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Production of biologically active Atlantic salmon interferon in transgenic potato and rice plants

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Interferons (IFNs) are cytokines that induce an antiviral state in vertebrate cells. The Atlantic salmon (*Salmo salar*) IFN gene (SasaIFN-α1) was introduced in potato and rice plants by *Agrobacterium*-mediated transformation to produce a biologically active fish IFN in these plants. The transgenes and their transcripts were detected by PCR and Northern blot analysis. Western blot analysis showed the existence of SasaIFN-α1in transgenic plants. The antiviral activity of the SasaIFN-α1 protein expressed in these plants was determined by the survival rates of pre-treated cultured fish cells against pancreatic necrosis virus infection. The survival rate of cells pre-treated with transgenic samples was up to 95% but was reduced to 30–47% when cells were pre-treated with non-transgenic samples. These results demonstrated an antiviral effect of the SasaIFN-α1 protein derived from transgenic plants. Plant-derived IFNs may be suitable as components of functional feeds because such IFNs are free of animal pathogens and can be produced at a lower cost compared with those from transgenic mammalian and bacterial cells. This is the first study describing the production of a biologically active fish IFN using transgenic plants.

[**Key words:** Interferons; *Salmo salar*; SasaIFN-α1; Transgenic plants; Antiviral activity; Infectious pancreatic necrosis virus; Functional feed]

Fish diseases caused by viruses, bacteria, fungi and parasites result in enormous economic losses in aquaculture. In particular, viral diseases are hard to control because of the difficulties in treating diseased fish by antibiotic chemicals. Thus, disease prevention is the most feasible way to control viral diseases, which includes sterilizing rearing water and facilities, disinfecting fertilized eggs, selecting virus-free parental fish and vaccination. However, these prevention methods require expensive facilities and high operating costs. Furthermore, few vaccines for fish viruses are commercially available (1).

Interferons (IFN) are cytokines that are key components of innate immune responses in mammals, birds, reptiles and fish and are the first line of defence against viral infections. In fish, IFN genes have been identified in zebra fish (*Danio rerio*) (2, 3), channel catfish (*Ictalurus punctatus*) (4, 5), Atlantic salmon (*Salmo salar*) (6, 7), rainbow trout (*Oncorhynchus mykiss*) (8), tiger puffer (*Takifugu rubripes*) (9), medaka (*Oryzias latipes*) (8) and spotted green puffer fish (*Tetraodon nigroviridis*) (9). Despite limited sequence homologies between fish, mammalian and avian IFNs, numerous functional studies have demonstrated that fish IFNs also possess characteristic antiviral properties (10).

Oral administration of IFNs produced from bacterial or eukaryotic cell culture systems are effective in animals and humans for treating autoimmune diseases (11, 12), allergies and infectious diseases (13–16). However, IFNs from different species were much less effective in inducing a detectable antiviral state than similar treatment of parental cells with homospecific IFNs (17). Thus, fish IFNs should also be effective for preventing fish diseases when they are administered orally. However, for the practical use of such vertebrate IFNs, some difficulties regarding cost and safety continue to exist. IFNs produced by bacterial and eukaryotic cell culture systems may contain bacterial endotoxins and animal viruses, respectively, as contaminants. Such IFN samples need to be purified and confirmed to be safe before commercialization. Compared with the bacterial and mammalian expression systems, plant expression systems have some advantages in terms of low production costs (18, 19) and safety. For example, plant-derived protein samples are basically free of animal pathogens and their products that are harmful to vertebrates. In addition, the post-translational modification of recombinant proteins in plants is comparable to that in vertebrates than that in bacteria. Mass production of recombinant proteins at a low cost is feasible using plant expression systems. Finally, a cold chain for storing transgenic plants is not necessary when the plants used are seed-propagated cultivars. Thus, plant expression systems are suitable platforms for producing biologically active IFNs.

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Various plant species have been utilized to produce biologically active IFNs using several plant engineering techniques for more than a decade (20). Human IFNs have been expressed in potato (*Solanum tuberosum*) (21, 22), tobacco (*Nicotiana tabacum*) (23, 24), rice (*Oryza sativa*) (25, 26), lettuce (*Lactuca sativa*) (27), cucumber (*Cucumis sativus*) and squash (*Cucurbita maxima*) (28). A chicken IFN was similarly produced in lettuce (29). However, no fish IFNs have been expressed in plants.

Among fish species, salmonids such as chum salmon (*Oncorhynchus keta*), king salmon (*Oncorhynchus tshawytscha*) and rainbow trout are some of the major aquaculture species. However, salmonids are susceptible to some lethal viruses, such as viral hemorrhagic septicemia virus, infectious hematopoietic necrosis virus, infectious salmon anemia virus and infectious pancreatic necrosis virus (IPNV). Atlantic salmon IFN produced using a mammalian (6, 7) or bacterial (30) cell culture system prevented IPNV infection in king salmon CHSE-214 cells. The fact that Atlantic salmon IFN had an antiviral effect on king salmon cells suggests that this IFN could also function in other salmonid species. In this study, for producing animal pathogen-free Atlantic salmon IFN at a low cost, we expressed this IFN in transgenic potato and rice plants and confirmed its antiviral activity. This is the first study describing the production of physiologically active fish IFN utilizing transgenic plants.

MATERIALS AND METHODS

Construction of plant binary vectors SasaIFN-α1 cDNA (GenBank accession no. AY216504) was synthesized using DNA synthesizer Expedite™ 8909 (Applied Biosystems, Foster City, CA). For subsequent vector manipulations, *Sma* I and *Sac* I restriction sites were introduced in the 5′- and 3′-termini of the cDNA fragment, respectively. In addition, synonymous mutations were introduced at positions 115 (C-A), 139 (C-T), 364 (A-T) and 496 (G-A) to disrupt the original *Sma* I and *Sac* I sites in cDNA. The mutated SasaIFN-α1 cDNA was cloned into pGEM-T (Promega, Madison, WI, USA). To construct pBE2113-SasaIFN-α1, the *Sma* I–*Sac* I GUS gene fragment of the plant binary vector pBE2113 (31) was replaced with the *Sma* I–*Sac* I fragment of the mutated SasaIFN-α1 cDNA. Likewise, the *Hin*d III–*Sac* I GUS gene fragment of pGPTV-HPT (32) was replaced with the *Hin*d III-*Sac* I fragment of pBE2113-SasaIFN-α1 (Fig. 1).

Potato plant transformation and regeneration *Agrobacterium*-mediated genetic transformation experiments were carried out using LBA4404 strain harbouring pBE2113-SasaIFN-α1. The potato cultivar 'May Queen' was used for genetic transformation according to the method described by Matsumura et al. (33). Potato tubers were surface-sterilized with 1% sodium hypochlorite for 15 min and then rinsed three times with sterile distilled water. The tubers were hollowed out using a sterile cork borer and sliced into 2-mm-thick discs using a sterile surgical blade. *A. tumefaciens* strain LBA4404 harbouring pBE2113-SasaIFN-α1 was cultured for 2 days at 28 °C in the dark and resuspended in Murashige–Skoog (MS) liquid medium (34). The tuber discs were soaked in the bacterial solution for 15 min and placed on solid MS medium containing 50 μg/l 3-indoleacetic acid (Wako Pure Chemical Industries, Ltd.), 0.1 mg/l gibberellic acid (Wako Pure Chemical Industries, Ltd.), 0.1 mg/l abscisic acid (Wako Pure Chemical Industries, Ltd.) and 2 mg/l zeatin riboside (Wako Pure Chemical Industries, Ltd.). After 3 days of culturing at 24 °C in the dark, inoculated tubers were transferred onto MS medium supplemented with 500 mg/l carbenicillin (Sigma-Aldrich, Inc.) and 50 mg/l kanamycin sulphate (Sigma-Aldrich, Inc.), followed by incubation at 23 °C under a 16/8-h (light/dark) photoperiod. The explants were sub-cultured every 2 weeks using the same medium to promote shoot formation. Regenerated shoots were placed onto MS medium supplemented with 500 mg/l carbenicillin and 50 mg/l kanamycin sulphate for selecting transformants.

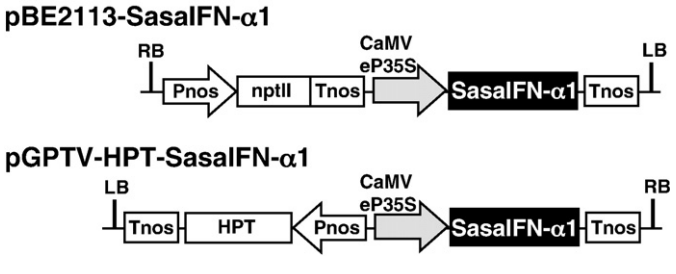

FIG. 1. Schematic diagrams of plant binary vectors pBE2113-SasaIFN-α1 and pGPTV-HPT-SasaIFN-α1. For expression in plants, SasaIFN-α1 cDNA is introduced in the plant binary vectors pBE2113 or pGPTV-HPT. RB/LB: T-DNA right/left border sequence, Pnos: promoter of nopaline synthase, NPT II: neomycin phosphotransferase gene, CaMV eP35S: Cauliflower mosaic virus 35S promoter with a duplicate enhancer sequence and Omega fragment (5′-untranslated sequence of Tobacco mosaic virus), HPT: hygromycin phosphotransferase, Tnos: polyadenylation signal of nopaline synthase.

Rice plant transformation and regeneration *Agrobacterium*-mediated genetic transformation experiments were carried out using the LBA4404 strain harbouring pGPTV-HPT-SasaIFN-α1. The rice cultivar 'Yukara' was used for genetic transformation. Mature rice seeds were dehusked, surface-sterilized with 2.5% sodium hypochlorite for 40 min and rinsed three times with sterile distilled water. The rice seeds were placed onto MS medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma-Aldrich, Inc.) to induce calli. Embryogenic calli derived from scutella were cultured in modified R2 medium (35) containing 2 mg/l 2,4-D with shaking and sub-cultured every 2 weeks. *A. tumefaciens* strain LBA4404 harbouring pGPTV-HPT-SasaIFN-α1 was cultured for 2 days at 28 °C and resuspended in liquid N6 medium (36). Scutellum-derived calli were soaked in the bacterial solution for 2 min and transferred onto modified solid N6 medium supplemented with 30 g/l sucrose, 10 g/l glucose, 2 mg/l 2,4-D and 10 mg/l 3′,5′-dimethoxy-4′-hydroxy-acetophenone (pH 5.2). After 3 days of culturing at 23–25 °C in the dark, the inoculated calli were rinsed with N6 liquid medium supplemented with 500 mg/l carbenicillin and 50 mg/l hygromycin B (Sigma-Aldrich, Inc.), followed by sub-culturing on the same medium at 23 °C under a 16/8-h (light/dark) photoperiod for 2 weeks. The proliferated calli were then transferred onto MS medium supplemented with 500 mg/l carbenicillin, 50 mg/l hygromycin B and 2 mg/l kinetin (Sigma-Aldrich, Inc.) for selecting transformants. Regenerated shoots were placed on MS rooting medium containing 500 mg/l carbenicillin and 20 mg/l hygromycin B. Rooted plants were transplanted in flowerpots filled with potting compost and grown to maturity in a greenhouse.

Genomic PCR analysis Insertion of the SasaIFN-α1 gene in the plant genome was confirmed by genomic PCR analysis. Potato genomic DNA was extracted from leaves and subsequent PCR analysis was performed using the Extract-N-Amp Plant PCR Kits (Sigma-Aldrich, Inc.) and SasaIFN α-1-specific primers (IFN-F; 5′-ATGTATACAGTGCAGAGTTGGACGTG-3′ and IFN-R; 5′-TCAGTACATCTGTGCTGCAAGGATATCC-3′). Rice genomic DNA was obtained from leaves using a MagExtractor system (Toyobo Co, Ltd.), and PCR was performed using ExTaq polymerase (Takara bio, Inc.) and the two primers. Regenerated non-transgenic potato and rice plants were used as negative controls. Thirty-five cycles of PCR were performed and the conditions were as follows: denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min and extension at 72 °C for 1 min. PCR products were run on a 1.5% TBE-agarose gel and visualized by ethidium bromide staining.

RT-PCR analysis Total RNA samples were purified from leaf tissues using an RNeasy Plant Mini Kit (Qiagen). RT-PCR using the IFN-F and IFN-R primers was performed using a Ready-To-Go RT-PCR Kit (GE Healthcare, Inc.). After reverse transcription at 42 °C for 30 min, amplification was carried out using a thermal cycler with 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min. The PCR products were separated on a 1.5% TBE-agarose gel and visualized by ethidium bromide staining.

Northern blot analysis Total RNA was extracted from leaves (potato and rice), tubers (potato) and seeds (rice) as described by Asif et al. (37). Total RNA (20 μg) from each sample was separated on a 1.2% agarose-formaldehyde gel in MOPS buffer and blotted onto a nylon membrane (Hybond-N+, GE Healthcare, Inc.) according to the manufacturer's instructions. Hybridization was performed at 50 °C for 16 h using the DIG Easy Hyb buffer (Roche, Inc.) and a DIG-labelled SasaIFN-α1 probe, which was synthesized by PCR from SasaIFN-α1 cDNA using PCR DIG Labelling Mix (Roche, Inc.) according to the manufacturer's instructions. Hybridization signals were detected using a DIG Luminescent Detection Kit (Roche, Inc.).

Production of recombinant proteins and preparation of antibody The mutated SasaIFN-α1 cDNA was subcloned between the *Bam*H I and *Xho* I restriction sites in the pFastBacHT-B (Invitrogen, Inc.) in which the 6× His-tagged SasaIFN-α1 gene was driven by the polyhedron promoter. Expression of 6× His-tagged SasaIFN-α1 was confirmed using a Bac-to-Bac baculovirus expression system (Invitrogen, Inc.) according to the manufacturer's instructions. The recombinant SasaIFN-α1 protein expressed using this expression system was purified on an Ni column according to the manufacturer's instructions (GE Healthcare, Inc.). Purified SasaIFN-α1 was subcutaneously and intradermally injected into female Wister rats every 2 weeks for 4 months. Each inoculum contained 50 μg of purified SasaIFN-α1 incorporated in Freund's complete adjuvant for the first injection and in Freund's incomplete adjuvant for subsequent injections. Rat hybridoma G2-7 cell line was prepared according to the method of Köhler and Milstein (38). Monoclonal antibodies against SasaIFN-α1 were purified from hybridoma supernatants using a protein L column (PIERCE, Inc.).

Western blotting for ifn detection Leaf tissues of the transgenic or non-transgenic potato plants were macerated on ice using a mortar and pestle with 5 volumes (w/v) of PBS containing 0.05% Tween 20. Macerated samples were centrifuged at 20,000×*g* for 10 min at 4 °C. The supernatants (soluble fractions) and pellets

(insoluble fractions) were individually collected and suspended in Laemmli sample buffer (Bio-Rad Laboratories, Inc.) supplemented with 5% 2-mercaptoethanol (Nacalai Tesque, Inc.). Transgenic rice seeds (T1) were ground with 10 volumes (w/v) of the same Laemmli sample buffer. These protein samples were loaded onto 10–20% SDS-PAGE gels and electroblotted onto Hybond-P membranes (GE Healthcare, Inc.) using Trans-Blot Cell (Bio-Rad laboratories, Inc.). SasaIFN-α1 on the membranes was detected using the anti-SasaIFN-α1 monoclonal antibody (1:500 dilution) and anti-rat IgM conjugated with peroxidase (1:10,000 dilution, Thermo Scientific, Inc.). Peroxidase signal was visualized using ECL plus Western Blotting Detection Reagents (GE Healthcare, Inc.).

Purification of SasaIFN-α1 The leaf extracts (10 g fresh mass) were homogenized in 20 mM Tris–HCl (pH 8.0) buffer containing 0.1% Triton X-100 and a proteinase inhibitor. The insoluble fraction was removed by centrifuging at 12 000 × *g* for 10 min, and the supernatants were then applied to columns with 7.5 ml DEAE Sepharose Fast Flow (GE Healthcare, Inc.) and equilibrated. After loading the lysates, the columns were washed and eluted with 0.1 M NaCl in 20 mM Tris–HCl (pH 8.0) containing 0.1% Triton X-100. The eluted protein solution was then concentrated up to 1 ml. The amount of purified protein was determined using the BCA protein assay reagent (PIERCE, Inc.).

Antiviral activity assay Leaf tissues (200 mg) harvested from transgenic potato or rice plants were homogenized in 1.0 ml PBS (−) supplemented with 0.05% Tween 20 and centrifuged at 15,000 rpm for 10 min at 4 °C. The supernatant was collected and sterilized by passing through a 0.45-μm filter. The sample was then serially diluted with PBS (−) and applied to the CHSE-214 cell for the antiviral activity assay as follows. The final concentrations of the plant samples diluted in the cultured medium were shown in the data.

CHSE-214 cells were cultured in a 24-well plate (Sumilon) at 20 °C containing 500 μl/well of Eagle's minimal essential medium (Nissui) supplemented with 10% foetal bovine serum (MEM-10). Twenty-four hours after plating, 100 μl of culture medium were replaced with 100 μl of the extracted plant sample and the cells were further cultured for 24 h under the same conditions. The cells were then washed once with Hanks' balanced salt solution (Nissui) and inoculated with IPNV at a multiplicity of infection (MOI) of 0.01. After 1-h incubation at 20 °C, the culture inoculum was replaced with MEM-10 and the cells were further cultured at 20 °C. Another set of CHSE-214 cells were similarly cultured and treated only with extraction buffer for use as a negative control.

Survived CHSE-214 cells after inoculation with plant samples and/or IPNV were measured by the uptake of crystal violet. The inoculated cells in a 24-well plate were washed once with PBS (−) and stained with crystal violet solution (1% crystal violet, 20% ethanol) for 10 min. The cells were washed three times with PBS (−) and the dye was recovered from the cells with an extraction solution (50% ethanol, 0.05 M sodium citrate and 0.05 M citrate). Absorbance of the extracted sample at 550 nm was measured to assess the rate of cell survival. High absorbance indicated a high survival rate. Antiviral activity of the plant samples were determined as the survival rates of CHSE-214 cells calculated from the following formula: survived cell number after inoculation with a plant sample and IPNV/survived cell number after inoculation with only the plant sample.

The antiviral activity of the plant samples was also examined by its inhibitory effects on the development of cytopathic effects (CPE). In general, CPE development represents virus infection in cultured cells. In CHSE-214 cells infected with IPNV, CPE is denoted as the appearance of rounded cells which are finally detached from the culture dish.

RESULTS

Transformation of potato and rice plants with SasaIFN-α1 cdna The binary recombinant plasmids pBE2113-SasaIFN-α1 and pGPTV-HPT-SasaIFN-α1 harbouring the SasaIFN-α1 cDNA were constructed (Fig. 1). Both vectors were designed to express IFN cDNA under the control of the CaMV 35S promoter. These plasmids were introduced into potato and rice plants, respectively, by *Agrobacterium*-mediated transformation. After infection with *A. tumefaciens*, 900 antibiotic-resistant potato calli were induced from 1000 tuber discs in 30–50 days. Similarly, 50 antibiotic-resistant rice calli proliferated from 300 scutellum-derived/embryogenic calli in 30 days. Potato and rice plants started to regenerate from the calli after sub-culturing for 5 months. Finally, 190 kanamycin-resistant potato plants and 11 hygromycin-resistant rice plants were obtained from the calli. No abnormal phenotypes were detected in these regenerated plants compared with regenerated non-transgenic plants.

Characterization of SasaIFN-α1 transgenic plants Integration of SasaIFN-α1 cDNA into potato and rice genomes was confirmed by genomic PCR, which showed amplification of a 528-bp fragment. Of 190 regenerated potato plants tested, 150 plants were positive for cDNA amplification. Similarly, 10 of 11 regenerated rice plants were positive (Fig. 2A). No amplicon was obtained using non-transgenic potato or rice plants. SasaIFN-α1 transcripts in the transgenic potato and rice plants were detected by RT-PCR. The salmon IFN transcripts

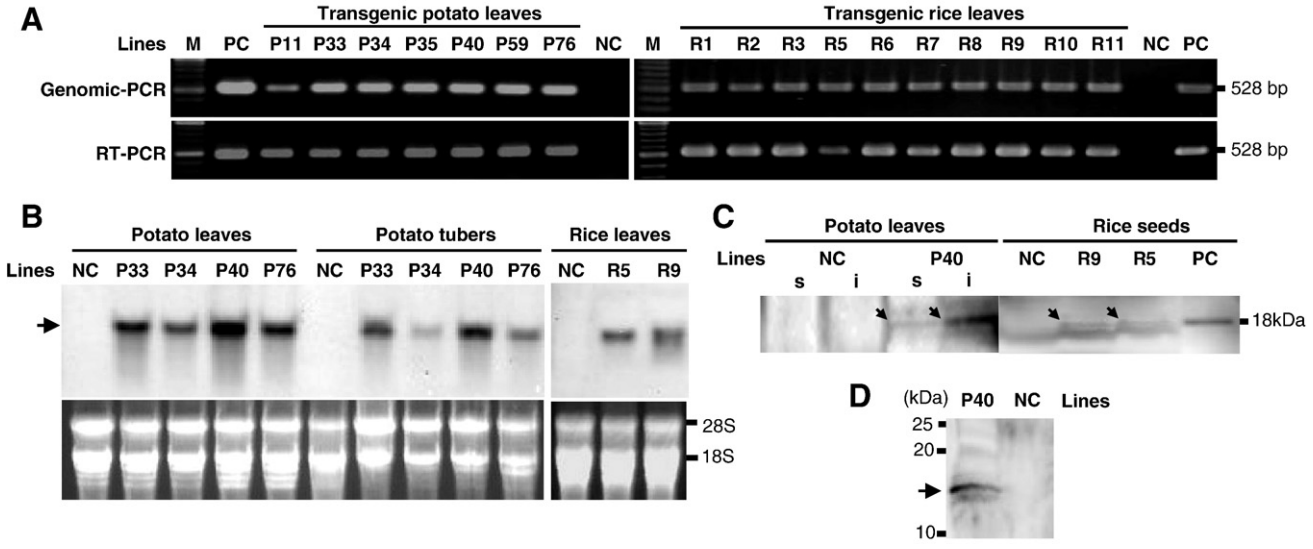

FIG. 2. SasaIFN-α1 expression in transgenic potato and rice plants. (A) Transgene and its transcripts were detected by PCR. M: 100-bp DNA ladder, PC: plasmid vector pBE2113-SasaIFN-α1 (left panel) or pGPTV-HPT-SasaIFN-α1 (right panel), NC: non-transgenic plants. (B) Total RNA extracted from transgenic potato/rice plants (lower panel) was subjected to Northern blot analysis using the DIG-labelled SasaIFN-α1 cDNA probe (upper panel). Arrowhead indicates the position of the SasaIFN-α1 mRNA. Ribosomal RNAs are indicated as 18S and 28S. NC: non-transgenic plants. Numbers above the panels indicate the transgenic plant lines. (C) Western blot analysis of SasaIFN-α1 proteins in transgenic potato leaves and rice T1 seeds. Proteins were resolved on 10–20% SDS-PAGE and used for Western blotting with anti-SasaIFN-α1 rat monoclonal antibody (IgM). Arrowheads indicate the position of the SasaIFN-α1 proteins. NC: non-transgenic plants. s: soluble fraction of crude leaf extracts. i: insoluble fraction of crude leaf extracts. PC: positive control of SasaIFN-α1. Numbers above the panels indicate the clone numbers of the transgenic plants. (D) Western blot analysis of the purified SasaIFN-α1 proteins from transgenic potato leaves. The sample containing 60 μg of soluble protein was loaded on each lane. The blots were treated as shown in (C). P40: transgenic line P40, NC: non-transgenic plants. Arrowhead indicates the position of the SasaIFN-α1.

were detected in 86 of 115 transgenic potato plants tested and in all transgenic rice plant cell lines examined (Fig. 2A). The non-transgenic potato and rice plants did not show SasaIFN-α1 transcripts. SasaIFN-α1 transcripts in transgenic potato and rice were also semi-quantified by Northern blot analysis. As shown in Fig. 2B, more SasaIFN-α1 mRNA was detected in the transgenic potato leaves of line P40 than in that in line P76. SasaIFN-α1 mRNA was also identified in the tubers of these two transgenic potato lines. Likewise, the mRNA level of SasaIFN-α1 cDNA was higher in line R9 than that in line R5 rice leaves (Fig. 2B). Although we could not detect the 18-kDa bands clearly in soluble fractions from the transgenic plants using Western blot analysis, the SasaIFN-α1 protein was barely detectable in the insoluble fractions of transgenic potato leaves and in the rice seeds using an anti-SasaIFN-α1 monoclonal antibody that specifically recognized the purified recombinant SasaIFN-α1 protein (Fig. 2C). In addition, we concentrated the SasaIFN-α1 protein from the soluble fractions of the massive transgenic potato leaves (line P40). A low amount of affinity-purified SasaIFN-α1 protein was detected from the soluble proteins by Western blot analysis (Fig. 2D).

Antiviral activities of recombinant IFNs The antiviral potential of the transgenic plants that expressed SasaIFN-α1 was examined using CHSE-214 cells and IPNV. Extracts from R5 and R9 transgenic rice plants showed maximum antiviral effects at concentrations of 10–20 and 5 mg/ml, respectively (Fig. 3A and Table 1). With these treatments, 80% and 92% of the virus-inoculated cells survived, respectively. In contrast, the survival rates of the virus-inoculated cells pre-treated with the non-transgenic samples were 40% (5 mg/ml sample), 47% (10 mg/ml sample) and 38% (20 mg/ml sample).

TABLE 1. Summary of the survival rates[a] of CHSE-214 cells inoculated with plant samples and/or IPNV.

	Line no.	Concentration of the leaf extract (mg/ml)							
		20.00	10.00	5.00	2.50	1.25	0.63	0.31	0.00
Rice	R5	0.80	0.80	0.71	0.59	0.49	0.43	0.44	0.38
	R9	0.81	0.87	0.92	0.72	0.52	0.42	0.36	0.30
	Control	0.38	0.47	0.40	0.41	0.38	0.36	0.39	0.37
Potato	P33	NT	NT	(0.81)	(0.72)	(0.83)	0.58	0.39	NT
	P34	NT	NT	(1.04)	(1.48)	(0.70)	0.62	0.38	NT
	P40	NT	NT	(1.45)	(0.95)	(0.77)	0.95	0.56	0.32
	P76	NT	NT	(1.02)	(0.96)	(0.74)	0.74	0.49	0.28
	Control	NT	NT	(0.97)	(1.13)	(0.93)	0.37	0.30	0.29

[a] Survival rates were calculated by dividing the absorbances of virus-inoculated cells by those of mock-inoculated cells. The absorbances were indicated in Fig. 3. Numbers in parentheses are not considered as reliable data because the potato samples were obviously toxic to the cells at those concentrations. NT, not tested.

The antiviral effects of R5 and R9 rice samples were similar to those of the non-transgenic samples when the samples were diluted to 0.31 mg/ml. The non-transgenic rice sample showed similar effects at all concentrations tested. When the cells were not inoculated with IPNV, the numbers of cells were similar in any treatments of the samples. Their antiviral effects persisted to some extent even though the samples were diluted to 0.63 mg/ml. Similarly, extracts from P33, P34, P40 and P76 transgenic potato plants showed antiviral effects (Fig. 3B and Table 1). Since these potato samples were obviously toxic to the cells when the cells were treated with the samples at 1.25–5 mg/ml (Fig. 3B and Table 1), the survival rates in these treatments were excluded from consideration.

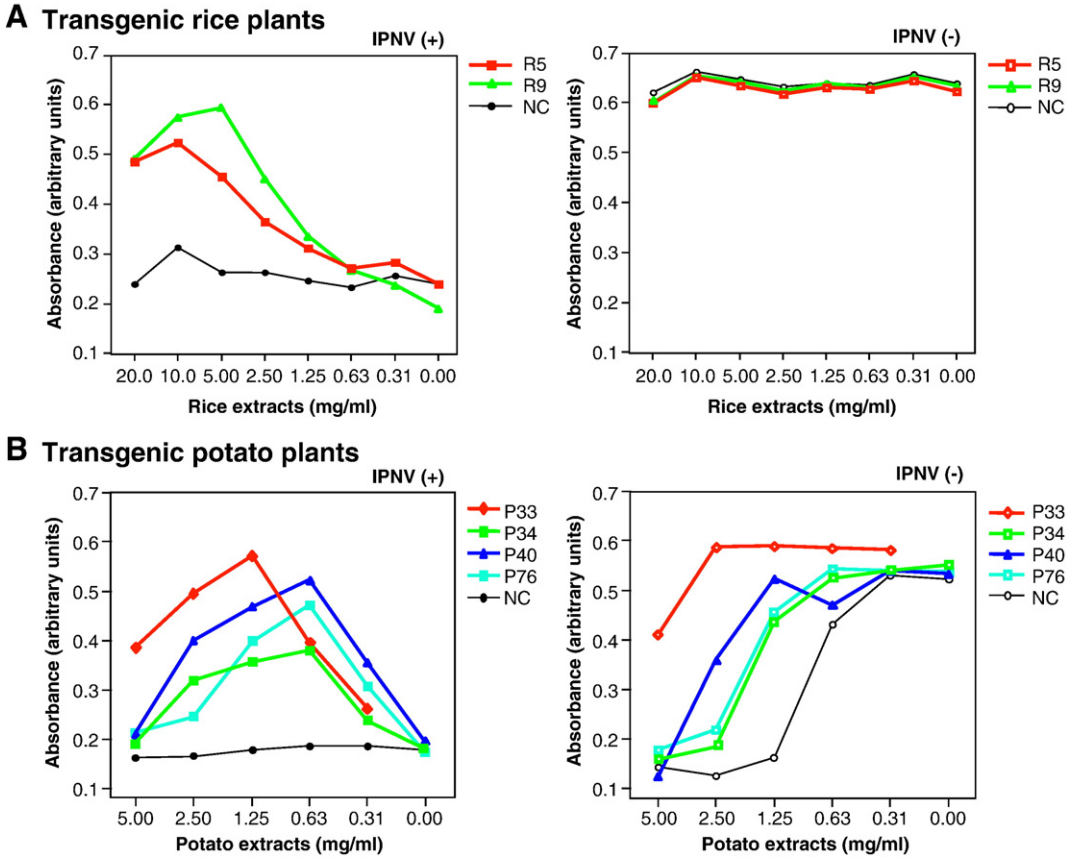

FIG. 3. Antiviral effects of the extracts from SasaIFN-α1-expressing plants. (A) CHSE-214 cells were pre-treated with extracts from transgenic rice (lines R5 and R9) leaves expressing SasaIFN-α1. Cells were then inoculated with infectious pancreatic necrosis virus (IPNV) at a multiplicity of infection (MOI) of 0.01 (left panel) or mock-inoculated (right panel). The antiviral effects of the samples were determined using a crystal violet assay. NC: non-transgenic plants. (B) CHSE-214 cells were pre-treated with extracts from transgenic potato (lines P33, P34, P40 and P76) leaves expressing SasaIFN-α1. Cells were then inoculated with infectious pancreatic necrosis virus (IPNV) at a multiplicity of infection (MOI) of 0.01 (left panel) or mock-inoculated (right panel). The antiviral effects of the samples were determined using a crystal violet assay. NC: non-transgenic plants.

TABLE 2. Antiviral activity of transgenic potato and rice plants expressing SasaIFN-α1 against IPNV.

Plant	Line no.	Antiviral activity (U/mg)[a]
Potato	P33	2.68
	P34	3.20
	P40	5.40
	P76	4.34
Rice	R5	0.43
	R9	0.82

[a] One unit of IFN is designated as the activity which gives 50% reduction of virally infected CHSE-214 cells compared with that of the controls. The units were calculated to identify the concentrations of the samples which fit the following formula using the absorbance data shown in Fig. 3. (TVirus − CVirus) / (TMock − CVirus) = 0.5, TVirus; absorbance of the cells inoculated with a transgenic sample and IPNV, CVirus; absorvance of the cells inoculated with a non-transgenic sample and IPNV, TMock; absorvance of the cells inoculated with a transgenic sample only.

Antiviral effects were obtained to some extent even though the potato samples had been diluted to 0.31 mg/ml. Compared with the rice samples, the potato samples had strong antiviral activity on the basis of fresh weight (Fig. 3B and Table 2). Antiviral activity of the potato samples was also indicated by their inhibitory effects on CPE development (Fig. 4).

DISCUSSION

The antiviral activity of IFNs has marked host specificity (17). On the other hand, oral administration of a low dose of human IFN-α to carps significantly increased phagocytic activity in the kidney and also displayed a significant upregulation in cytokine gene expression (39). However, they did not demonstrate the antiviral activity. We tested a high concentration of human IFNs (up to 8333 IU/ml) for antiviral activity to fish cells but they did not demonstrate a significantly difference in survival rates between IFNs-treated fish cells and non-treated fish cells (data not shown). Therefore, it is important to use fish IFN molecules for immunotherapy of fish.

We successfully expressed SasaIFN-α1 cDNA in rice and potato plants using an *A. tumefaciens*-mediated transformation method. We chose SasaIFN-α1 as the material because some studies have shown that SasaIFN-α1, an rSasaIFN-α1 subtype, has antiviral activity (6, 7). The crude extracts from the IFN-expressing plant tissues were found to have antiviral activity based on an in vitro assay using cultured fish cells. This is the first study describing the production of physiologically active fish IFN using transgenic plants.

Of 190 antibiotic-resistant potato plants obtained in this study, 150 plants carried SasaIFN-α1 cDNA (transgenic plants). In addition, 86 of 115 transgenic plants produced SasaIFN-α1 transcripts. Similarly, 10 of 11 antibiotic-resistant rice plants were positive for SasaIFN-α1 cDNA and also produced SasaIFN-α1 transcripts. These high transformation and transcription efficiencies suggest that SasaIFN-α1 is harmless to plant cells. This possibility is supported by the fact that no abnormal phenotypes were detected in the SasaIFN-α1-expressing rice and potato plants. These results encourage us to increase IFN contents in transgenic cells using a strong promoter.

In this study, when CHSE-214 cells were exposed to high concentrations of the potato leaf extracts, the inoculated cells soon died irrespective of a virus infection. The same phenomenon was reported in human amnion WISH cells and the toxicity of the samples could be removed by dialysis (21).

The antiviral activity of the potato extracts was higher than that of the rice samples based on the amounts of samples used for treatments (Table 2). It is difficult to determine the exact reason for these differences. However, one possible explanation is that recombinant SasaIFN-α1 was not extracted efficiently from the rice leaves because of the hardness of the rice leaf cell wall. Another possibility is that the CaMV 35S promoter worked more effectively in potato than in rice. The latter possibility is supported by our

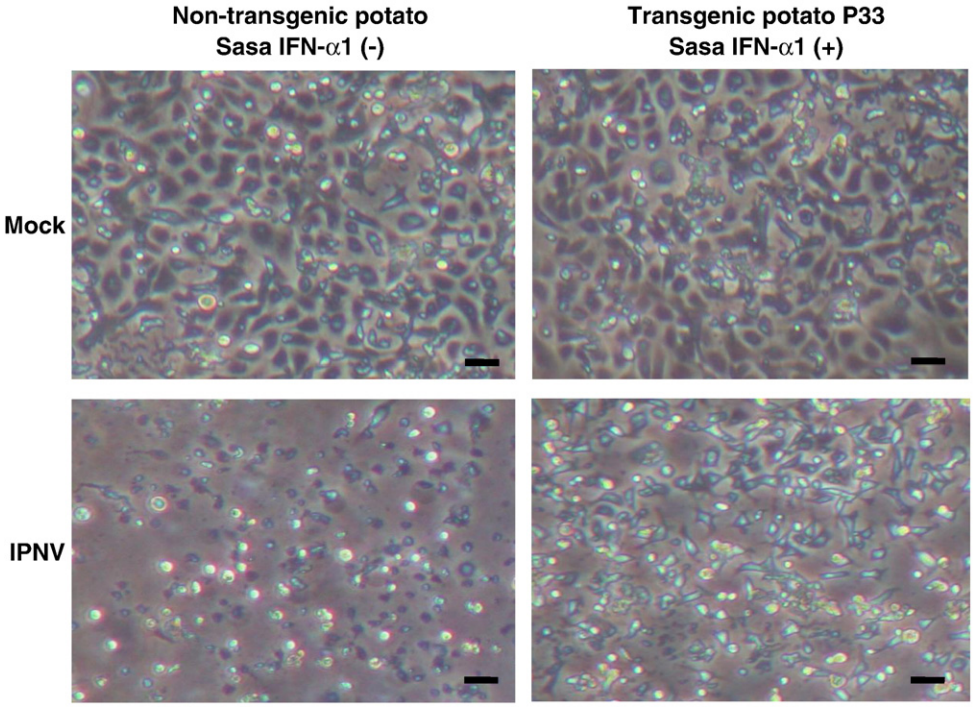

FIG. 4. Effect of SasaIFN-α1-expressing potato extracts on CPE development. CHSE-214 cells were pre-treated for 24 h with extracts from transgenic (P33) or non-transgenic potato leaves and then inoculated with IPNV. CPE suppression denotes the decreased level of virus multiplication. The data of P33 is shown here as a representative result. The scale bars represent 50 μm.

Northern and Western blotting data, which showed that the transgenic potato produced more SasaIFN-α1 transcripts than the transgenic rice (Fig. 2B).

The SasaIFN-α1 protein was barely detectable in the insoluble fractions and was not found in the soluble fractions from the transgenic plants. These expression levels seemed to be significantly lower than those mentioned in previous reports: tobacco expressing human IFN-β cDNA produced 170 ng hIFN-β/g of fresh leaf (23); cultured rice cells transiently expressing human IFN-γ produced 700 ng hIFN-γ/g of fresh weight (26). Because the SasaIFN-α1 protein expressed in *Escherichia coli* was also detected in the insoluble fractions (30), SasaIFN-α1 may have a tendency to form an insoluble complex. In this work, the antiviral activity was observed in the soluble protein fraction but immunoblot analysis revealed extremely low levels of expression of SasaIFN-α1 protein in the soluble protein fraction of transgenic plants (Fig. 2C and D). In any event, the additional work for expressing more SasaIFN-α1 as a soluble protein in plants is required.

In plant cells, proteins are commonly modified by adding oligo-mannose-type sugar chains at the N-termini. In mammals, many glycoproteins are mannosylated and further sialylated at the N-termini, which is important for stabilization and maintenance of glycoproteins in vivo (40). A series of functional recombinant IFNs produced in plant, mammalian and bacterial cells discussed earlier indicate that such N-terminal modifications have no deleterious effects on IFN activity when orally administered to mammals or their cultured cells. Recombinant fish IFNs also exhibited antiviral activity for cultured fish cells irrespective of the N-terminal modifications (30, this study). However, it remains to be addressed whether such N-terminal modifications of fish IFNs will also have no significant effects on IFN activity when fish IFN is administrated orally.

For producing a functional protein for animals, choosing an appropriate expression system is important for reducing production costs. Because functional protein production using plants has many advantages as mentioned earlier, such systems have been recently spotlighted as “molecular farming under sunlight” (41, 42). One of these advantages is that a cold chain is not required for the storage and transportation of transgenic cells when plant storage organs are used to express target proteins. Our transgenic potato and rice plants expressed SasaIFN-α in their storage organs, tubers and seeds, respectively (data not shown), as well as in their leaves. Although IFN activity of the samples from the transgenic potato tubers and rice seeds were not examined in this study, these organs should be applicable for the commercial production of fish IFN as a component of a functional feed.

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