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Expression of *Leucaena Leucocephala* de Wit Chitinase in Transgenic Koshihikari Rice

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***Abstract*—**The cDNA encoding the 326 amino acids of a Class I basic chitinase gene from *Leucaena leucocephala* de Wit (KB3, Genbank accession: AAM49597) was cloned under the control of CaMV35S promoter in pCAMBIA 1300 and transferred to Koshihikari. Calli of Koshihikari rice was transformed with agrobacterium with this construct expressing the chitinase and β-glucouronidase (GUS). The frequencies of calli 90 % has been obtained from rice seedlings cultured on NB medium. The high regeneration frequencies, 74% was obtained from calli cultured on regeneration medium containing 4 mg/l BAP, and 7 g/l phytagel at 25°C. Various factors were studied in order to establish a procedure for the transformation of Koshihikari Agrobacterium tumefaciens. Supplementation of 50 mM acetosyringone to the medium during coculivation was important to enhance the frequency to transient transformation. The 4 week-old scutellum-derived calli were excellent starting materials. Selection medium based on NB medium supplement with 40 mg/l hygromycin and 400 mg/l cefotaxime were an optimized medium for selection of transformed rice calli. The percentage of transformation 70 was obtained. Recombinant calli and regenerated rice plants were checked the expression of chitinase and gus by PCR, northern blot gel, southern blot gel, and gus assay. Chitinase and gus were expressed in all parts of recombinant rice. The rice line expressing the KB3 chiitnase was more resistant to the blast fungus Fusarium monoliforme than control line.

***Keywords*—**chitinase, Leucaena leucocephala de Wit, Koshihikari, transgenic rice.

I. Introduction

ENDOCHITINASES (EC 3.2.1.14) are among thr pathogen response proteins that defend plants against bacterial an fungal infection. They are induced by pathogens infection, as well as by the stress hormone ethylene, and have fungicidal activity in vitro, particularly in the presence of β-1,3-glucanase [1]. It has also been demonstrated that transgenic plants that overexpress chitinases have increased resistance to fungal attack [2,3], supporting their role in anti-fungal defense. Indeed, many chitinases have been shown to be upregulated in response to fungal elicitors, ethylene and other stresses [1,4-7]. However, chitinases have also been implicated in other roles, such as regulation of legume response to rhizobial nod factors, which contain chito-oligosacharides [8], and in regulation of normal plant development, perhaps by hydrolysis of similar oligosaccharide substrates [9,10]. Thus, though antifungal activity is an important function of chitinases, it is not their only function, which may be why plants produce many chitinases with different regulatory patterns [2,11).

The cDNA for class I basic endochitinases from *Leucaena leucocephala* de Wit were found to encode 31.9 and 32.2 kDa proteins that contain N-terminal chitin-binding domains followed by chitinase domain [12]. The leucaena chitinase KB3 (32.2 kDa) was expressed as a thioredoxin-fusion protein in Origami (DE3) *E. coli* expression vector pET32a was found to hydrolyze colloidal chitin, purified chitin, swollen chitin, glycolchitin and chitosan. The 46 kDa recombinant protein also inhibited the growth of *Collectotrichum* sp., *Pestalestiopsis* sp. *Anthanose collectotrichum*, *Fusarium* sp., *Fusarium monoliforme*, *Fusarium oxysporum*, *Pestalestiopsis* sp., *Cercospora* sp., *Drechslera* sp., and *Sclerotium* sp., whereas *Cladosporium* sp. was not inhibited at the concentrations tested. In comparison with other plant chitinases, purified recombinant *L. leucocephala* chitinase inhibited these fungi at similar or better levels. For instance, chitinases from Arabidopsis thaliana and sorghum seed could inhibit *Fusarium moniliforme* and *Fusarium oxysporum* at concentrations of 8 mg and 5 mg per disc, respectively, while leucaena chitinase inhibited at only 1 mg per disc [12, 13). Though differences in strains used and experimental differences may account for some of these differences, *L. leucocephala* chitinase appears to be comparable to or better than other plant chitinases in fungal inhibition, and may be a good candidate for this application. As transgenic plants expressing chitinases have been shown to have improved fungal resistance [2,14,15], it may be useful to express the *L. leucocephala* chitinase cDNA in crop plants.

In this study, we transformed Koshihikari japonica rice calli with the cDNA for the class I basic chitinase KB3 of Leucaena leucocephala de Wit (326 amino acids) regulated by the Caulifower Mosaic Virus (CaMV35S). This gene was expressed in rice plant to resistant of the transformant to fungal pathogen Fusarium monoliforme was assessed.

II. Materials and Methods

A. Rice seedlings Materials and Fungi

Koshihikari seedlings were obtained from the Rice Research and Development Institute of Thailand at Chiang

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Mai. Fusarium monoliforme strain was obtained from the Plant Pathology and the Microbiology Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

B. Enzymes and Reagents

Agar, peptone, and yeast extract were products from DIFCO. Restriction enzymes, deoxyribonucleotides, 5-bromo-4-chloro- 3-indolyl-b-D galactoside (X-Gal), Taq polymerase, and T4 DNA ligase were products from Promega (Madison, WI, USA). pBI121 was obtained from Fujimura laboratory, University of Tsukuba, Ibaraki, Japan, pCAMBIA 1300 and pCAMBIA 1301 were obtained from CAMBIA, Canbera, Australia. All chemical were products from Ajax. All antibiotics were products from Sigma (St. Louis, MO, USA). The northern blot and southern blot (North2South Direct HRP Labeling and Detection) kit was from PIERCE (Rockford, Il, USA). All other chemicals were products of Ajax FineChem.

C. Plasmid constructs and DNA transformation

A BamHI-SacI subclone of pUC19 containing 326 amino acids chitinase gene [12] was cloned into BamHI and SacI sites of pBI121. The pBI121 plasmid contains the cauliflower mosaic virus (CaMV) 35S promoter and Nos terminator. The resulting plasmid was digested with HindIII and EcoRI and subcloned into the *Hin*dIII and *Eco*RI sites of pCAMBIA 1300. The resulting plasmid was checked by digestion with *Bam*HI-*Sac*I, *Hin*dIII-*Eco*RI, *Bam*HI-*Eco*RI, and *Hin*dIII-*Sac*I. The recombinant pCAMBIA 1300, control pCAMBIA 1300 and pCAMBIA 1301 plasmids were transformed into Agrobacterium tumefaciens strain EHA 105 by a standard method [16]. Agrobacterium tumefaciens containing the plasmid recombinant pCAMBIA 1300, control pCAMBIA 1300, and pCAMBIA 1301 plasmids were selected by growing in the LB medium containing 50 mg/l kanamycin and 50 mg/l hygromycin.

D. Callus culture and plant transformation

Calli of Koshihikari was induced on NB medium containing 2 mg/l 2,4-D for 1, 2, 3, 4, 5, and 6 weeks (study on effect of calli age). The medium were changed every 5 days. Agrobacterium cells which contained pCAMBIA 1301 containing the GUS gene, control pCAMBIA 1300, and pCAMBIA 1300 containing the CaMV35S promotor, leucaena chitinase gene, and NOS expression cassette insert were cultured in the AB medium containing 50 mg/l kanamycin at 28?C for 3 days. Half a colony of agrobacterium was inocaulted in 30 ml of sterileR2L (R2 macro + micro + LS vitamin + 10 g/l glucose + 2.5 mg/l 2,4-D, pH = 5.8 filtration) containing 50, 100, 250, and 500 mM of acetosyringone (study on effect of acetosyringone concentration) and shaken at 28°C until the OD =1 at 600 nm and diluted to 0.01 with R2L (3-5x10^9 cell/ml). The calli of Koshihikari was suspended in the agrobacterium for 10 min with occasional shaking, removed, and transferred onto R2S (R2 + 50, 100, 250, and 500 mM of acetosyringone) plates, and incubated at 25°C in the dark for 2-3 days.

E. Selection of hygromycin resistant colonies

Calli that had been cocultured with the agrobacterium for 2-3 daus was transferred to NB medium containing 2.5 mg/l 2,4-D, 300, 400, 500, and 600 mg/l cefotaxime (study on effect of cefotaxime concentration), 7 g agarose gel type I, and 30, 40, 50, and 60 mg/l hygromycin (study on effect of hygromycin concentration). Koshihikari was maintained in darkness for 3 weeks in controlled environmental with 80% relative humidity at 25°C and 28°C (study on effect of temperature).

F. Regeneration of rice plants

Hygromycin-resistant Koshihikari rice plant was regenerated on NB + 2.5 mg/l 2,4-D + 1, 2, 4, 5, and 6 mg/l of BAP (study on effect of BAP concentration) + 5, 6, 7, 8, and 9 g/l of phytagel (study on effect of phytagel concentration) for 2 weeks in dark and transferred on NB + 0.5 mg/l NAA for 1 week in darkness and 3 weeks in light. The transgenic rice plants were transferred to grow in soil under ambient conditions.

G. GUS assay

Detection of GUS activity for find part of plant expression, was according to the method of Budelier, et al. [17]. Calli and transgenic plants tissues were stained in staining solution, which contained 50 mg of 5-bromo-4-chloro-3-indolyl-b- D-glucuronide (X-gluc) in 50 ml of 100 mM sodium phosphate pH 7.0, 0.5 mM potassium hexacyanoferate, 10 mM EDTA. The tissues were incubated at 37°C for 3 h and fixed in FAA (42% ethanol, 5% glacial acetic acid, and 10% formalin).

H. PCR Analysis

To checked for the chitinase gene, DNA was extracted from leaf, root, stem, and seedlings at 4 weeks by the CTAB method. The mixtures were amplified with Taq polymerase under the following conditions, denaturation at 95°C for 5 min and 30 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. The 5'KB3 primer (ATCAAATCCAAGAGATGG) and the 3'KB3 primer (CTCGAGGAGGACGTCGATGAG) To checked for the hygromycin resistance gene, PCR was done by the same method with the HRG5' primer (ATGAAAAAGCCTGAACTCACC) and the HRG3' primer (TTCGGTCGGCATCTACTCTAT).

I. Northern blot analysis

Total RNA was extracted from leaf, roots, stems, and seedlings at 4 weeks by the CTAB method. Total RNA was run on 0.8 agarose gel and transferred onto nylon membranes. The membranes were hybridization to a chitinase gene probe (KB3 chitinase) labeled with the North2South Direct HRP Labeling and detected according the kitdirections.

J. Southern blot analysis

Genomic DNA was extracted from leaf, root, stem, and seedlings at 4 weeks by the CTAB method. The genomic DNA was digested by *Bam*HI and *Sac*I, *Bam*HI and *Eco*RI, and *Eco*RI and *Hin*dIII. The resulting digests were separated by electrophoresis on a 0.8 agarose gel and transferred onto nylon membranes. The membranes were hybridized to the chitinase gene probe and detected by the same method as in northern blotting.

K. Fungus infection assays

A suspension of Fusarium monoliforme spores ($1.5x10^5$ spores/ml) was spread on the upper surface of leaf of 20 four-months-old control and 20 Koshihikari plants. Control plants were mock-inoculated in the same way for 1-2 weeks. obtaining any security clearances.

III. Results and Discussion

Plasmid constructs and DNA transformation

The HindIII-EcoRI containing CaMV35S promoter, chitinase, and Nos poly A was introduced into rice by Agrobacterium tumefaciens mediated transformation. The 1.1 kb chitinase fragment (326 amino acids, KB3) was cloned into BamHI-SacII of the polylinker region of the plasmid pBI121. It contains a CaMV35S promoter, and Nos poly A. The recombinant palsmid containing CaMV35S promoter, chitinase, and Nos poly A was subcloned into HindIII-EcoRI of the polylinker region of the plasmid pCAMBIA 1300 that shown in fig. 1 and introduced into A. tumefaciens strain EHA 105. The A. tumefaciens was used for infection of rice calli.

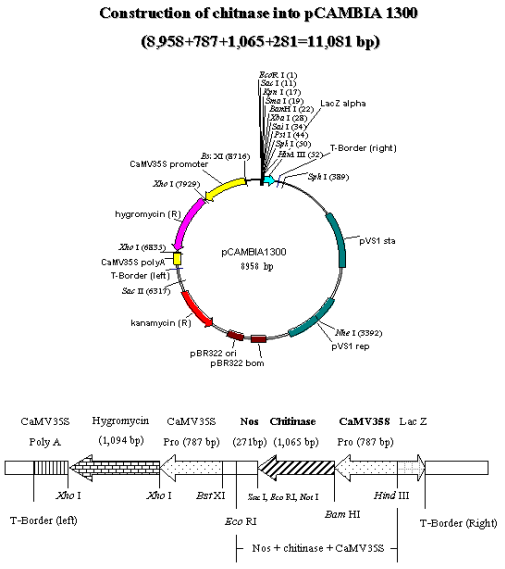
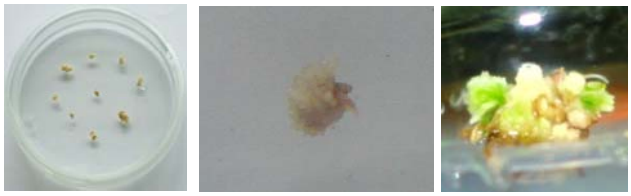


Fig. 1 The recombinant plasmid pCAMBIA 1300 containing CaMV35S promoter, leucaena chitinase, and Nos poly A.

Callus culture and transformation

Calli of Koshihikari was induced on NB medium (fig. 2A) supplemented with 2 mg/l 2,4-D. The calli were formed from the scutella tissue of mature seeds within 3-4 weeks of culture initiation. After 4 weeks out of 50 of Koshihikari mature seeds, 45 seeds produced calli corresponding to 90 % calli induction that shown in table 1. A. tumefaciens strain EHA 105 habouring pCAMBIA1300 containing CaMV35S, chitinase, and Nos poly A; pCAMBIA 1301 containing a GUS-expression for comparing, were used for co-cultivation of calli. The transformation efficiency, defined as number of GUS transformations divided by number of explants inoculated, as the percentages, were 51.22 of Koshihikari. GUS-position blue spots and yielded were shown in fig. 2B and table 1, respectively. Control treatment never yielded GUS-position blue spots. Calli age for regenerated was between 42% and 70 % from 1 to 6 weeks. The highest frequencies of calli age for regenerated was 4 weeks of 70 % that shown in table 2. The small globular calli were cocultivated with Agrobacterium tumefaciens strain EHA 105 on R2S medium supplement with 50, 100, 250, and 500 mM of acetosyringone plate incubate at 25°C dark for 2-3 days. The highest frequencies of acytosyringone concentration for remove agrobacterium is R2S medium supplement with 50 mM of acetosyringone of 74% that shown in table 3.



A B C

Fig. 2 4 weeks of rice calli. (A) is Koshihikari. (B) is GUS transformations in rice calli of Koshihikari was checked. It shown blue spot. (C) is regenerated of transgenic Koshihikari.

TABLE I
THE PERCENTAGE OF CALLI PRODUCED, GUS ASSAY, AND HYGROMYCIN RESISTANCE.

Numbers of seeds	Number of cali	percentage	GUS-blue spots	percentage
50	45	90	23	51.11

TABLE II
EFFECT OF AGE OF CALLI ON THE FREQUENCY TRANSFORMATION.

Age of calli (weeks)	The initial number of calli	Nuber of regenerated calli	percentage
1	50	21	42.00
2	50	19	58.00
3	50	30	60.00
4	50	35	70.00
5	50	27	54.00
6	50	19	38.00

TABLE III
EFFECT OF ACETOSYRINGONE ON THE FREQUENCY TRANSFORMATION.

Age of calli (weeks)	The initial number of calli	Nuber of regenerated calli	percentage
50	50	37	74.00
100	50	33	66.00
250	50	22	44.00
500	50	0	0

It was produced shoots and roots.

Selection of hygromycin resistant calli

The co-cultivated calli were transferred to the selection medium (R2S) containing 30, 40, 50, and 60 mg/l hygromycin to inhibit growth of non-transformed rice cells and supplemented with 300, 400, 500, and 600 mg/l cefotaxime to inhibit A. tumefaciens growth. The highest frequencies of hygromycin concentration is R2S medium containing with 40 mM of hygromycin of 70% Koshihikari that shown in table 4. The highest frequencies of cefotaxime for inhibit A. tumefaciens growth is R2S medium supplement with 400 mg/l of cefotaxime of 70% Koshihikari that shown in table 5. Calli was grown in controlled environmental chamber in dark at 25°C and 28°C. The optimum temperature was 25°C of 70% that shown in table 6.

TABLE IV
EFFECT OF HYGROMYCIN RESISTANCE
ON THE FREQUENCY CALLI SELECTION.

Hygromycin concentration (mg/l)	The initial number of calli	Nuber of regenerated calli	percentage
30	50	28	56.00
40	50	35	70.00
50	50	30	60.00
60	50	18	36.00

TABLE V
EFFECT OF CEFOTAXIME RESISTANCE
ON THE FREQUENCY CALLI SELECTION.

Cefotaxime concentration (mg/l)	The initial number of calli	Nuber of regenerated calli	percentage
300	50	31	62.00
400	50	35	70.00
500	50	30	60.00
600	50	9	18.00

TABLE VI
EFFECT OF TEMPERATURE
ON THE FREQUENCY CALLI SELECTION.

Temperature (°C)	The initial number of calli	Nuber of regenerated calli	percentage
25	50	35	70.00
28	50	29	58.00

Regeneration of rice plant

The calli of cells that recovered from the selection medium were regenerated on RN containing 0.5 mg/l NAA; 1, 2, 4, 5, and 6 mg/l of BAP; 5, 6, 7, 8, and 9 g/l of phytagel, and 40 mg/l hygromycin. These were incubated in dark for 1 week and in light (16 h.) for 3 weeks. Shoots and roots were regenerated shown in fig. 2C. The highest frequencies of BAP concentration is RN medium containing with 4 mg/l of BAP of 74% Koshihikari that shown in table 7. The highest frequencies of pytagel to regenerated plant is RN medium supplement with 7 g/l of pytagel of 74% Koshihikari that shown in table 8.

TABLE VII
EFFECT OF BAP CONCENTRATION
ON THE FREQUENCY REGENERATION.

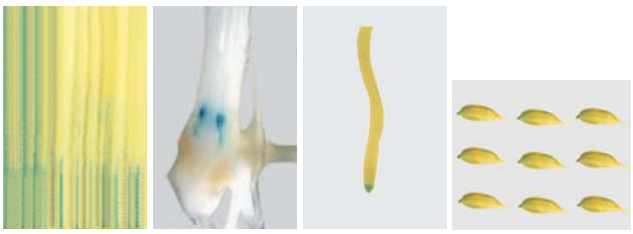
BAP concentration (mg/l)	The initial number of calli	Nuber of regenerated calli	percentage
1	50	26	52.00
2	50	30	60.00
4	50	37	74.00
5	50	17	34.00
6	50	8	16.00

TABLE VIII
EFFECT OF PYTAGEL CONCENTRATION
ON THE FREQUENCY REGENERATION.

Pytagel concentration (mg/l)	The initial number of calli	Nuber of regenerated calli	percentage
6	50	20	40.00
7	50	37	74.00
8	50	13	26.00
9	50	5	10.00

GUS assay

To find part of rice expression with pCAMBIA 1301 for comparing chitinase gene expression with pCAMBIA 1300 containing chitinase gene. Twenty plants were indenpendently regenerated from fourty randomly selected hygromycin resistant calli. Gus activity was detected in the leaf of 10 plants by X-Gluc staining. Gus activity of different organs was determined transgenic Koshihikari plants was monitored by fluorogenic assay after the addition of buffered MUG substrate to calli and all parts of Koshihikari transgenic plants that shown in the fig. 3. Every parts of transgenic rice shown blue color in leaf, stem, root, and seedlings but they shown a small blue spot in roots of transgenic rice.



A B C D

Fig. 3 Transgenic rice of Koshihikari was Gus assay. They shown blue color in leaf, root, stem, and seedlings. (A), (B), (C), and (D) were leaf, stem, root, and seedlings of Koshihikari, respectively.

PCR

Molecular analysis by PCR amplification confirmed that the leucaena chitinase gene was present in leaf, root, stem, and seedlings at 4 weeks, compared control pCAMBIA 1300 and pCAMBIA 1301 (non-transformed). Fig. 4 show that one bands corresponding to the expected leucaena chitinase fragments of 1,100 bp was detected in all part of Koshihikari transgenic plants but non-transgenic plants did not show that band. It was compared gus expression in transgenic rice, chitinase gene expressed in all of part of transgenic rice which the same gus gene expressed in all of part of transgenic rice. For confirmed that the hygromycin resistance gene was present in leaf, root, stem, and seedlings at 4 weeks, compared control pCAMBIA 1300 and pCAMBIA 1301. Figure 4A show that one bands corresponding to the expected hygromycin resistance fragment of 1,300 bp was detected in all part of Koshihikari transgenic plants and non-transgenic plants because of hygromycin resistance gene containing in pCAMBIA 1300 and pCAMBIA 1301. The hygromycin resistance gene was checked and shown 1,100 bp in fig. 4B.

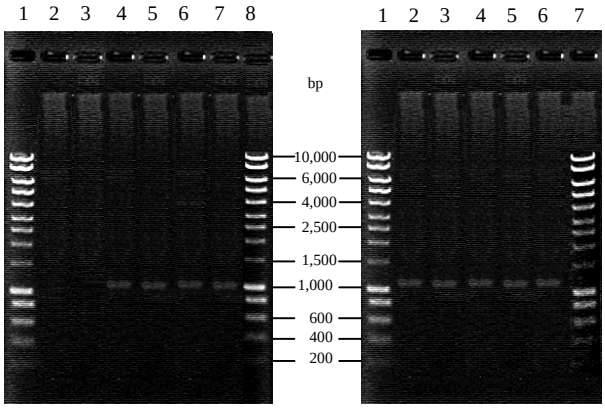


Fig. 4 (A) Leucaena chitinase gene was analyzed by PCR. Leucaena chitinase band shown about 1,100 bp. Lane 1, and 8 are hyperLadder I DNA marker, lane 2-3 PCR product of nontransgenic lane 4-7 PCR product of leaf, root, stem, and seedlings from 4 month of transgenic rice, respectively. (B) is hygromycin resistance gene was analyzed by PCR. Hygromycin band shown about 1,100 bp. Lane 1, and 7 are hyperLadder I DNA marker, lane 2 is control pCAMBIA 1300. Lane3-6 PCR product of leaf, root, stem, and seedlings from 4 month of transgenic rice, respectively.

Northern blot analysis

To assay RNA expression of leucaena chitnase gene in the Koshihikari plants, RNA gel blot analyzes was formed. Total RNA was isolated from transgenic Koshihikari plants harboring either the control plasmid pCAMBIA 1300. Fig. 5A shows that, in each case, the level of hybridization of chitinase mRNA was high in leaf, stem, and seedlings but it was lowest in root of Koshihikari. It was compared gus expression in transgenic rice, chitinase gene expressed in all of part of transgenic rice which the same gus gene expressed in all of part of transgenic rice.

Southern blot analysis

To assay DNA expression of leucaena chitnase gene in the Koshihikari plants, DNA gel blot analyzes were formed. Genomic DNA which digested by *Bam*HI and *Sac*I, *Bam*HI and *Eco*RI, and *Eco*RI and *Hin*dIII were isolated from transgenic Koshihikari plants harboring either the control plasmid pCAMBIA 1300. Fig. 5B shows that band digested with BamHI and SacI was smaller than band digested with BamHI and EcoRI and band digested with *Eco*RI and *Hin*dIII which have 1,100 bp, 1,300 bp, and 2,100 bp, respectively. In each case, the level of hybridization of chitinase mRNA was high in leaf, stem, and seedlings but it was lowest in root of Koshihikari. It was compared gus expression in transgenic rice, chitinase gene expressed in all of part of transgenic rice which the same gus gene expressed in all of part of transgenic rice.

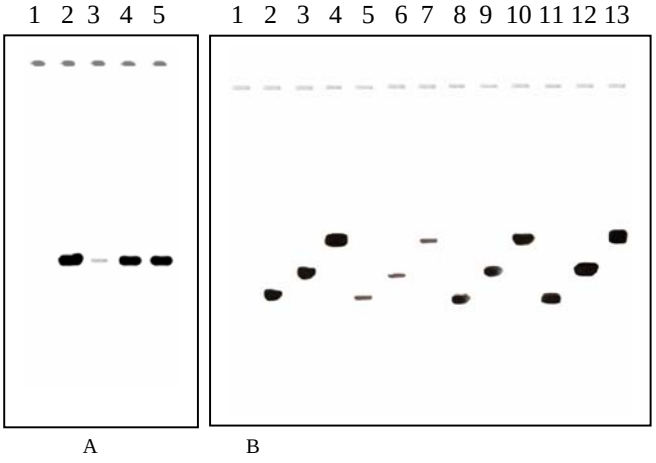


Fig. 5 (A) is northern blot was analyzed from transgenic rice 4 months. Lane 1 is control pCAMBIA 1300 non-transformed, lane 2-5 northern blot of leaf, root, stem, and seedlings. (B) is Southern blot was analyzed from transgenic rice 4 months. Lane 1 is control pCAMBIA 1300 non-transformed, lane 2,5,8,11 southern blot that digested by *Bam*HI and *Sac*I of leaf, root, stem and seedlings, respectively. Lane 3,6,9,12 southern blot that digested by *Bam*HI and *Eco*RI of leaf, root, stem and seedlings, respectively. Lane 4,7,10,13 southern blot that digested by *Eco*RI and *Hin*dIII of leaf, root, stem and seedlings, respectively.

Fungus infection assays

We compared the time course of disease symptoms in non-transformed and chitinase transformed plant infected with Fusarium monoliforme spores and the percentage of the total leaf area infected was scored for up two weeks after inoculation. Non-transformed control plants was observed cell

death. In contrast, in all chitinase transformed plant of Koshihikari was clearly suppressed in the leaf blade, indicating that leucaena chitinase gene resistance against rice blast fungus, Fusarium monoliforme (fig. 6).

A B

Fig. 6 Plant chitinase of Koshihikari was resistance against rice blast fungus, Fusarium monoliform. (A) is transgenic plant chitinase and (B) is non-transgenic plant chitinase.

IV. Conclusion

Agrobacterium-mediated transformation can be used to transformed chitinase gene into japonica rice, Koshihikari using EHA 105 (pCAMBIA 1300 and 1301). Plant regeneration of Koshihikari frequency of 90 % has been obtained from calli cultured on NB medium. The phytagel concentration of the regeneration medium is another important factor for induction of somatic embryogensis and high frequency plant regeneration from calli Koshihikari that used the pytagel concentration of the regeneration medium was 7g/l. From the present study, it was demonstrated that inclusion of acetosyringone to medium during cocultivation is important for increasing the frequency of transient transformation. The highest frequency, 74%, has been obtained from calli cocultivated on NB medium supplemented with 50 mM acetosyringone. The choice of explants as starting material and the addition of suitable concentration of hygromycin to the selection medium were other important factors for the successes in producing a high efficiency of transformed rice plants. The 4 weeks-old scutellum-derived embryogenic calli were shown to be starting material in these transformation experiments. Selection medium based on NB medium supplemented with 40 mg/l hygromycin and 400 mg/l cefotaxime are an optimized medium for select transformed rice cells form a mixed population of transformed and untransformed cells. Regeneration medium base on RN medium supplement with 4 mg/l BAP and 7 g/l pytagel are optimized medium for regenerated rice of 74%. Calli and transgenic rice were checked the expression of chitinase and gus by PCR, northern blot gel, southern blot gel, and gus assay. Chitinase and gus shown to express in all part of transgenic rice, such as calli, leaf, roots, stem, and seedlings but they shown little expression in root of transgenic rice. For antifungal of rice chitinase, they show antifungal the Fusarium monoliforme.

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