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Overexpression of chitinase gene with a GC-rich synthetic enhancer in tobacco plant (*Nicotiana tabacum* L.)

Overekspresi gen kitinase dengan enhancer sintetis kaya GC pada tanaman tembakau (Nicotiana tabacum *L.*)

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Ringkasan

*Perakitan tanaman perkebunan toleran terhadap serangan cendawan patogenik dilakukan dengan mengoverekspresikan gen penyandi kitinase. Untuk itu elemen DNA peningkat ekspresi (*enhancer *E52) yang berupa oligonukleotida 52 pb dan kaya kandungan basa purin (GC) disisipkan di ujung 5' konstruk 35S-*chi. *Penyisipan E52 tersebut dilakukan secara lebih terarah pada situs ganda* Hind*III*-Sal*I dari MCS pCAMBIA2301. Melalui situs* Hind*III yang terletak tepat di ujung 3' E52, konstruk 35S-*chi *kemudian disambungkan dengan E52 pada pCAMBIA tersebut. Transformasi DNA rekombinan ke dalam sel tembakau dikerjakan melalui perantaraan* Agrobacterium tumefaciens *LBA4404. Sel tanaman transgenik diseleksi dan diregenrasi dalam media yang mengandung 3% sukrosa, 0,5 mg/L diregenerasikan pada media MS padat benzilaminopurin (BAP) dan 50 mg/L kanamisin. Pada media ini tunas transgenik tembakau mulai terbentuk setelah 5 minggu penanaman. Analisis tingkat aktivitas enzimatis menunjukkan bahwa aktivitas kitinase pada tembakau transgenik 40 hingga 80 kali lebih tinggi daripada non-transgenik. Pengujian hibridisasi protein menggunakan antibodi anti-kitinase,* dot blot *dan* western blot, *membuktikan bahwa* enhancer *tersebut dapat meningkatkan ekspresi transgen* chi *pada tanaman tembakau.*

Summary

Development of estate crops tolerant to pathogenic fungi is conducted by overexpressing *chi* gene. For this purpose, a synthetic enhancer consisting of 52 base pairs and GC-rich was inserted at immediate 5' end of a 35S-*chi* cassette. Insertion of the E52 was directed at *Hind*III-*Sal*I restriction sites of the pCAMBIA2301 MCS. With *Hind*III restriction site located just after the 3' end of the E52 sequence, the 35S-*chi* construct was then ligated with the E52 of the pCAMBIA. Transformation of the resulting recombinant DNA into tobacco cells was mediated by *Agrobacterium tumefaciens* LBA4404. The transgenic cells were selected and regenerated on a solid MS medium supplemented with 3% sucrose, 0.5 mg/L benzylamino purine (BAP) and 50 mg/L kanamycin. Tobacco shoots were initiated after 5 weeks inoculation on the selection media. Enzymatic analysis demonstrated that chitinase activity of transgenic tobacco was 40 to 80 folds higher than that of the control plant. Analysis of enzymatic activity using hybridization with anti-chitinase antibody indicated that the level of chitinase activity in the transgenic tobacco carrying the enhancer is higher than that without enhancer. These data suggest that the enhancer improved the expression of *chi* transgene in tobacco.

[*Key words*: Gene expression, recombinant DNA, transgenic tobacco]

Introduction

An attempt to develop estate crops tolerant to fungal deseases has led to utilization of chi gene. This gene encodes chitinase, an enzyme capable of degrading chitin which is a cell wall constituent of most pathogenic fungi. Genetic engineering of plant species using *chi* gene for resistance to pathogenic fungi was reported on rice (Lin *et al.*, 1995), tobacco and canola (Anonim, 2000). With similar approach, estate crops tolerant to fungal diseases such as *Ganoderma* sp. in oil palm, and *Hemilia vastatrix* of Arabica coffee should be regenerated with chitinase transgene.

The resistance of plant to pathogenic fungus is determined by the effectiveness of the enzyme in degrading the fungal chitin. In addition to the specificity, the enzymatic activity is also affected by the amount of the protein expressed in the plant. The more chitinase protein present in the plant, the more resistant the plant to the pathogenic fungi. Therefore plants possessing the chitinase transgene are not always tolerant to the pathogenic fungus due to low expression of the transgene or low activity of the enzyme (Lin *et al.*, 1995; Siswanto, 1999).

DNA fragments having a high content of purin bases (GC) and specific motives, at some degree capable of enhancing gene expression in eukaryotic cells through interaction with *trans-acting element* (Kay *et al*.,1987; Balcells & Coupland, 1994; Wilde *et al*., 1994). This type of interaction is required for the cells to form initiation complex between proteins of transcription factors and the DNA promoter. With this complex, RNA polymerase binds to the DNA and starts the transcription process at the start site (Santoso, 1995). Involvement of regulatory DNA to enhance such gene expression was also reported currently on flowering stage of *Arabidopsis* (Deholos & Sieburth, 2000).

This research is aimed to construct recombinant DNA carrying *chi* under a synthetic enhancer and examine the expression of the transgene in tobacco plant. The enhancer is a 52-bp oligonucleotide and GC rich. The expression was assayed at RNA level with Northern dot blot analysis using DIG-labeled DNA probe and at protein level using immunoassay.

Materials and Methods

Cloning of plasmid DNA was performed with *Escherichia coli* JM109 of Promega (Madison, WI). Gene construct of 35S-chi in pBS-G11 was obtained from Dr. Swapan Datta at IRRI, The Philippines. Plasmid vector of pCAMBIA2301 was kindly provided by Dr. R.A. Jefferson of CAMBIA, Australia. Restriction endonucleases, alkaline phosphatase and ligase were from Promega. Kits for plasmid preparation of *Quantum MiniPrep* and *Prep-A-Gene* were from BioRad (Hercules, CA), Acetosyringone and other reagents were from Sigma Chem. Co. (St Louis, MO) or Gibco BRL (Gaitherburg, MD).

Construction of recombinant plasmid

Recombinant DNA carrying 35S-chi construct under the 52-bp enhancer was made through two steps. First, insertion of the enhancer (E52) in between *Hind*III and *Sal*I sites at the MCS of pCAMBIA2301. Confirmation for the right construct was conducted using the restriction enzymes flanked the E52 fragment followed by electrophoresis with high concentration of agarose gel (Santoso & Haqqi, 1998). Functionality of the E52 was previously tested by examining the expression of *gus* in transgenic tobacco (Chaidamsari & Santoso, 1999). The second step was insertion of 35S-*chi* that was done by ligating the 35S-*chi* fragment excised from the pBS-G11 using *Hind*III, with *Hind*III-cleavaged PCAMBIA 2301/E52 recombinant. The 35S-*chi* fragment was purified from the 1.5-kb preparative gel slice using *Prep-A-Gene* kit. To improve efficiency of ligation, the *Hind*III-digested pCAMBIA was dephosphorylized using calf intestine alkaline phosphatase (CIAP). After ligation at 4^{o}C for overnight, the recombinant DNA was then transferred into JM109 competent cells, which were prepared following the method of Schnable (1991). Afterward, the transformation products

were plated on selection media containing kanamycin 50 mg/L.

To screen plasmid-carrying bacterial clones, plasmid DNA was prepared from selected colonies using Speedy method (Schnable, 1991) then electrophorezed using 0.7% agarose gel. To confirm the identity for true recombinants harbored in the selected bacterial clones, plasmid prepared with *Quantum MiniPrep kit* was examined for its map of restriction sites. Restriction enzymes of *Sph*I or *Pst*I were used to confirm the presence of E52, *Hind*III for the presence of the 35S-*chi* insert and *Bam*HI to determine the orientation of the DNA fragments.

The presence of chitinase gene in the DNA fragment was reconfirmed with Southern blotting using non radioisotop-labeled probe of DIG. The probe was synthesized with linearized 35-*chi* DNA template. The transfer of DNA from the agarose gel to the nylon membrane was perfomed based on capillary force as described in Sambrook *et al.* (1989). Procedures for hybridization, detection of hybridized DNA with antibody and staining were accomplished as recommended in the user manual. Specvific DNA bands detected on the membrane were recorded.

Plant genetic transformation and regeneration

Genetic transformation of tobacco plant of local variety was mediated by *Agrobacterium* LBA4404 with a slightly modified method from Sain *et al*. (1994). Acetosyringone at final concentration of 100 mg/L was added at the steps of inoculation and co-cultivation. After co-cultivation, the leaf explants were rinsed with MS liquid media containing 500 mg/L cefotaxime (Murashige & Skoog, 1962) to remove *Agrobacterium* stuck on the leaf surface. The clean transformed explants were then inoculated on MS solid media containing 500 mg/L cetofaxime, 100 mg/L kanamycin, 0.5 mg/L BAP and 3% sucrose.Planlets with 1-2 pair of leaves were cut at the bottom of the stem and transferred onto the MS solid media with no hormone to induce roots. The planlets were subcultured every 8-10 weeks. The culture for plantlet regeneration was incubated at 25^{o}C under a 12-h photoperiod provided by a bank of 40-W cool white fluorescent tubes.

Chitinase assay

Procedure to quantitatively determine the activity of chitinase was previously described by Siswanto (1999). Enzymatic extract containing 0.3-0.9 mg protein was added with 1 mM substrate of p-nitrophenyl β-D-glucosamine and phosphate buffer 50 mM pH 6.5 up to total volume of 250 μL. The reaction mixture was incubated at 37°C for 0 and 15 minutes. After stopping the reaction with 125 μL TCA 20% and centrifugation 12.000 g for 10 minutes, 0.3 mL of the supernatant was added with 0.7 mL NaOH 0.5 M, and absorbance was measured at λ 405 nm for 30 minutes. Chitinase activity was calculated with a standard curve based on the concentration of pure p-nitrophenyl phosphate (pNP).

Preparation of antibody

Preparative antigen containing 0.6 mg protein/mL was emulsified with complete Freud's adjuvant. The immunogene was then injected intramuscularly to rabbit (Young New Zealand Albino). After 14 days the rabbit was bled for 1 mL serum. The procedure was adopted from Dunbar & Schwoebel (1990). The harvested antiserum was stored in deep freezer until ELISA test.

For ELISA, antigen was 10-1000X diluted with carbonate buffer pH 9.6 then pipeted into micro-well plates and incubated at 4°C for overnight. After 3x washing with PBS-Tween 0.05 % and drying for 5 minute, the wells was added with 50 μL Ab, incubated for 1.5 hours at room temperature with slow shaking, then washed. Afterward 50 μL anti-rabbits IgG conjugate peroxidase was added into the wells, incubated for 1.5 hours with slow shaking, then washed. After added with 100 μL 2,2'-azino-bis(3-ethylbenzthia-zoline-6-sulfonic acid), the mixtures were shaken at room

temperature for color development. The absorbancy was measured at λ 405 and 492 nm.

Immunology analyses of proteins

Procedure for dot blot analysis was modified from Timmons & Dunbar (1990). Diluted Ag was spotted onto nitrocellulose membrane. After drying, the membrane was immersed into TEN-TC buffer and incubated with slow shaking for 30 minute then with diluted Ab for 2 hours. After 3x washed with PBS Tween, the membrane was incubated with anti-rabbits IgG peroxidase conjugate for 1.5 hours. After washing, the membrane was stained by incubation with 3,3'-diaminobenzidine tetrahydrocholride (DAB) until color developed, then washed with aquadest. For Western blot analysis, the experimental protocol was basically similar, except the protein bands were transferred from SDS-PAGE gel onto nitrocellulose membrane.

Northern dot blot

A total RNA was isolated from tobacco leaves using miniscale method slightly modified from Wadsworth *et al.* (1988). Solution of total RNA was spotted slowly onto nylon membrane drop by drop. After air-drying, the RNA on membrane was fixed by baking at 80^{o}C for 1-2 hours. Hybridization using DIG-labeled probe was conducted as described in instruction manual. The DIG-labeled probe was prepared using DNA template of *chi* gene excised from pBS-G11. The detail of the protocol was also described in the manual.

Results and Discussion

Ligations of the DNA fragments of E52, 35S-*chi* gene, and linearized vector provide several possible recombinants. To select the right recombinant, their restriction site map was examined. For this purpose, plasmid DNAs was prepared from three bacterial clones using *QuantumPrep* kit, then digested with three restriction enzymes *Sph*I, *Bam*HI and *Hind*III. Electrophoresis of the digested plasmids showed varying DNA banding pattern (Figure 1). Digestion with *Sph*I of clone 1, clone 2 and clone 3 resulted in dominant DNA bands of 6.7, 6.7 & 5.0, and 8.2 & 7.8 kb respectively. Digestion of the clones with *Bam*HI provided DNA bands of >11.6, 11.6, and 11.6 & 1.2 kb. Whereas bands from digestion with *Hind*III respectively were 11.6 & 1.4, 11.6, and 11.6 and 1.4 b. The 1.4 kb-DNA of clone 3 with *Hind*III is more intense than that of the others. The DNA fragments sizing of 8.2 kb of the *Sph*I digestion, 1.2 kb of the *Bam*HI and 1.4 kb of the *Hind*III were likely those carrying the chitinase gene which is 1.1 kb (Lin *et al.*, 1995).

Analysis of the DNA banding patterns suggested that the tested three recombinant plasmid clones possess different restriction maps or characteristics (Figure 2). Clones 1 and 3 could be recombinant of pCAMBIA- *chi*. Whereas clone 2 is pCAMBIA-E52 without 35S-*chi* construct. Based on the result from *Bam*HI digestion, it is assumed that the orientation of 35S-*chi* in clone 3 is in opposite orientation of clone 1. In the clone 3, 35S promoter of 35S-*chi* located across the E52, whereas in clone 1 the E52 resides just next to the 35S promoter.

Recombinant plasmids confirmed to harbor E52 and 35S-*chi* were then transferred into explant slices of tobacco via *Agrobacterium* LBA4404. Selection regeneration on the kanamycin-containing media supplemented with 0.5 mg/L BAP resulted in plantlets able to growth on the selection media (Table 1). Shoot initiation from the transformed explants on the selection media was seem slower than from untransformed control explants on the media without kanamycin. Green spots representing shoot initiation were observable after about 6 weeks. Furthermore the number of regenerated transgenic planlets from transformed explants was less than that from the control regeneration. Whereas untransformed explants which had been cultured for 6 weeks on the selection media regenerated no shoot.

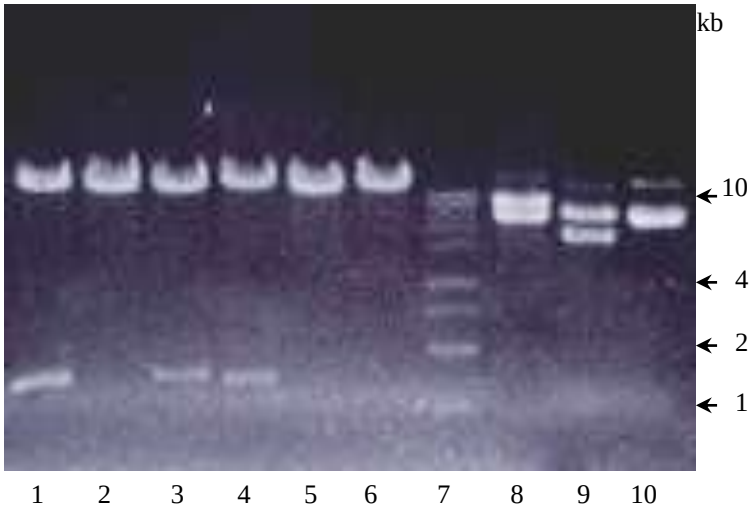

Figure 1. Banding pattern of recombinant DNA after digestion with restriction endo-nucleases. Lanes 1-10 are clones 1-*Hind*III, 2-*Hind*III, 3-*Hind*III, 1-*Bam*HI, 2-*Bam*HI, 3-*Bam*HI, 1-kb ladder, 1-*Sph*I, 2-*Sph*I, and 3-*Sph*I respectively.

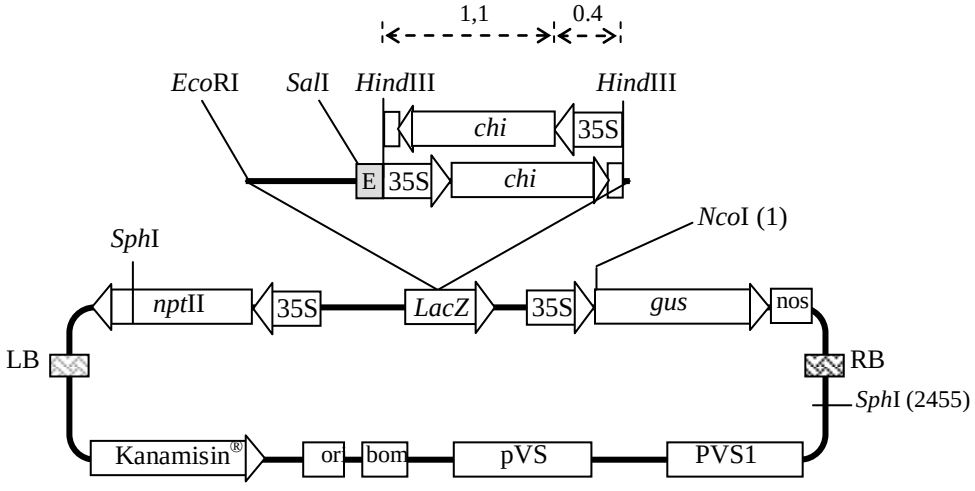

Figure 2. Restriction site map of pCAMBIA2301 plasmid before and after recombination. E, RB and LB are enhancer E52, right and left borders of the T-DNA.

Table 1. Selection of 35S-chi carrying cells and the regeneration for the tobacco transgenic plantlets on the selection media.

Leaf explant & tratment	Kanamycin (mg/L)	Initiation at	Number Shoot/planlet
Control	0	4 weeks	5 - 8
Control	50	N/A*)	-
Transformed with 35S-*chi*	50	6 weeks	2 - 4
Transformed with E52/35S-*chi*	50	6 weeks	2 - 4

Note: *) N/A = not applied because the explant died

Table 2. Activities of chitinase in the leaves of transgenic tobacco, assayed using two types of chromogenic substrates

Enzyme source	Chitinase activity (mM pNP/mg protein/hour)	
	$(pNP\text{-}GlcNAc)_2$	$(pNP\text{-}GlcNAc)_3$
Control	0.15	0.14
Transgenic	12.39	5.70

The plant cells growing on the selection media containing lethal dose of kanamycin, must theoretically be the cells bearing and expressing the transgenes in between the T-DNA borders, which includes *NPT*II gene encoding the kanamycin resistance. Expression of the *NPT*II gene indicated by the ability of the transformed cells to grow and regenerate on the selection media. To examine the expression of the disease resistance *chi* gene, enzymatic assays were done on the activity of chitinase. The axtivity of chitinase in transgenic planlets were 40-80 fold higher than that of the non-transgenic planlets (Table 2). With double 35S cCMV promoter, *chi* transgene from *Trichoderma viride* was expressed in plant *Vitis vinifera* at varying levels 10-100 fold higher than chitinase activity in the untransformed control plant (Kikkert *et al.*, 1998). This concludes that the transgene of the rice *chi* was integrated into the chromosomal DNA

and expressed properly on the transgenic tobacco planlet.

The increase of chitinase activities in the transgenic tobacco was likely the consequence of increase amount of chitinase protein expressed from the *chi* transgene. To prove this pressumption, the amount of the protein was measured with protein dot blot using anti- lane chitinase antibody. The result of the blotting analysis demonstrated that the intensities of the spots from transgenic tobacco transformed with E52-35S-*chi* was the highest, followed by 35S-*chi* and no colored spot from non-transgenic plantlet. The data suggested that expression of *chi* transgene construct with E52 was the highest, meaning that the DNA sequence E52 enhanced the expression of the *chi* constructed under its influence. The expression was also confirmed with Western blot analysis (the right panel of Figure 3). More color on the representing expression improved by E52 enhancer. This enhancement function of E52 DNA sequence was also reported in our previous publication for reporter gene of *gus* (Chaidamsari & Santoso, 1999). The recombinant DNA used in the previous study harboring the enhancer at position about a half kb away from the 5' end of the 35S promoter driving the expression of the reporter gene. These experimental data collected so far indicate that E52 enhances gene expression in a fashion of a bit position independent. This is in agreement with general rule for DNA enhancer of gene expression. In monocotyledon, a regulatory sequence shown to modulating high gene expression was characterized as a GC-rich regulatory sequence (Zhu, 1995).

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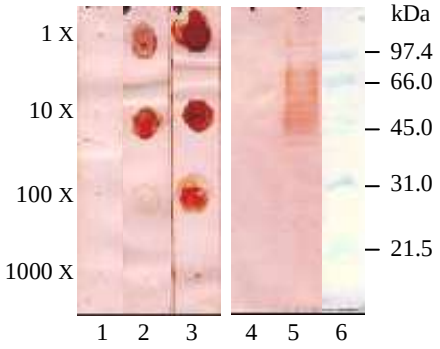

Figure 3. Hybridization of proteins. Left panel, lanes 1-3 are dot blots negative control, extracts from 35S-*chi*-, E52-35S-*chi*-transformed tobacco. Right, lanes 4-6 are western blot of the 35S-*chi*-, E52-35S-*chi*-trans-formed plants and molecular marker respectively.

Figure 4. Dot blot of leaf tobacco total RNA. Spots 1 - 5 are from non-transgenic tobacco, positive control pBS-G11, transgenic 1, 2 and 3 respectively.

Plants including tobacco possess chitinase activity with metabolic function obviously similar to those of most species. At molecular level, protein hybridization approach has degree of less specificity. To reconfirm the overproduction of chitinase activities as well as proteins were merely as the result of chitinase transgene expression, a simplified Northern blot was conducted to examine the expression of the gene at RNA level. The result of RNA dot blot analysis is presented in Figure 4. The presence of color dots on some spots representing transgenic tobacco indicates that the transgene *chi* was expressed in the transgenic plantletsPlants including tobacco possess chitinase activity with metabolic function obviously similar to those of most species. At molecular level, protein hybridization approach has degree of less specificity. To reconfirm the overproduction of chitinase activities as well as proteins were merely as the result of chitinase transgene expression, a simplified Northern blot was conducted to examine the expression of the gene at RNA level. The result of RNA dot blot analysis is presented in Figure 4. The presence of color dots on some spots representing transgenic tobacco indicates that the transgene *chi* was expressed in the transgenic plantletsLess intensity of color is expected in non-radioisotope-label probe. Since the spotted samples were solutions of about one µg total RNA prepared miniscally from tobacco plantlets, it is reasonable that the spot color is not as strong as if ^{32}P labeled probes were used (Santoso & Thornburg, 1998). Beside, total RNA is dominated by population of rRNA, whereas the mRNA is usually less than 1% of the total RNA. Furthermore chitinase mRNA maybe only one over thousands of mRNA population in the total RNA solution. Nevertheless, the chitinase transcript was still detectable confirming that the chitinase transgene was really overexpressed in the transgenic tobacco harboring E53-35S-*chi* construct.

Regulation of gene expression can occur at several different points. In eukaryotic cells, it is mostly at transcription process and sometime at translation step (Anonim, 2001).

Examinations at enzymatic activity, protein and transcript levels suggest that the regulation of the transgene *chi* under 35S promoter and E52 enhancer does not seemingly rule out the common mechanism. Like those involved in the metabolism of pyrimidine nucleotide (Santoso & Thornburg, 1998), expression regulation of such fungal resistance transgene also occurs at transcription level. A current report on

Arabidopsis also indicated the presence of regulation at transcription process particularly at initiation of transcription for expression of genes in the process of cell differention (DellaPenna, 2001).

Conclusion

1. Transformation of 35S-*chi* constructs into tobacco explants via *Agrobacterium* LBA4404 carrying pCAMBIA/35S-*chi* with or without E52 resulted in plantlets with increased chitinase activities.
2. DNA *cis*-acting element consists of 52 bp and GC rich was able to enhance the expression of *chi* transgene in tobacco plants.

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References

Anonim. (2000). News report articles search results: chitinase. Information system for Biotechnology. http://www.isb.vt.edu/

Anonim (2001). Gene regulation in eukaryoites. http://www.google.com /enhancer'gene'.

Balcells, L. & Coupland G. (1994). The presence of enhancer adjacent to the *AC* promoter increases the abundance of transposase mRNA and alters the timing of *Ds* excision in *Arabidopsis*. *Plant Mol. Biol*., **24,** 789-798.

Chaidamsari, T. & D. Santoso (1999). Expression of gus gene in tobacco (*Nicotiana tabacum* L.) by insertion of an enhancer. *Menara Perkebunan,* **67** (1), 17-25.

Deyholos, M.K. & L.E. Seiburth (2000). Separable whorl-specific expression and negative regulation by enhancer elements within the *AGAMOUS* second intron. *Plant Cell,* **12,** 1799-1810.

DellaPenna, D (2001). Plant metabolic engineering. *Plant Physiol.*, **125,** 160-163.

Dunbar, B.S. & E.D. Schwoebel (1990). Preparation of polyclonal antibodies. *In* Deutscher, M.P. (ed.) *Guide to protein purification*. San Diego, Academic Press, pp. 663-669.

Kay R., Chan, A., Daly, M. & McPherson, J. (1987). Duplication of CaMV 35*S* promoter sequences creates a strong enhancer for plant genes. *Science,* **236,** 1299-1302.

Kikkert, J.R., G.M. Reustle, G.S. Ali, P.G. Wallace & B.I. Reisch (1998). Expression of a fungal chitinase in *Vitis vinifera* L. 'Merlot' and 'Chardonnay' plants produced by biolistic transformation. *Proc. of The VIIth International Symposium on gravevine breeding and genetics*, Montpellier, France, July 6-10, 1998.

Kushalappa, A.C. (1989). Introduction. *In* Kushalappa, A.C. & Eskes, A.B. (eds). Coffee rust: epidiology, resistance, and mangement. CRC Boca Raton. 13-80.

Lin, W., Anuratha, C.S., Datta, K., Potrykus, I., Muthukrishnan & Datta, S.K. (1995). Genetic engineering of rice for resistance to sheath blight. *Biotechnology,* **13,** 686-691.

Murashige T. & Skoog F. (1962). A revised medium for rapid growth bioassays with tobacco tissue culture. *Physiol. Plant.*, **15,** 473-497 .

Sambrook, J., E.F Fritsch & T. Maniatis (1989). Molecular cloning, a laboratory manual, Book 3, 2nd ed. Cold Spring Harbor Laboratory, New York.

Sain SL, KK Oduro & DB Furtek (1994). Genetic transformation of cocoa leaf cells using *Agrobacterium tumefaciens*. *Plant Cell Tiss & Org. Cult.*, **37,** 243-251.

Santoso, D. & M.R.A. Haqqi (1998). Kloning DNA elemen peningkat ekspresi (EPE) gen. Makalah Seminar Nasional XIV Perhimpunan Biokimia & Biologi Molekuler. Bandung, 12 Desember 1998.

Santoso, D. & R.W. Thornburg (1998). Uridine 5'-monophosphate synthase is trans-criptionally regulated by pyrimidine levels in *Nicotiana plumbaginifolia*. *Plant Physiol.*, **116,** 815-821.

Schnable, P.S. (1991). *Genetic engineering lab manual, genetics 520 L*. Department of Genetics, Iowa State University, 47p.

Siswanto (1999). *Perakitan tanaman transgenik kopi arabika tahan terhadap penyakit karat daun.* Riset Unggulan Terpadu VII. Bogor, Unit Penelitian Bioteknologi Perkebunan

Siswanto (2001). Perakitan tanaman transgenik kopi arabika tahan penyakit karat daun. Laporan kemajuan tahap I dan kertas kerja RUT VIII tahun ke 2. Bogor, Unit Penelitian Bioteknologi Perkebunan, 52 p.

Timmons, T.M. & B.S. Dunbar (1990). Protein blotting and immunodetection. *In* Deutscher, M.P. (ed.) *Guide to protein purification*. San Diego, Academic Press, pp. 663-669.

Wilde R.J., S.E. Cooke, W.J. Brammar & W. Schuch (1994). Control of gene expression in plant cells using a 434:VP16 chimeric protein. *Plant Mol Biol.*, **24,** 381-88.

Wadsworth, G.J., M.G. Redinbaugh & J.G. Scandalios (1988). A procedure for the small-scale isolation of plant RNA suitable for RNA blot analysis. *Anal Biochem.*, **172,** 279-283.

Zhu, Q. (1995). Rice chitinase promoter. PATN Patent Bibliographic Information. *Patent Number 05399680*