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Expression of Chicken Cystatin for Improving Insect Resistance in Rice

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To become mature and infectious, many viruses and insects require proteolytic cleavage, which can be specifically inhibited by proteinase inhibitors. Oryzacystatin (OC), the first-described cystatin originating from rice seed, consists of two molecular species, OC-I and OC-II, both of which have antiviral activity. These intrinsic rice cystatins show a narrow inhibition spectrum and ordinarily are present in rice seeds at insufficient levels for inhibiting the cysteine proteinases of rice insect pests. In addition, our comparison of inhibitory activity (Ki value) showed that chicken cystatin (Ki 5×10^{-12} M) was more powerful than other cystatins, such as OC-I (Ki 3.02×10^{-8} M) and OC-II (Ki 0.83×10^{-8} M). Chicken cystatin also possesses a wide inhibitory spectrum against various cysteine proteinases. Here, we introduced the insecticidal chicken cystatin gene into rice plants to improve their insect resistance. Four highly expressive, independent transgenic lines were identified. Molecular analyses revealed that the transferred gene was expressed stably in the independent transgenic lines. Therefore, introducing the insecticidal cysteine proteinase inhibitor gene into rice plants can be part of a general development strategy for pest control.

Keywords: chicken cystatin, insect resistance, rice

One of the major recent highlights in crop plant research has been the exploitation of inherent antiviral activities and insect resistance from proteinase inhibitors. The last decade has witnessed enormous progress in research on the structures, functions, and evolutionary relationships of proteinases. As the regulatory mechanism for proteolytic cleavage continues to be clarified, the primary focus is on cysteine proteinase, one of the four classes of proteinase. In addition, the discovery of crystal structures from chicken cystatin and human stefin B has provided a new mechanism for interaction between cysteine proteinases and their inhibitors (Turk and Bode, 1991; Desmazes et al., 2001).

Cystatins inhibit the activity of most cysteine endopeptidases of the papain type, as well as the exopeptidase dipeptidyl peptidase I (Irie et al., 1996; Strizhov et al., 1996; Brown and Dziegielewska, 1997; Janowski et al., 2001). They comprise a homologous group of protein inhibitors. The first of these to be studied in detail was chicken cystatin, derived from egg whites (Barrett, 1986; Colella et al., 1989; Verdot et al., 1999). Three distinct protein families, or types, are recognized within the cystatin superfamily. Type I cystatins (also called "stefins") are the simplest in structure, being single chains of about 100 amino acids each, with no disulfide bonds or carbohydrates. Type II cystatins, which include the eggwhite protein, have about 115 amino acids and two disulfide loops, but also lack carbohydrates.

The Type III cystatins are plasma kininogens, in which each molecule contains three divergent copies of the typical cystatin sequence. However, each of the kininogen types differs in its activity as well as its structure. These complex inhibitor molecules contain disulfide bonds as well as carbohydrate groups.

Each cystatin molecule has a single reactive site for all the peptidases it inhibits, but Ki values vary greatly among the different combinations of cystatins and enzymes, with calpains being inhibited by only one of the segments of the kininogens. The cystatins share many important characteristics, but their differences in molecular structure suggest unique routes of biosynthesis and a variety of physiological functions (Barrett, 1986; Diaz et al., 2001).

In general, cystatins protect cells from inappropriate endogenous or external proteolysis, and participate in the regulatory mechanism of intracellular or extracellular protein breakdown. Plant cystatins, or phytocystatins (PhyCys), are cysteine proteinase inhibitors that contain the Q × V × G motif. They have been classified in the cystatin superfamily of proteins (Machleidt et al., 1989; Arai et al., 1991; Abe et al.,

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1992; Fujimoto et al., 1993; Salm et al., 1994; Ebert et al., 1997). In rice plants, the primary sequences of the PhyCys have a high degree of homology with members of the cystatin family, but they resemble stefins in their absence of disulfide bonds and cysteine residues.

Two cystatins occur in the mature seeds of japonica rice (*Oryza sativa* L.): 1) oryzacystatin I (OC-I), and 2) oryzacystatin II (OC-II). The amino acid sequences of OC-I share 55% identity with those of OC-II. Likewise, the sequences of both oryzacystatins are significantly homologous with those in the cystatin superfamily members of animal origin, especially the Type II cystatins (Kondo et al., 1990; Arai et al., 1991; Chen et al., 1992; Michaud et al., 1995; Nagata et al., 2000). Because both also lack disulfide bonds, as is the case with Type I cystatins, they each seem to be chimerical of Type I and II cystatins. OC-I and OC-II are distinct in two respects: 1) their specificities against cysteine proteinases; and 2) the expression patterns of their mRNAs in the ripening stage of rice seeds. OC-I inhibits papain more effectively (Ki 3.0×10^{-8} M) than does cathepsin H (Ki 0.79×10^{-6} M), whereas OC-II inhibits cathepsin H (Ki 1.0×10^{-8} M) better than does papain (Ki 0.83×10^{-6}).

The mRNA for OC-I is expressed maximally at two weeks post-flowering, but is not highly detected in mature seeds. In contrast, the mRNA for OC-II is constantly expressed throughout the maturation stages, and is clearly present in mature seeds (Kondo et al., 1990; Abe et al., 1992; Masoud et al., 1993). However, factors for insectresistance, as would be expected in plants, are expressed at relatively low levels because the copy of the gene is low. These factors are almost completely lacking in the leaves, stems, and roots of rice plants, and exist at levels of only about 0.001 - to 0.002% in the seeds. This amount does not provide adequate protection against insect attacks. Furthermore, some species of insects feed exclusively on leaves and seeds, so any defense mechanism against these pests would require overexpression of the cystatin.

A Type II cystatin, isolated from chicken lungs, is a powerful protein inhibitor of papain (Ki 5×10^{-12} M) and other cysteine proteinases, and is more powerful than either OC-I (Ki 3.0×10^{-8} M) or OC-II (Ki 0.83×10^{-6}) (Table 1). Therefore, we proposed in this study that introducing this chicken cystatin into several japonica rice varieties could improve insect resistance. In addition, we investigated the antiviral effect of chicken cystatin. Our expectation was that the introduction of an insecticidal proteinase inhibitor gene into cereal plants might be used in a general strategy for controlling insect pests, as was suggested by Auerswald et al. (1992), Chen et al. (2000), and Golab et al. (2001).

MATERIALS AND METHODS

Plant Materials and Culture Media

Seeds of japonica varieties of rice (*O. sativa* L.) were obtained from Chonnam University, Korea, and from Jilim and Yanbian in China. We used various media for culturing the rice cells and for transformation (Table 2).

Callus Induction and Agrobacterium-Mediated Rice Transformation

Mature seeds were manually husked, then sterilized

Table 1. Ki of wild-type maize cystatin-II, cowpea cystatin, and oryzacystatin-I, plus a range of mutagenized forms of OC-I against two cysteine proteinases -- papain and the *Caenorhabditis elegans* cysteine proteinase gep-1 -- that show the changes in Ki of the wild-type protein.

Cystatin	Mutagenized Amino	change	Ki (nM) acid(s)	
			Papain	gcp-1
Maize cystatin-II	NONE		6.5 = 0.08	7.2 ± 0.16
Cowpea cystatin	NONE		6.1 = 0.10	7.2 ± 0.18
Oryzacystatin-I	NONE		7.0 = 0.13	8.0 ± 0.14
850C-I	Met85		–	7.1 ± 0.17
860C-I	Asp86		0.5 = 0.18	0.6 ± 0.16
830C-I	Por83	C,E,F,G,H,R,L,K,Q,S,Y,A	–	
840C-I	Trp84	C,E,F,G,H,R,L,K,Q,S,Y,p,A	–	–
860C-I	Asp86	C,E,F,G,H,R,L,K,Q,S,Y,P,N	–	–

Values are means = SEM.

Abbreviations: C, cysteine; E, glutamine; F, phenylalanine; G, glycine; H, histidine; P, proline; R, argine; L, leucine; K, lysi ne; Q, glutamine; S, serine; Y, tyrosine; -, indicates the inhibitor was not tested against that protease.

Table 2. Media used for *Agrobacterium*-mediated rice transformation.

Medium	Components
AAM	AA salts and amino acids, MS vitamins, 500 mg/L casamino acids, 68.5 g/L sucrose, 36 g/L glucose, 100 μM acetosyringone; pH 5.2
AB	3 g/L K_2HPO_4, 1 g/L NaH_2PO_4, 1 g/L NH_4Cl, 0.3 g/L $MgSO_47H_2O$, 0.15 g/L KCl, 0.10 g/L $CaCl_2$, 0.0025 g/L $FeSO_47H_2O$, 5 g/L glucose; pH 7.0 - 7.2
2N6	N6 medium, 2 mg/L 2,4-D, 30 g/L sucrose, 1 g/L casamino acids, 2 g/L Phytagel (Sigma): pH 5.6 - 5.7
2N6-ASB	N6 medium, 2 mg/L 2,4-D, 30 g/L sucrose, 1 g/L casamino acids, 2 g/L Phytagel, 10 g/L glucose, 100 μM acetosyringone, 1 mM betaine; pH 5.2
2N6-CH30	2N6 medium, 250 mg/L cefotaxime, 30 mg/L hygromycin B, 2g/L Phytagel; pH 5.6 - 5.7
2N6-BA	N6 medium, 1 mg/L 2,4-D, 0.5 mg/L BAP, 2 g/L casamino acids, 20 g/L sucrose, 30 g/L sorbitol, 250 mg/L cefotaxime, 40 mg/L hygromycin B, 2 g/L Phytagel; pH 5.6 - 5.7
MSR16	MS medium, 100 mg/L myo-inositol, 50 g/L sucrose, 20 g/L sorbitol, 0.1 mg/L NAA, 1 mg/L kinetin, 16 g/L agar; pH 5.6 - 5.7
MS	MS medium, 100 mg/L myo-inositol, 2 g/L Phytagel; pH 5.6 - 5.7

AA salts and amino acids (Toriyama and Hinata, 1985).
MS vitamins (Murashige and Skoog, 1962).
N6 medium (Chu et al., 1978).
MS medium (Murashige and Skoog, 1962).

with 70% ethanol for 1 min, followed by treatment with 50% Clorox for 1 h, with vigorous shaking. After the seeds were rinsed five times with sterilized water, they were cultured in the dark on the 2N6 medium for four weeks at 25°C. Actively proliferating calli were subcultured onto fresh 2N6 media for 4 to 5 d. We used rapidly growing embryogenic calli for subsequent experiments.

Agrobacterium cells were cultured for 3 d at 30°C in an AB liquid medium (Table 2) that contained ampicillin. The bacteria were collected by centrifugation. Cell pellets were washed with 1 mL of the AAM medium, and then suspended in the same medium, which now contained 100 μM acetosyringone (Aldrich Chem. Co.). This bacterial suspension was mixed with the rice calli and incubated for 10 min. The calli were transferred onto a 2N6-ASB medium, then co-cultivated with *Agrobacterium* in the dark at 25°C for 3 d.

Selection of Transgenic Calli and Plant Regeneration

The cultured rice cells and *Agrobacterium* were co-cultivated with chicken cystatin. Afterward, the *Agrobacterium* cells were thoroughly removed by washing the culture several times with sterile water that contained 250 mg/L cefotaxime. The rice cells were then placed on a 2N6-CH30 medium. After three to four weeks of culturing in darkness at 25°C, the proliferating hygromycin-resistant calli were transferred to a 2N6-BA medium and cultured for two more weeks in the dark at 25°C.

To regenerate whole plants, we transferred actively growing calli to an MS16 medium and incubated them at 26 - 30°C under continuous light (40 μmol $m^{-2}s^{-1}$).

Vector Construction of the Chicken Cystatin Gene for Rice Transformation

We amplified the chicken cystatin gene via PCR, then cloned it into a Topo vector (Invitrogen) and sequenced it. This cystatin DNA first was digested with BamHI and NotI, and then was subcloned under the control of the ubiquitin promoter in the plant vector pMJ-U (as constructed at Myeongji University).

Southern Analysis

Rice genomic DNA (1 μg) from leaves was digested with either BamHI or EcoRI, then separated on a 0.8% agarose gel by electrophoresis and blotted onto a nylon membrane (Hybond-N+, Amersham), using the alkaline transfer method (Sambrook et al., 1989). The membrane was hybridized with [α-^{32}P]d-CTP-labeled PCR that amplified the chicken cystatin cDNA probe. Afterward, it was washed with 2x SSC and 0.1% SDS at 65°C for 1 h, followed by an additional washing with 0.2x SSC and 0.1% SDS at 65°C for 1 h. Finally, the membrane was exposed for 1 d to Kodak X-ray film, using two intensifying screens at −80°C.

Northern Analysis

Total RNA was isolated from rice seedlings using the RNAeasy Plant Kit (QIAGEN), according to the manu-

facturer's protocol (Verwoerd et al., 1989). Northern analysis was carried out as described by Agrawal et al. (2000) and Rakwal et al. (2001). Again, hybridization with the [α-^{32}P]d-CTP-labeled PCR, for 18 h at 65°C, amplified the chicken cystatin cDNA probe. The resulting membrane was washed with 2X SSC and 0.1% SDS at 65°C for 1 h, then exposed for 3 d on Kodak X-ray film, using two intensifying screens at −80°C.

RT-PCR Assay

Total RNA was isolated from chicken lung tissue, using an RNAeasy Kit (QIAGEN) according to the manufacturer's protocol. Total RNA samples were Dnase-treated with RNase-free DNase (Stratagene, La Jolla, CA, USA) prior to RT-PCR, to ensure that the samples were free of contaminating DNA. First-strand cDNA was synthesized in a 50-μL reaction mixture with a StartaScript™ RT-PCR Kit (Stratagene), according to the manufacturer's protocol, and using 10 μg total isolated RNA. The consensus primer pair, whose design was based on the chicken cystatin amino acid sequence, was used to amplify mRNA, with the first-strand cDNA prepared above serving as template. The resulting amplified fragment of 436 bp was purified from the gel (QIAquick gel extraction kit, QIAGEN), ligated into a TOPO vector (Invitrogen), and sequenced with a dye-terminator cycle sequencing reaction kit and an automated DNA sequencer.

Nucleotide Sequencing

Plasmid DNA for sequencing reactions was prepared by the alkaline lysis method, using a QIAprep Spin Miniprep kit (QIAGEN). The reaction was performed with the ALF Express AutoRead Sequencing kit (Amersham Pharmacia Biotech), using the kit's fluorescent dye-labeled M13 universal or reverse primer. The nucleotide sequences were obtained via electrophoresis on an ALF Automatic Sequencer (Perkin-Elmer).

RESULTS AND DISCUSSION

Identification of Oryzacystatin

Both types of oryzacystatin that occur in rice - OC-1 and OC-2 - inhibit activity of papain as well as insect digestive cysteine proteinases. However, the two differ remarkably in their specificities against cysteine proteinases and in their mRNA expression patterns during seed ripening. In this experiment, we

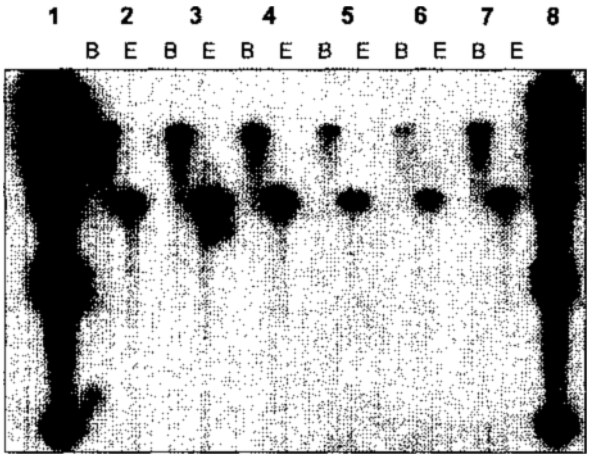

Figure 1. Southern blot analysis of oryzacystatin from rice leaves. Genomic DNA was isolated from leaves of black rice (Jilim), black rice (Yanbian), and scented rice. The digestion was performed using the restriction enzymes indicated on the tops of the lanes. B, BamHI; E, EcoRI. Lane 1, I-kb marker; Lane 2, Jilim (1-week-old leaves); Lane 3, Jilim (2-week-old leaves); Lane 4, Yanbian (1-week-old leaves); Lane 5, Yanbian (2-week-old leaves); Lane 6, scented rice (1-week-old leaves); Lane 7, scented rice (2-week-old leaves); Lane 8, I-kb marker.

determined whether the oryzacystatin gene was expressed in rice, first by digesting the genomic DNA with BamHI or EcoRI, then by blotting and hybridization. We observed one band in our Southern blot analysis, which indicated the presence of the oryzacystatin gene (Fig. 1). In a comparative experiment, we also detected this the oryzacystatin gene in black rice and scented rice from Jilim and Yanbian, China. However, we could not confirm the exact copy numbers for OC-I and OC-II because of their close homology with each other.

Oryzacystatin Expression in Rice Leaves

Expression of cysteine proteinase inhibitors (cystatins) in rice or other crop species may improve their resistance to pathogens and insects that naturally possess cysteine proteinase. Kondo et al. (1990), Nagata et al. (2000), and Rakwal et al. (2001) have reported that the mRNA for OC-I is maximally expressed two weeks after flowering, but is not detected in mature seeds. In contrast, mRNA for OC-II is consistently expressed throughout the maturation stages, and is clearly present at seed maturity.

To determine the expression patterns of oryzacystatins in the rice from Jilim and Yanbian, China, we isolated RNAs from their mature leaves and seeds.

Figure 2. Northern blot analysis of oryzacystatin in total RNA isolated from rice leaves. Lane 1, Jilim (1-week-old leaves); Lane 2, Jilim (2-week-old leaves); Lane 3, Yanbian (1-week-old leaves); Lane 4, Yanbian (2-week-old leaves); Lane 5, scented rice (1-week-old leaves); Lane 6, scented rice (2-week-old leaves).

Expression was seen in the leaves, but not in the seed (Fig. 2). Furthermore, our northern analysis revealed no difference in expression levels between the black and the scented rice leaves.

Cloning and Sequence Analysis of Chicken Cystatin Gene

We compared the sequencing results for the chicken cystatin gene (Fig. 3) with those of other cysteine proteinase inhibitors (including OC-I and OC-II, for which alignment data are not shown). A particular sequence, Q × V × G, was found to be totally conserved among several of the animal cystatins, as well as among phytocystatins (see Barrett, 1986). This conserved sequence had been identified at positions 76 to 80 in the chicken cystatin molecule (Auerswald et al., 1992; Chen et al., 2000; Golab et al., 2001; Janowski et al., 2001).

Furthermore, we found conserved sequences for Gly to Ala (positions 32 to 33 in the N-terminus) and for the Pro-Trp residue (positions 126 to 127 in the C-terminus), which agreed with reports by Arai et al. (1991), and Turk and Bode (1991). Therefore, the sequence for the chicken cystatin gene was homologous with those of other cysteine proteinase inhibitors (see Desmazes et al., 2001; Diaz et al., 2001; Golab et al., 2001).

For our rice transformation, the PCR fragments of chicken cystatin were subcloned into the expression vector pMJ-U under the control of the ubiquitin promoter (Fig. 4). To elevate the frequency of expression for the chicken cystatin gene in our transgenic rice plants, we combined the matrix attachment region (MAR) sequence with that gene, and named this expression vector pMRcys. The recombinant plasmid pMRcys was then transferred into cultured rice cells through a Agrobacterium-mediated transfer system.

Chicken cystatin

Translation =

"**M**AGARGCVVLLAAALMLVGAVLGSEDRSRLLGAPVPVDENDEGL

QRALQFAMAEYNRASNDKYSSRVVRVISAKRQLVSGIKYILQVEIGR

TTCPKSSGDLQSCEFHDEPEMAKYTTCTFVVYSIPWLNQIKLLESKCQ"

ccactccgctccgcagccttaaaatggcggccaggcgtgtagtgcggtgtgtggagcc**at**

ggcaggagctcggggttgcgtagtgctgctggccgcggccctaatgctcgtcggcgctgt

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ggagtagctgtttgtcttctcgcagctttcccaataaagcaactccagtgtcaaattaaatgaat

tgtgtccctataagtgactactgtaaacattaaaactcaattcatcatt

Figure 3. Nucleotide sequences of chicken cystatin isolated from the lung.

Transgenic Rice Calli and Regenerated Rice Plants Expressing Insect-Resistance Cystatin from Chicken Lung Tissue

Transformed calli and regenerated rice plants were obtained via *Agrobacterium*-mediated transformation. That method for producing transgenic tissue is well-established for dicotyledonous species, but is controversial for monocots. Using an in-vitro regeneration protocol, we were able to generate calli from rice seeds and then efficiently regenerate those transformed calli into whole plants (Fig. 5). Calli induced from seed cells were co-cultivated with *Agrobacterium tumefaciens* that harbored pMRcys (Fig. 5A), and then were cultured on a hygromycin-containing medium. Cell proliferation was observed on the selection medium. Most of the green calli that recovered from the first round of selection also proliferated on the second medium. Plants were readily regenerated when the resistant cells were transferred to a regeneration medium that also contained hygromycin (Fig. 5, B - D).

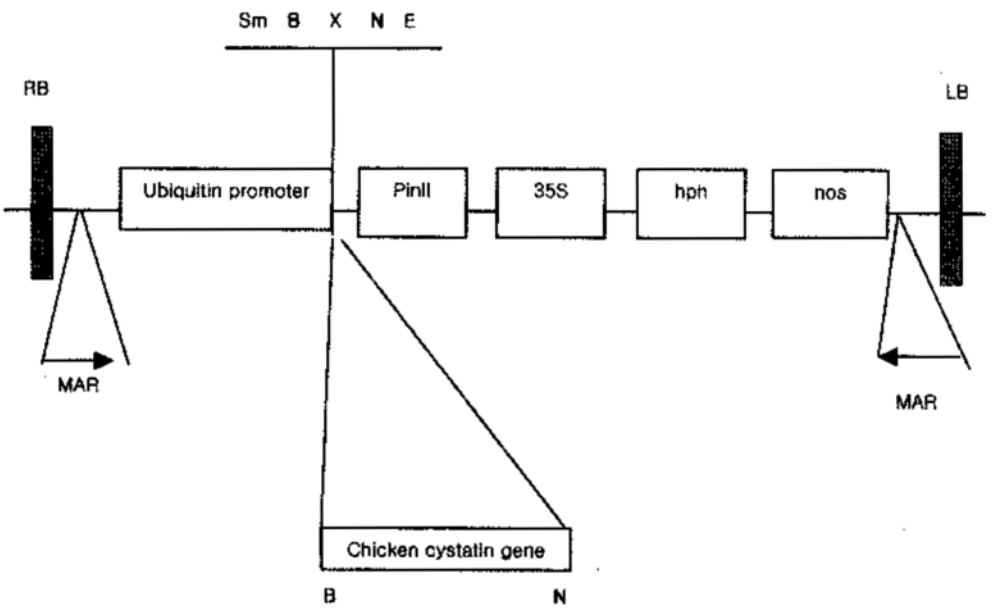

Figure 4. Map of pMRcys for cloning of chicken cystatin for rice transformation. Sm, SmaI; B, BamHI; X, XbaI; N, NotI; E, EcoRI. 3' Pin II, Pin II; 35S, CaMV promoter; hph, hygromycin gene; nos, 3' NOS.

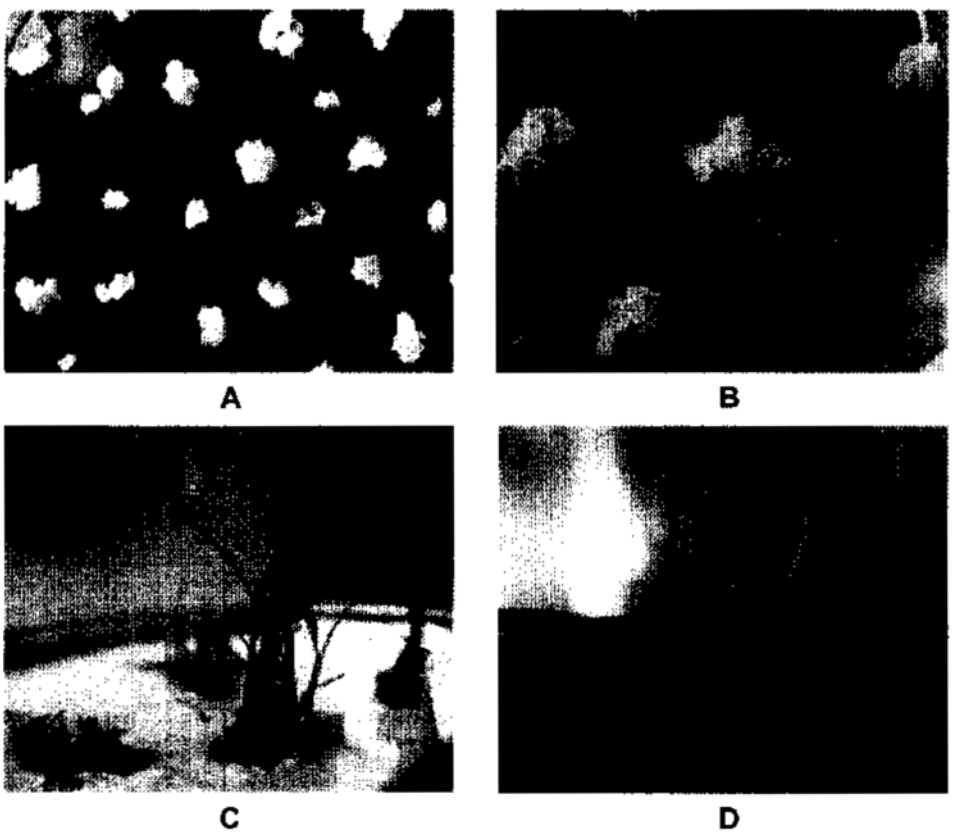

Figure 5. Regenerated transformants of the cystatin gene from cultured rice cells; rice transformants using the chicken cystatin gei construct with pMRcys vector. **A.** calli after co-cultivation with *Agrobacterium* harboring the pMRcys vector containing the chick cystatin gene. **B.** hygromycin-resistant green shoot proliferating from the calli. **C.** 5-week-old culture rice after transformation. 10-week-old transgenic rice plant in soil after transformation.

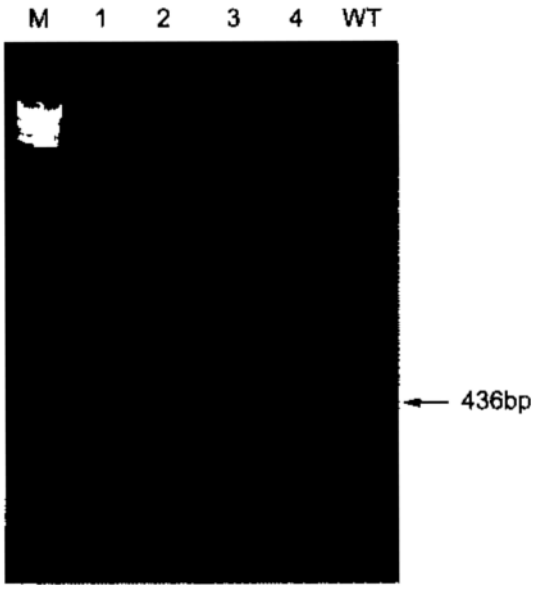

Figure 6. Confirmation of chicken cystatin expression in transgenic rice plants by RT-PCR. Lane M, HindIII size markers; Lanes 1 to 4, transgenic rice expressing the cystatin gene; WT, wild type.

To detect expression of the chicken cystatin gene in transformed rice plants, as was reported by Colella et al. (1989) and Rakwal et al. (2001), we isolated total RNA from the transgenic rice to provide verification by RT-PCR. The transformants gave signals with the expected 436-bp band (Fig. 6).

In our experiments, transformation efficiency for calli and whole plants was higher than was previously reported (Lee et al., 1999). Our transformed plants were successfully cultivated to maturity under greenhouse conditions. Therefore, we propose that insect-resistant, transgenic rice plants can be obtained from rice seeds transformed with *A. tumefaciens*, which harbors pMRcys that contains the chicken cystatin gene. We also suggest that, through further feeding bioassay experiments, we will be able to demonstrate that chicken cystatin is more inhibitory than potent cysteine proteinase inhibitors that show broad specificity, e.g., the engineered OC-I and OC-II.

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