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GENETIC TRANSFORMATION OF TOBACCO PLANT USING *INHIBITOR OF MERISTEM ACTIVITY* (*IMA*) GENES

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ABSTRACT

Inhibitor of Meristem activity (*IMA*) gene encoded a Mini Zinc Finger protein (MIF). *IMA* gene regulates flowering and ovule development. It controls *WUSCHEL* gene in meristem center and determined nucellus during the development of ovule. *IMA* gene is activated by the gene expression of type-D inhibited the proliferation of cell during floral termination, controlled the amount of carpels during flower development, and has a role in the primordial ovule initiation. *IMA* gene plays crucial roles during shoot apical meristem organization which associated with cell division, differentiation, and growth hormone. *IMA* gene has been successfully constructed into vector pGWB402 and introduced into *Agrobacterium tumefaciens* LB4404 through electroporation means. *IMA* gene is transferred to *Nicotiana tabacum* by using cotyledons *in vitro* culture. The transformed plant tissue is selected by addition of kanamycin in culture medium. The validation of transgene is detected by PCR using CaMV 35 S forward primer and *IMA* gene reverse specific primer. Transgenic plants show band size 500 bp indicating *IMA* gene has been integrated into the plant genome of *N. tabacum*.

INTRODUCTION

INHIBITOR OF MERISTEM ACTIVITY (*IMA*) gene encodes mini zinc finger protein family which is signed by a short sequence and usually contains zinc finger domain (Sicard *et al.*, 2008a). *It* encodes 90 amino acid which characterize as MIF protein family (Hu and Ma, 2006). *IMA* gene has some roles in regulating flowering and ovule development. Expression of *IMA* gene was localized during cell division stage of fruit development (Joubès *et al.*, 2001). Also, *It* inhibits cell proliferation during floral termination, controls the number of carpels during floral development, and acts as a repressor of the meristem organizing center *WUSCHEL* gene which functions as controller of stem cell homostasiesis through interaction with CLAVATA1 (Leyser and Furner, 1992) and CLAVATA2 (Jeong *et al.*, 1999). WUSCHEL itself plays crucial role during nucellus development (Laux *et al.*, 1996).

Ectopic expression of tomato *IMA* gene in *Arabidopsis thaliana* showed that this gene inhibited the growth phase and obtained stunted plant (Sicard *et al.*, 2008a). The results indicated that *IMA* gene involved in signal transduction pathways of some hormones and is an important factor regulating meristem activity pathway related to cell division, hormone, and differentiation (Sicard *et al.*, 2008a).

Overexpression analysis of *IMA* gene has done in *A. thaliana* as a model plant and in tomato as an original plant. Additionally, *A. thaliana* is a subtropics plant which is difficult to grow in tropical country for example Indonesia so that further analysis of *IMA* gene needs other model plant which able to grow in Indonesia. Tobacco is a dicotyledonous plant and the natural host for *A. tumefaciens* (Mayo *et al.*, 2006). Types of tobacco that are often used in genetic transformation is *N. tabacum* (Batti and He, 2009; Mayo *et al.*, 2006) and *N. benthamiana* (Anggraito, 2012). The species *of N. tabacum* such as Samsun (Stanic *et al.*, 1999), SRI (Batti and He, 2009), Bright yellow (An, 1985), Xanthi (Su *et al.*, 2012), and KasturI (Miswar *et al.*, 2005) has been successfully transformed by intermediaries *A. tumefaciens*. The genetic transformation of tobacco has been used for various purposes such as unco-vering the regulation of biological systems of plants, bioremediation for mercury, and a model plant for biotic and abiotic stresses analysis.

Genes such as *Citrate synthase* gene (De la Fuente *et al.*, 2013) and *Copper/Zinc Superoxida*

Dismutase (*CuZnSOD*) gene (Hannum, 2012) related to environmental stress also has been successfully introduced on tobacco. Tobacco plants have excellence as the model plants because of a large amount of seeds, short life cycle, and high efficiency transformation and easily are induced by doing *in vitro*. Therefore, the aims of this research are to construct *IMA* gene under CaMV 35S constitutive promoter and to integrate the gene into tobacco plant genome.

MATERIALS AND METHODS

PLANT MATERIALS

To obtain cotyledons of *Nicotiana tabacum* L, seeds (collected from Biology Research Indonesia Netherland, Bogor, Indonesia) were germinated and grown for 2 weeks in MS medium by tissue culture.

To recombination process used material by Gateway® Technology kit from Invitrogen (pDONR™ vectors, destination vector, *BP Clonase*™ enzyme, *proteinase K* enzyme, *LR Clonase*™ enzyme), competent cells, Luria Agar (LA) media, plasmid kit from Fermentas (Gene JET™ Plasmid Miniprep Kit) used for plasmid isolation, tobacco (*N. tabacum*) used as a model plant, and *A. tumefaciens* strain LBA4404.

Construction of pGWB402 vector to IMA gene

Amplification of fragment *IMA* gene; *IMA* gene fragment isolated from tomato (*Solanum lycopersicum*) in previous study amplified by using PCR according to (Sicard *et al.*, 2008b). The amplification used specific primer pair (gateway *IMA* forward and gateway *IMA* reverse) and then inserted into cloning site of pDONR.

***IMA* gene fragment was inserted into pDONR vector**; *IMA* gene fragment reacted with pDONR plasmid according to the protocol of Invitrogen (USA). *IMA* gene recombination in donor vector began in preparing as much as 2 µl DNA, 1µl donor vector (pDONR™ 221), and Tris-EDTA buffer (TE) until the volume became 8 µl. Afterward, 2 µl *BP Clonase*™ enzyme was added lastly and then incubated at 25°C for 2 hours. After incubation was complete, 1 µl proteinase K enzyme was added and incubated again at 37°C for 15 minutes. Recombination product has taken as many as 5 µl and then transformed into *E. coli* to replicate the plasmid that contains *IMA* gene. *E. coli* colonies grown on LA media were confirmed by colonies PCR method. Recombinant plasmid isolated from bacterial colonies and inserted into destination vector. 2 µl recombination plasmid and 1 µl destination vector was mixture in micro tube and TE buffer solution added to fulfill the volume until it became 8 µl. Next, 2 µl *LR Clonase* enzyme added and incubated at 25°C for 2 hours. After incubation was complete, 1 µl proteinase K enzyme added and incubated again at 37°C for 15 minutes. Recombination products as much as 5µl transformed into *E. coli*. DNA plasmid isolated from bacterial colonies and then the recombinant plasmid confirmed by using colonies PCR method and transformed into *A. tumefaciens* LBA4404.

Inserting recombination pDONR vector into *E. coli*; Recombinant pDONR introduced into competent cells (*E. coli* strain DH5α) by heat shock transformation at 42°C (Sambrook *et al.*, 1989). Colonies that were grown on the Luria Bertani Agar (LA) medium containing 50 ppm of kanamycin have taken for colonies PCR analysis. The process of PCR conducted by using a toothpick to take the colonies and then suspended in 5 µl H_2O. Next, the suspension heated at 95°C for 10 minutes and cooled at 15°C for 5 minutes. The suspension is used as a template for PCR reaction (Suharsono *et al.*, 2008) using M13 forward and M13 reverse primers. Then, recombinant pDONR plasmid isolated and purified by using high-speed plasmid mini kit-Geneaid.

Cloning vector pENTR/pDONR recombinant to pGWB402 vector; LR reaction stage used *LR Clonase* enzyme to insert pENTR/pDONR into pGWB402 vector in which is the process as according to gateway cloning protocol (Invitrogen, 2003).

Inserting pGWB402-*IMA* vector into *E. coli* strain DH5α; the results of LR reaction inserted into *E. coli* strain DH5α. Colonies comprising pGWB402-*IMA* plasmids verified by colonies PCR method using 35S CaMV forward and *IMA* reverse primers. DNA isolation containing pGWB402-*IMA* used *high-speed plasmid mini kit-Geneaid*.

pGWB402-*IMA* transformation into *A. tumefaciens* LBA4404; Expression vector of pGWB402-*IMA* isolated from *E. coli* DH5α inserted into *A. tumefaciens* LBA4404 through electrophorator. The results of electrophorator plated on LA medium containing 100 ppm streptomycin, 50 ppm spectinomycin, and 50 ppm kanamycin. Plasmids isolated from the colonies that grew by using the high speed geneaid plasmid mini kit. In order to confirm the success of transformation, PCR plasmids used

by specific 35S CaMV forward and *IMA* reverse primers.

Inserting *IMA* gene into *N. tabacum* SR1 genome; In the process of preparation of explants *N. tabacum*, seeds sterilized by using 70% alcohol for 1-2 minutes and washed by using sterile water five times. Then the seeds soaked in a disinfectant solution containing Na hypochlorite solution (1 ml Na hypochlorite, 10 ml Tween-20, and 20 ml water) for 10 minutes. Seeds washed again with sterile water five times and planted at MS0 media. Tobacco leaves obtained from sterile seeds used as an explant in the transformation.

Transformation, selection, and regeneration of *N. tabacum*; *A. tumefaciens* LBA4404 comprising pGWB402-*IMA* expression vector cultured in liquid LB medium. It contained 50 ppm kanamycin, 100 ppm streptomycin, and 50 ppm spectinomycin. Cultural bacterium that has objective density (OD) = 0.8-1 harvested and then diluted in liquid co-cultivation medium until its OD = 0.1-0.3 in which the medium contained 40 ppm acetosyringone. 1cm^2 explants where immersed in the culture solution of *A. tumefaciens* for 15 minutes and put on the shaker. Those explants dried by using sterile tissue paper and then planted on solid co-cultivation media containing 40ppm aceto-syringone. The co-cultivation was placed in a dark room for 56-72 hours. Furthermore, the explants washed again by using sterile water containing 200 ppm cefotaxime and then planted on callus induction media. After 3-4 weeks, callus that had been formed transferred into shoot medium containing 60 ppm kanamycin. The shoots that had been formed and kept on shoot induction media for 2-3 weeks until the plantlets have formed. Those plantlets subsequently were moved to MS0 media for rooting.

Integration analysis of *IMA* gene in transformant plants; DNA genome of putative transgenic *N. tabacum* isolated by using cetyl trimethylammonium bromide (CTAB) method (Sambrook *et al.*, 1989). The amplified PCR product checked by electrophoresis comprising 1% (w/v) agarose gel. The successful integration of *IMA* gene verified by PCR using 5 µl Kappa master mix, 3 pmol forward primer, 3 pmol reverse primer, 10 ng DNA genome of *N. tabacum*, and ddH$_2$O until the total volume was 10 µl. The PCR cycling is pre-PCR 95°C for 4 minutes; denaturation 94°C for 30 seconds; annealing at a temperature of 55°C or 45 seconds; and elongation at 72°C for 5 minutes. This cycle was repeated 35 times with a post-PCR temperature of 72°C for 5 minutes and temperature of 20°C for 5 minutes. *IMA* gene detection was made using primers CaMV-35S 5'AGGGATGACGCACAATCC3' (forward) and 5'TCATTTAGTAGTAGAAGAAG3' (reverse).

RESULTS AND DISCUSSION

Construction of pGWB402 vector to IMA gene: *IMA* gene fragment which successfully amplified by using specific *IMA* primers. The first stage in the gene construction was gateway specific primer design. *IMA* primers designed based on gene sequences which have been obtained from previous research (Sicard *et al.*, 2008b). Those primers designed by adding four nucleotide bases (Guanine), followed by *att*B (bacterial attachment site), and then added with 18-25 nucleotide sequences of specific *IMA* gene (Invitrogen, 2003). The site of *att*B referred to the binding site of attachment site where the lambda DNA integrated in the chromosome of *E. coli*.

The reaction product introduced into *E. coli* DH5α by using heat shock transformation method. Recombinant colonies selected in a medium with 50 ppm kanamycin and verified by colonies PCR method using M13 forward and reverse primers. Colonies containing pENTR/DONR-*IMA* had reached ~300 bp of DNA band after amplification (Figure 1). The fragments were reacted with pENTR/pDONR vector (Figure 2).

pENTR/DONR-*IMA* plasmid used for the recombination reaction using pGWB402 *LR Clonase* enzyme. The results of LR reaction transformed into *E. coli* DH5α by heat shock method. Verification of recombination between pENTR/DONR-*IMA* and pGWB402 carried out by colonies PCR method using 35S forward and *IMA* reverse primers.

M 1 2

← 300 bp

Figure 1: The results of PCR of gene fragments that were added gateway specific primer forward and reverse (column 1.2) and 100 bp ladder marker (M)

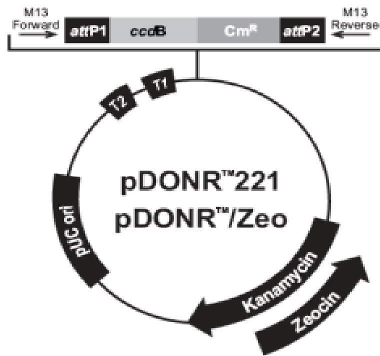


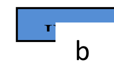
Figure 2: Genetic map of pDONR^{TM}221 with kanamycin resistance marker

The results of PCR amplification using 35S forward dan *IMA* reverse primers showed that the size of band was 500 bp. This result indicated that *IMA* gene integrated in area which flanked by attL1 and attL2 sequences of pEN TR/pDONR vector. *IMA* gene in this vector regulated by 35S CaMV constitutive promoter.
The assembling of expression vector has done by gateway cloning technique based on the principles of two vectors recombination, namely pENTR/pDONR as donor vector and pGWB402 as recipient vector. Gateway cloning could be done by using two kinds of enzymes, *BP* and *LR Clonase* according to the homologous sequences in both vectors.

Recombination between pENTR/DONR-*IMA* vector and pGWB402 vector used *LR Clonase* enzyme, so that *IMA* gene sequence was placed between *att*L1 and *att*L2 to replace *ccd*B area of pGWB402. This site facilitated gateway cloning techniques in the selection of a recombinant expression vector (pGWB402-*IMA*). *E. coli* DH5α inserted by non-recombinant pGWB402 cannot grow because *ccd*B will be expressed as toxic protein to *E. coli* DH5α. In the other hand, *E. coli* comprising recombinant plasmid (pGW B402-*IMA*) will grow easily because *ccd*B sequences have replaced by *IMA* gene sequences (Figure 3).

Transformation of recombinant pGWB402-IMA to A. tumefaciens LBA4404: Transformation using electroporator greatly help the acceleration process of introducing a recombinant vector into *A. tumefaciens*. This process began with the preparation of competent *A. tumefaciens* and plasmid DNA vector carrying the recombinant vector. The selection process of *A. tumefaciens* LBA4404 brought pGWB402-*IMA* used a media containing 100 ppm streptomycin, 50 ppm kanamycin, and 50 ppm spectinomycin. pGWB402-*IMA* itself contained resistance gene of kanamycin (*npt*II) inside the T-DNA and spectinomycin (*spc*^{R}) outside the T-DNA (Figure 3a). *A. tumefaciens* comprising pGWB402-*IMA* can be grown in LB medium containing streptomycin, kanamycin and spectinomycin. pGWB402 also has a kanamycin resistance gene (*npt*II) and spectinomycin (*spc*^{R}) (Figure 3b).

a



b

Figure 3: Genetic map of pGWB402 and *ccd*B gene (a) and *IMA* gene which were flanked by 35S Promoter and Tnos (b)

The colonies of *A. tumefaciens* were confirmed by colonies PCR method using 35S forward and *IMA* reverse primers (Figure 4).

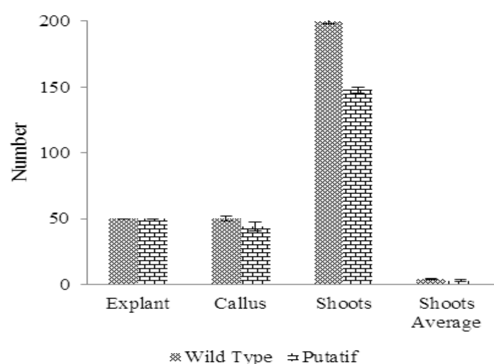
← 500

Figure 4: PCR amplification to verify DNA by using 35S forward and *IMA* reverse primers

Transformation of A. tumefaciens LBA4404 into N. tabacum: The development of *in vitro* technology facilitated inserting specific genes into plant genome by using *Agrobacterium* as vector. Tobacco as model plant can help to determine the level of specific genes expression. In this research, *A. tumefaciens* LBA4404 containing pGWB402-*IMA* was used for genetic transformation of *N. tabacum*.

The explants were cocultivated in a dark room by using *A. tumefaciens* culture in OD=0.1 for 56-72 hours. This treatment gave the occupation

to *A. tumefaciens* to grow well and to transfer its T-DNA region into *N. tabacum* genome. After co-cultivation, the explants were transferred to shoot induction medium containing Indole Acetic Acid (IAA) and 6-Benzyl Amino Purine (BAP) hormone. The explants at the age of 10-14 days in the shoot induction medium were transferred to selection medium containing 60 ppm kanamycin. Resistant plants which were able to grow in the media containing 60 ppm kanamycin are the putative transgenic plants. The percentage of callus formed either in non-transgenic plants and putative transgenic plants respectively are 100% and 95%. The average of shoots formed on the non-transgenic plant is 4 shoots/ callus. For putative transgenic plants, the average of shoots is 2.63 shoots/ callus (Figure 5).



gure 5: Number of explants, callus and shoots formed during transformation

The treated explants show that shoots formation is lower than control explants. It was because the *Agrobacterium* which grew too fast until it inhibited the forming of callus. The solution of this problem can be anticipated by treatment reducing bacterial OD to 0.1, increasing the concentration of cefotaxime in growing medium, and washing the explants by using sterile water containing cefotaxime. The plantlets grew on selection media containing kanamycin further were acclimatized and planted until it produced seeds. After treatment was done, 40 plantlets could grow on selection medium containing kanamycin. In this study did not use saline. At *Dianthus caryophyllus* L. root growth is reduced after the addition of 150 mM NaCL (Haouala and Jaziri, 2009). The successful integration of *IMA* gene was tested through PCR analysis by using 35S-F and *IMA*-R primers. 38 transgenic tobacco plants obtained as the result. Morphologically, non-transgenic and transgenic plants showed their differences in plant height and growth velocity. Non-transgenic plants grew normally while the putative transgenic plants did not grow normally (Figure 6a and b). 5 putative transgenic plants were selected for further analysis (Figure 6c). Actin gene was used as DNA internal controller by using specific actin gene primers Figure 6d).

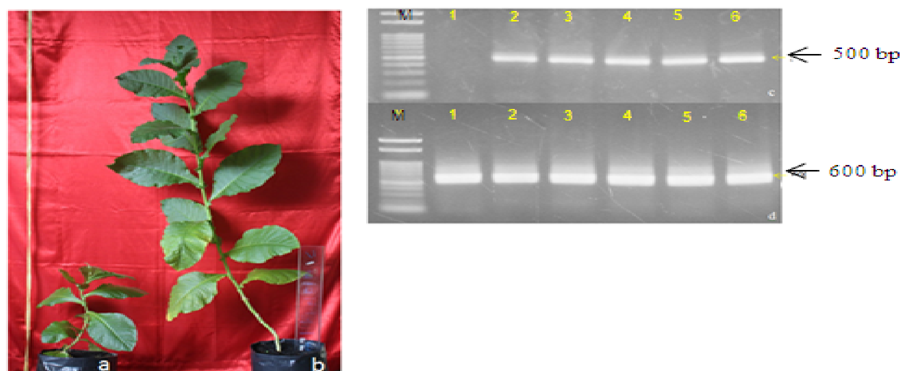


Figure 6: (a) The morphology of transgenic plant, (b) the non-transgenic plant, (c) DNA genome of transgenic plants, positive for 35S-*IMA* (column 2-6), non-transge plant (column 1), (d) PCR *actin* gene as DNA internal controller

The success of genetic transformation from *A. tumefaciens* to the genome of *N. tabacum* influenced by the addition of acetosyringone (Chen *et al.*, 2014) and pH of the co-cultivation medium (Godwin *et al.*, 1991). Acetosyringone enhances the virulence of Agrobacterium for dicotyledonous plants and can induce VirG. VirG itself plays a role in the transferring process of T-DNA (Zupan *et al.*, 1996). *IMA* gene transformation to other plants have been be done, eg *Jatropha curcas* (Paserang *et al.*, 2015).

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