was performed by using QI Aprep Spin Miniprep Kit, and the insertsanalyzed by direct PCR of transo formantcolonies.

**Trip rental mating to introduce the binary vector**

The constructed plasmids p CHISOD were introduced into *Agro bacterium tumefactions'* LBA4404 by the Trip rental mating method (Liberty *et al.* 2008). Transformation using three kinds of bacteria: *E.coli* DH5α carrying the construct p CHISOD, *E.coli* HB101 was carrying helper RK 2013 and *Agrobacteriumtumefaciens*strain LBA4404.

Selection was performed by growing the 10-µl aliquot of bacterial suspension on solid LB selection medium containing the antibiotic streptomycin 50 mg/L, kanamycin 50 mg/L, hygromycin 10 mg/L and incubated at 28°C for 32 hours. *Agro bacterium* LBA4404 which carrying the construct phased is expected to grow on this selection medium.

Verifying the recombinant plasmids CHISOD in *Agro bacterium* carried out through colony PCR by using SOD specific primers. The expected size of the *SOD* fragment was approximately 564bp.

**Agro bacterium-mediated genetic transformation**

The *Melastoma*seeds were surface sterilized with 70% (v/v) ethanol for 30 s, and rinsed in sterile distilled water. Seeds were then treated with a 40% (v/v) commercial bleach (containing 5.25% of sodium hypochlorite) supplemented with 0.1% of Tween-20 for 10 min with occasional agitation, followed by washing in sterile distilled water 4 - 5 times.

The sterilized seeds were germinated on fresh, half strength Murashige and Skoog (MS) (Murashige & Skoog 1962) basal medium without any plant growth regulators. The seeds were germinated at 24 °C under 16 light/8 h dark photoperiod. Leaf in approximately 0.5—0.6 cm

fron 6 to 8 weeks old plantlets were used as explants materials for transformation.

**Preparation of the bacterial suspension for co-cultivation**

The *A. tumefaciens*strain LBA4404 harboring the binary vector p CHISOD was used for Tran's formation experiments. Streak out a single colony of *A. tumefactions'* LBA4404 containing p CHISOD on solid LB (Luria Bertani) medium containing spectinomycin20 mg/l and hygromycin (10 mg/l) which is appropriate antibiotic for plasmid selection. *A. tumefaciens*cells were then collected by centrifugation at 4000 rpm for 5 min at 4°C, and the pellet obtained was resuspended in about 20 ml of liquid CIM medium (Callus-Inducing-Medium) + Acetosyringone.

**Transformation and regeneration of *Melastomamalabathricum***

Young healthy well-expanded leaves were choose from the 8-weeks-old *in vitro* plantlets. Leaf at a time was transfer to large glass Petri dish (14.4 cm diameter) containing sterile water. Manipulating the explants in water for avoids desiccation. The excised leaf explants (0.5x0.5 cm) were inoculated by soaking in *Agro bacterium* suspension with agitation at 200 rpm on a rotary shaker at room temperature. Infected explants were blot-dried on sterile filter papers. Then transfer the explants, abaxial side up, to a Petri dish containing solid Co medium (Co cultivation-Medium) for 3 days in the dark at 26 ± 2°C.

After 3-4 days of co-cultivation in total darkness, the infected explants were rinsed three times with sterilized water, followed by two times with sterilized water with 300 mg/L cefotaximeto remove excess Agro bacterium suspension. The explants were then blotted dry on sterile filter paper and placed onto the CIM (Callus-Induction Medium) containing hy gromyc in for selection of transformed cells for maintaining the explants from Agro bacterium overgrowth. Explants in Petri dishes were cultured in the dark at 26 ± 2°C.

After 3 weeks, calli were sub cultured onto the SIM (Shoot-Inducing Medium). Hygromycin-resistant shoots obtained were subcultured several times onto fresh SEM (Shoot-Elongation-Media). Plantlets with well-developed shoots were transferred into RIM (Root-Inducing-Medium) for root development.

The rooted plantlets were then transferred into half strength M&S medium supplemented with 0.5 mg/L NAA for root elongation. Plantlets with well-developed roots were acclimatized

and grown under greenhouse conditions with a 16/8 -h (light/dark) photoperiod.

**Molecular characterization of transgenic plants**

Genomic DNAs were isolated from leaves of hygromycin-resistant *Mel stoma* plants by the cetyltrimethylammoniumbromide (CTAB) method (Doyle & Doyle 1990; Shearson 2002). PCR analysis was carried out using200–300 ng of *M. malabathricum* genomic DNA employing 2x KAPA2G Fast Ready mix (Kapa Bios stem) in a 20-µl reaction volume according to manufacturer's instructions. PCR amplification was performed by initial denaturation at 94°C (5 minhold), followed by 35 cycles at 94°C (30s), annealing 56°C (for *ALS*)/ 58°C (for *SOD*) (45s) and 72°C (90 s), and followed by a final extension of 5 min at 72°C. Plasmid used as a positive control, with non-transgenic plant DNA as a negative control.

## 4. Results and discussion

**PCHIE binary plasmid construction**

The *SOD* fragment has been successfully isolated from p GEM -T easy-1 by using a PCR strategy with specific primers (Figure 3). Both vector and the insert had been successfully isolated for expression vector construction. After ligation process, the resulting recombinant plasmids were transformed into. *Coli* strain DH5α.

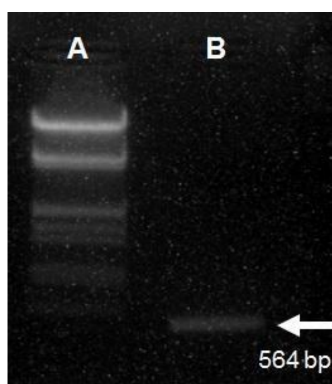


**Figure 3:** *Agarose gel profile of SOD insert (lane B) (lane A M= λ EcoRI/Hind III marker)*

After heat-shock transformation to introduced p CHIE into *E .coli* DH5α, we performed direct colony PCR on selective media using specific primer for *SOD*. The results show that eight randomly selected colonies carried the ligation product. Figure 4 shown 564 BP band which

correspond to the *SOD* coding region which indicated that *SOD* had been successfully inserted and cloned into the pCHISOD binary vector. The chased would then introduced into the *Agro bacterium* LBA4404 cells.

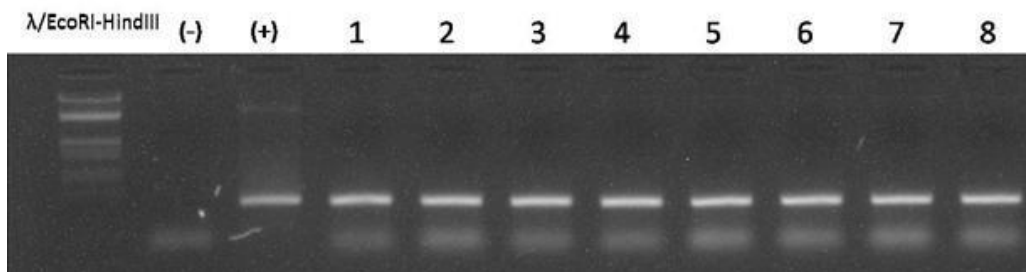


**Figure 4:** *PCR amplification of the cloned 564-bpfragment of the SOD gene was performed by direct colony PCR using specific primer for SOD (Lane 1-8 transformed E. coli carrying p CHISOD)*

In order to establish a system in which *A. tumefactions'* could transfer T-DNA to the *M. malabathricum* leaf explants, we constructed the binary vector p CHISOD. Binary plasmid p CHISOD were successfully been introduced into *A. tumefactions'* LBA 4404 by trip rental mating procedure. Trip rental matting's (Simon *et al.* 1983) were used to mobilize plasmid p CHISOD into *A. tumefactions'* LBA 4404 with the helper plasmid pRK2013. Bacterial colonies grown in the presence of selective agent 20 μg/ml streptomycin and 10 μg/ml hygromycin were

*A. tumefactions'* harboring SOD gene which confers resistance to streptomycin and hygromycin.

**Regeneration of transgenic Mel stoma transformed with SOD gene**

The present study describes results dealing with the establishment of a transformation protocol for the introduction of alien genes into the *M. malabathricum* genome. The transfer could depend on the induction of the bacterial virulence genes, which expression could mediate transfer of T-DNA into the explants.

Leaves explants from mature seeds were used for *Agro bacterium*-mediated transformation (Fig. 6A), since leaves of *M. malabathrcium* were found more susceptible to *Agro bacterium* infection than other explants such as hypocotyls, or petioles (Li et al., 2006).Li *et al.* (2008) made an attempt using cotyledon disks of *J. curcas*, however, the transformation efficiency was low and use of cotyledon explants may results in genetic variability and loss of the characteristic of the target plant (Bhatia et al., 2005).

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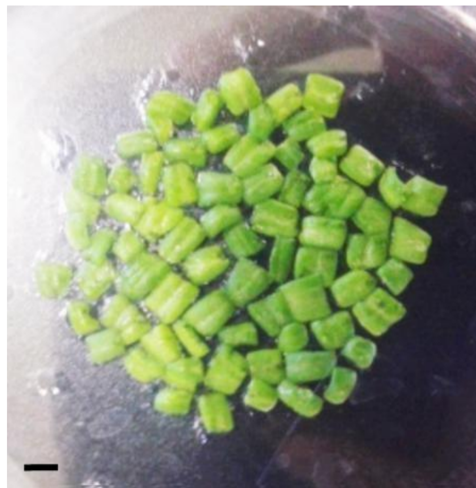
A B

**Figure 5:** *(A) Mel stoma malabathricumseed on MS medium (B) Four weeks old M. malabathricum plantlet on MS medium*



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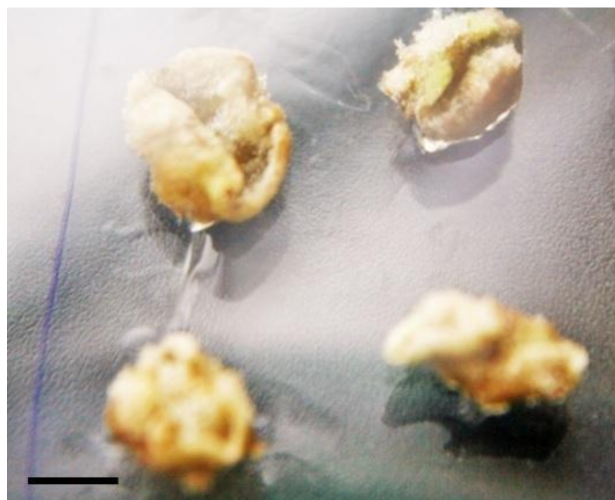

B

**Figure 6:** (A) *M. malabathricum*leaves explants on co-cultivation medium

(B) *M. malabathricum* leaves on selection medium (hygromycin 50 mg/L). Bar equals 1.0 cm

When the leaves of *M. malabathricum*seed lings were cultured for 5 d on half strength MS medium supplemented with NAA, the cut of leaf segments displayed expansion. The edge of most leaves curled when cultured for 6 d. After 8 d, callus were formed in some leaves and distributed in leaf vein and leaf edge, and the formation of callus peaked at 11 d. However, the formation of callus peaked at approximately 9 d. Along with increase of the culture time, callus gradually enlarge.

The callus is the primary step in the stimulation of shoot reproduction via indirect mode and adventitious organs regeneration. The regenerative processes in cell and tissue cultures may be provoked by plant grow deregulators where auxin is the best choice. From woody explants, callus is achieved via TDZ at concentrations≥0.1 µM (Huntsman & Preece 1993).

We used p CHIE to construct binary vectors. This vector hasan *HPT* gene, which enable selection of trans formants by hygromycin and bacterial selection by spectinomycin.The induction and differentiation of the callus from the leaf explants infected by *A. tumefactions'* and cultured for 50 d on MS medium supplemented with plant growth regulators, 200 mg/L cefotaxime and 15 µg/mL hygromycin, are shown in Figure 6 (A and B). A total of fifteen resistant buds were obtained.

As it is well known, genetic transformation mediated by *A. tumefactions'* is a complex process and transformation efficiency is affected by many factors, in which the type and concentration of antibiotics is very important to enhance the frequency of genetic transformation.

**Molecular confirmation of putative transgenic plants**

In the present investigation, we report the genetic transformation of *Mel stoma* leaf explants using the binary Ti vectors. The established transformation protocol proved effective for the selectable marker genes, *HPT gene* using cotyledon as explants. PCR analyses with genomic DNAs were not ready to conduct because insufficient calls formed up until now. This protocol should use the DNA isolated from putative transformed shoots, were used as template DNA.

## 5. Conclusion

In conclusion, the present work indicated that the transgender SOD have been sub cloned into p CHIE binary vector, by removed the e GFP gene and replaced with SOD to generate p CHISOD binary plasmid. The construct p CHISOD were successfuly introduce into *Agrobacterium tumefaciens* LBA4404 by Triparental mating technique.

## 6. Acknowledgment

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