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Transient expression of chicken alpha interferon gene in lettuce*

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Abstract: We investigated the possibility of producing chicken alpha interferon (ChIFN-α) in transgenic plants. The cDNA encoding ChIFN-α was introduced into lettuce (*Lactuca sativa* L.) plants by using an agro-infiltration transient expression system. The *ChIFN-α* gene was correctly transcribed and translated in the lettuce plants according to RT-PCR and ELISA assays. Recombinant protein exhibited antiviral activity in vitro by inhibition of vesicular stomatitis virus (VSV) replication on chicken embryonic fibroblast (CEF). The results demonstrate that biologically active avian cytokine with potential pharmaceutical applications could be expressed in transgenic lettuce plants and that it is possible to generate interferon protein in forage plants for preventing infectious diseases of poultry.

Key words: Chicken alpha interferon (ChIFN-α), Expression, Transgenic lettuce, Bioactivity
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INTRODUCTION

Alpha interferon (IFN-α) possesses powerful and wide-range antiviral properties (Schultz *et al*., 1995a; Ruttanapumma *et al*., 2005) by regulating the pathogenesis, virulence and transmission of virus (Kochs *et al*., 2007; Cauthen *et al*., 2007). In response to virus, the expressions of interferon regulatory factor 3 target genes (IRF3), IFN-stimulated genes, alpha IFNs and IFN-dependent antiviral gene establish the antiviral state (Fredericksen *et al*., 2008). Chicken alpha interferon (ChIFN-α) plays an important role in chicken's defense against viral infection and the treatment of viral infection such as avian influenza virus (AIV) (Xia *et al*., 2004; Wei *et al*., 2006), new castle disease virus (NDV) (Marcus *et al*., 1999), infectious bursal disease virus (IBDV) (Mo *et al*., 2001), infectious bronchitis virus (IBV) (Pei *et al*., 2001), Marek's disease virus (MDV) (Jarosinski *et al*., 2001) and Rous sarcoma virus (RSV) (Plachý *et al*., 1999). Many viruses have evolved diverse mechanisms to combat the host defense mounted by IFNs (Ahmed *et al*., 2003). For example, influenza virus with a full-length nonstructural protein NS1 can antagonize the induction of interferon protein and cause disease and death in chickens (Zhu *et al*., 2008). Therefore, enhancing interferon-inducing capacity and increasing interferon protein levels in chicken may help to ameliorate virus pathogenicity and improve immunity of birds.

It is important to produce alternative and desired broad-spectrum antiviral substance that is natural, effective, convenient and with low costs in poultry. Many viruses are threatening the health and welfare of poultry and have even struck the poultry industry and brought tremendous economic losses to the world, such as the case of the highly pathogenic avian influenza (HPAI) in recent years. At present, vaccina-

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tion is the great asset for outbreak containment of avian disease (Habib *et al*., 2006; Rajput *et al*., 2007). However, the intensive use of vaccines is driving virus to increase its virulence such as the case of the MDV with greater virulence in vaccinated chickens (Witter, 1997). Vaccine use is also restricted by some factors such as cryopreservation and injection technique which is difficult in immunity of migratory birds that are known to act as vectors in the dispersal of AIVs (Jourdain *et al*., 2007). In addition, problems of antibiotic and drug residues in animal-derived food are a great concern of consumers. Therefore, it is wise to use naturally produced IFN in poultry industry. ChIFN-α has shown powerful antiviral activity. Nevertheless, the cost of IFN production with fermentation method or extraction from animal tissue cell is too high to be commonly used in poultry industry. Therefore, a more economic approach for IFN is needed. IFN protein from transgenic plant is obviously an attractive alternative to meet above requests.

Since 1994 (Sekellick *et al*., 1994), the genes encoding ChIFN-α have been successfully expressed in *Escherichia coli* (Schultz *et al*., 1995b; Wei *et al*., 2006), COS cells (Schultz *et al*., 1995b) and baculovirus (Ruttanapumma *et al*., 2005) but not in plants. Plants can supply a better protein secretion and low risk of contamination by animal viruses or bacterial endotoxins (Denecke *et al*., 1995; Boston *et al*., 1996; Ohya *et al*., 2001). They are also the most economical production system of recombinant functional proteins (Khoudi *et al*., 1999; Vaquero *et al*., 1999). Human alpha/beta interferon (*HuIFN-α/β*) genes have been studied in cucumber, squash, lettuce and rice plants, and considerable evidence has accumulated to indicate that *HuIFN-α/β* can be correctly expressed in plant cells and exhibit apparent interferon activity by resistance viral infection (Zhu *et al*., 1994; Arazi *et al*., 2001; Sawahel, 2002; Ohya *et al*., 2005; Li *et al*., 2007). Nonetheless, the feasibility of animal interferon expression has not, to our knowledge, been evaluated in plants. Poultry IFN immune system is an innovative field for antagonizing viruses and ameliorating animal health by direct oral feeding with plant feedstuff.

We investigated *ChIFN-α* gene expression and bioactivity of recombinant protein in transgenic lettuce plants, although the expression level was low. Here we present the transformation and expression analysis of *ChIFN-α* by β-glucuronidase (GUS) staining, RT-PCR, ELISA and cytopathic effect (CPE) inhibition assay.

MATERIALS AND METHODS

Transformation vector and cultivation of *Agrobacterium tumefaciens*

IFN cDNA (GenBank accession No. U07868) was amplified from plasmid pBSK-BNANSI with a *ChIFN-α* gene from chemical synthesis (Generay Ltd., Shanghai, China) by using the following primers: forward primer 5′-GTT CTA GAA TGG CTG TTC CAG CTT CTC-3′, and reverse primer 5′-GGG GTA CCC TAT TAC TAG GTC CTG GTG-3′. *ChIFN-α* gene obtained was cloned into plasmid pSH to make the plant expression vector pSFIFN-α and transfered to *Agrobacterium tumefaciens* strain EHA105. The resulted vector comprises cauliflower mosaic virus 35S promoter, the selectable NPT gene and report GUS gene, *ChIFN-α* gene (*IFNR*), *Brassica napus* napin signal peptide (*tp*) (Crouch *et al*., 1983) and NOS terminator (Fig.1). Recombinant *Agrobacterium* was maintained in a modified liquid yeast extract and beef (YEB) (Negrouk *et al*., 2005) with 100 mg/L kanamycin sulfate (Amresco, USA) and 20 mg/L rifampicin (Sigma, USA). *Tumefaciens* was grown to log phase and condensed to an OD_{600} (optical density at 600 nm) of about 2.1 for transformation of vacuum infiltration.

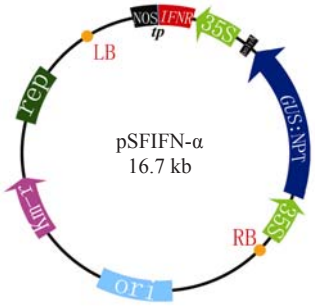

Fig.1 Recombinant plasmid for expression of *ChIFN-α* in plants

Transformation of lettuce plants

Lettuce plants of the same age were harvested, rinsed with distilled water, and drained for 8 h at room temperature before vacuum infiltration. The specific

transformation of lettuce was performed according to the described method by Negrouk *et al*.(2005) except that 0.065 MPa of vacuum degree was used in transformation. After infiltration, lettuce samples were cultivated for 72 h (16-h light/8-h dark photoperiod) at 22 °C (Vaquero *et al*., 1999).

Histochemical analysis of GUS

The lettuce leaves of 72 h culture were incubated with GUS assay staining solution containing the following components: 50 mmol/L NaCl, 100 mmol/L Tris-HCl (pH 7.5), 2 mmol/L potassium ferricyanide, 1 mmol/L X-gluc (Gold Biotechnology, USA) and 20% (v/v) methanol in dark at 37 °C for 3 h, and then dehydrated by 90% (v/v) ethanol.

Expression detection of *ChIFN-α* gene by RT-PCR

Total RNA from the transformed lettuce leaves was used in reverse transcription and polymerase chain reaction (RT-PCR) analysis with a one-step RNA PCR kit (TaKaRa, Dalian, China) by the primers as described above.

ELISA assay of ChIFN-α in lettuce leaves

Soluble protein was extracted for ELISA analysis by using 3 ml of extraction buffer (100 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA, 50 mmol/L ascorbic acid, 10.0% (v/v) glycerol, 10.0 mmol/L β-mercaptoethanol and 0.5% (w/v) SDS) per gram of leaf material. The IFN expressed was measured using a ChIFN-α ELISA kit (RapidBioLab, California, USA) following the manufacturer's instruction. Experiments of standard curve and every sample were all repeated thrice.

Chicken embryonic fibroblast IFN-α assay

Total soluble protein was extracted from the transgenic lettuce by using ammonium sulphate precipitation method (100% saturation) and lyophilized in a freeze dryer (Eyela, Tokyo, Japan). Biological activity of ChIFN-α expressed was measured for lethality to vesicular stomatitis virus (VSV) by using a CPE inhibition assay (Schultz *et al*., 1995b). Chicken embryonic fibroblasts (CEFs) from 9-day-old chicken embryos were treated with IFN preparations from transgenic lettuce, then challenged with VSV at a 100 times 50% tissue culture infectious dose (100 $TCID_{50}$) after overnight culture, and continued to grow until destruction of the untreated virus-infected cells was apparent (at 48 h post-infection). The IFN titer in arbitrary units per ml (AU/ml) was expressed as a reciprocal of the dilution giving the monolayer 50% virus protection. The CPE inhibition assay was repeated thrice.

RESULTS AND DISCUSSION

Lettuce plants of infiltration with expression vector pSFIFN-α were positively transformed in the histochemical staining reaction for GUS gene expression. *ChIFN-α* in the lettuce had marked mRNA expression, and there was a specific fragment near 686 bp indicating the expected gene by RT-PCR amplification (Fig.2). The recombinant protein was found to neutralize the chook IFN-α antibody in sandwich ELISA, and the expression level was estimated to be 0.393 μg protein/kg tissue or 0.0004% of the total soluble protein according to the standard curve:

$$Y=0.0010+0.0013X,\ R^2=0.9913,$$

where, Y and X represent OD_{450} reading and concentration of expression protein, respectively. Antiviral activity of ChIFN-α expressed by transgenic lettuce was calculated to be about 8.2×10^2 AU/mg total proteins on CEF.

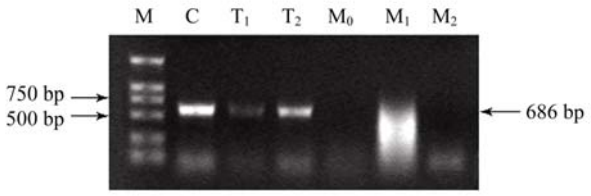

Fig.2 RT-PCR analysis of the expression of *ChIFN-α* mRNA in infiltrated lettuce by ethidium bromide-stained agarose gel analysis

M: Marker; C: Plasmid; T_1, T_2: Different transformed lettuce plants with pSFIFN-α; M_0: Water; M_1: RNA of transgenic lettuce with no reverse-transcriptase; M_2: Non-transgenic lettuce plants

In this study, the antiviral ChIFN-α was first introduced into plants by a transient expression system that offers a rapid testing of the exogenous gene con-

struction and the recombinant protein expression within a week before generating stably transformed plants (Negrouk *et al*., 2005). The IFN expression was in good agreement with the experimental results of *HuIFN* gene in transgenic plants from others (Ohya *et al*., 2001; Sawahel, 2002; Li *et al*., 2007). However, the amount of IFN expressed in this system may be not sufficient for a plant bioreactor or an oral vaccine; therefore, further studies are needed to develop a more robust system.

To determine the IFN activity expressed by plants, the CEFs treated with ChIFN-α from transgenic lettuce were challenged by VSV. The results show that recombinant ChIFN-α could induce antiviral activity in CEFs, and that the maximum level of IFN activity was about 8.2×10^2 AU/mg total soluble protein, demonstrating a higher interlaboratory agreement on the expression of *HuIFN-α/β* gene in plant cells (Ohya *et al*., 2001; Li *et al*., 2007). However, the bioactivity of recombinant IFN from transgenic forage plants in animals needs to be further studied.

CONCLUSION

In the present study, *ChIFN-α* was correctly transcribed and expressed in lettuce plants, and the recombinant IFN obtained was active for conferring protection against VSV infection. These findings can be valuable for prevention of infectious diseases in poultry by agricultural feed with avian cytokines.

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