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Expression of Attacin-like Antibacterial Protein NUECIN in *Pleurotus ostreatus*

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ABSTRACT: *Pleurotus ostreatus*, the oyster mushroom, is one of the most important edible mushrooms. It is especially susceptible to bacterial blotch disease, which is caused by *Pseudomonas tolaasii*. In order to develop bacterial blotch disease-resistant transgenic mushroom, *NUECIN* cDNA, a gene for an antibacterial peptide cloned from *Bombyx mori*, was overexpressed in *Pleurotus ostreatus*. *NUECIN* cDNA was fused to the *β-TUBULIN* promoter of oyster mushroom and co-transformed with the pTRura3-2 vector into the uracil auxotrophic mutant strain. Twelve transformants containing the NUECIN gene were identified by genomic PCR and Southern blot analysis. *NUECIN* gene expression was confirmed by Northern blot analysis. Three transformants showed the transcriptional expression of the gene. However, we could not detect expression of the protein in the transformants. This study showed the possibility of transgenic mushroom development for disease resistance.

KEYWORDS : *Pleurotus ostreatus*, NUECIN, transgenic mushroom

Introduction

Bacterial blotch disease caused by *Pseudomonas tolaasii* is one of the most important bacterial diseases in oyster mushroom. Many studies have been carried out on the prevention and control of the disease in the environmental and agrochemical aspects. Diverse agrochemicals or cultivation methods have been used to control the bacterial attacks to oyster mushrooms and prevent this disease (Healey et al., 1989; Wong et al., 1979). Conventional breeding is also considered as a useful method for preventing and controlling the disease by the development of new oyster mushroom cultivars with enhanced resistance to the bacterial disease. However, this approach has not yet developed the resistant cultivars.

Recently, the transformation systems for oyster mushroom have become available (Joh et al., 2003; Kim et al., 1999; Honda et al., 2000). Hence, producing a transgenic oyster mushroom has emerged as another way to develop bacterial disease-resistant oyster mushroom. Several researches have reported on the development of bacterial disease-resistant genetically modified (GM) plants (Osusky et al., 2000; Sharma et al., 2000). Oyster mushroom that contains antibacterial proteins of non-oyster mushroom origin can also be a possible way to develop a bacterial disease-resistant strain.

ATTACINs are potent antibacterial proteins in the immune hemolymph of *Hyalophora cecropia*. With the molecular weight ranging from 20 to 23 kDa, four basic and two acidic attacins have been reported. ATTACIN inhibits the synthesis of bacterial outer membrane and activates the antibacterial activity of cecropin and lysozyme. NUECIN is a family of ATTACIN identified from *Bombyx mori*. It shows antibacterial activity for both gram-negative and gram-positive bacteria. NUECIN expressed in *Escherichia coli* has antibacterial activity against *P. tolaasii*, which is a causative bacteria of bacterial blotch disease of oyster mushroom (Yun et al., 1997).

In this work, we tried to express NUECIN in *P. ostreatus* and test the possibility of developing bacterial disease-resistant transgenic oyster mushroom. Our experiments showed the possibility of developing transgenic mushroom for strain improvement.

Materials and methods

Strains and plasmids

The uracil auxotrophic mutant strain ASI2029-8 was used as the host. The pTRura3-2 vector was obtained

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from Dr. Christian Barreau (Berges and Barreau, 1991, Kim et al., 1999). The *NUECIN c*DNA clone was obtained from the National Academy of Agricultural Sciences in Korea (Yun et al., 1997).

Vector constructs

The *β-TUBULIN* promoter of *P. ostreatus* was reported by Kim et al. in 2001. It was subcloned into the pBluescript KS(+) vector digested by *Xba*I and *EcoR*I following PCR amplification using T7 (5'-GTAATACG ACTCACTATAGGGC-3') and Tubeco (5'-cgcgaattctacg gtagaaag-3') primers, which have *Xba*I and *Eco*RI sites. This resultant vector was named as pTubpro. The NUECIN cDNA gene digested by *Eco*RI and *Xho*I restriction enzymes was eluted. After digestion of the pTubpro vector with *Eco*RI and *Xho*I restriction enzymes, the pTubpro and the eluted *NUECIN* cDNA were ligated and transformed into the *E.coli* strain XL1-Blue MRF'. This recombinant DNA was called pTubnuecin.

Transformation

The mycelia were incubated in MCM (mushroom complete media) broth supplemented with uridine shaken with glass beads twice a day for 6 days. The mycelia were harvested from the liquid cultures by filtration. After being washed in 0.6 M sucrose, the mycelia were suspended in 10 ml lysis buffer, 2 mg ml^{-1} cellulase "ONOZUKA" RS (Yakult Pharmaceutical), 0.5 mg ml^{-1} zymolyase (Kirin Brewery), 0.4 mg ml^{-1} chitinase (Calbiochem-Behring), and 3 $\mu\ell$ ml^{-1} glucuronidase (Sigma), and gently shaken at 25℃ for 3 hrs. The protoplasts were filtered through a glass filter (G3), centrifuged at 2,000g, and suspended in 10 ml of STC (0.6 M sucrose, 10 mM Tris-HCl, pH 7.5, and 10 mM CaCl2). The protoplasts were then centrifuged again at 2,000g and suspended in STC to a concentration of 5 x 10^7 protoplasts ml^{-1} (Kim et al., 1997).

For the transformation, 5 μg of plasmid DNA and 50 $\mu\ell$ of PTC (60% PEG 3350, 10 mM Tris-HCl, pH 7.5, and 10 mM $CaCl_2$) were added to 200 $\mu\ell$ of the protoplast suspension. The mixture was kept on ice for 20 minutes. A further 600 $\mu\ell$ of PTC was then added to the mixture, followed by incubation for 20 minutes at room temperature. The protoplasts were directly plated on MCM media containing 0.6 M sucrose to allow them to regenerate (Joh et al., 2003).

Genomic DNA isolation and Southern blot analysis

Genomic DNA was prepared by a modification of the simplified method described by Graham (1994). The mycelia in MCM plates were harvested and freeze-dried. The freeze-dried mycelia were then ground to powder using a toothpick in a 1.5 ml Eppendorf tube, and 500 ul of extraction buffer (2% CTAB, 100 mM Tris-HCl, pH 7.4, 1.4 M NaCl, and 20 mM EDTA) was added. The mixture was incubated at 60℃ for 1 hour. After centrifugation at 15,000 rpm for 10 min, the supernatant was mixed well and extracted with phenol/$CHCl_3$/isoamyl alcohol. One-tenth volume of 3 M CH_3COOH (pH5.2) was added to the aqueous phase, and nucleic acid was precipitated with two volumes of absolute alcohol.

For Southern blot analysis, 5 μg genomic DNA was digested with restriction enzymes. The amplified product of *NUECIN* cDNA was labeled with α-^{32}P-dCTP and hybridized at 65℃. The membrane was exposed to X-ray films.

Northern blot analysis of transformants

Total RNAs were isolated from the mycelia of transformants by a TRI reagent according to the manufacturer's instructions. The total RNA was then transferred to a nylon membrane, and the amplified product of *NUECIN* cDNA was labeled with α-^{32}P-dCTP. The membrane was hybridized and washed at 42℃ using the Northern Max™ kit (Ambion Co.). Following the washing step, the membrane was exposed to X-ray films (Agfa CP-BU) at -70℃ .

Western blot analysis of transformants

The total protein of transformants was subjected to electrophoresis in 15% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was prehybridized in skim milk for 1 hour, hybridized using 1/3,000 diluted nuecin antibody, and washed in TBS buffer (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl). Secondary antibodies were hybridized and washed, and then the membrane was soaked in luminol, H_2O_2, and p-iodophenol solution. This membrane was exposed to X-ray films.

Results

Isolation of transformants co-transformed with pTubnuecin and pTRura3-2

In fungi, the cotransformation efficieny is relatively higher than other eukaryotes. In basidiomycetes such as *Coprinus cinerius*, cotransformation method has been used. We want to use the promoter derived from *Pleurotus* but we don't have the vector which has marker and cloning cassette containing the proper promoter and terminator. So, we tried the cotransformation using the vector for selection of transformation and vector for gene expression.

After co-transformation using pTubnuecin and pTRura3-2, a total of 52 colonies were isolated on minimal media in the first screening. These showed that the pTRura3-2 vector was inserted into the chromosome of *Pleurotus ostreatus*. Among these, pTubnuecin transformants were isolated by PCR using Nuecinfr and Nuecinre primers. Only 12 strains out of the 52 transformants in which the pTRura 3-2 vector was integrated into the chromosome were amplified by Nuecinfr and Nuecinre primers (Fig. 1). These showed that 12 strains were co-transformed by both pTRura3-2 and pTubnuecin. The co-transformation efficiency was about 23% according to these results. In order to analyze the chromosome integration pattern of *NUECIN cDNA*, Southern blot analysis was performed (Fig. 2). Most of the transformants showed multiple band integration, but three transformants #16, #56, #59 might have single-copy integration.

NUECIN cDNA was transcriptionally expressed in transformants

Expression of the heterologous gene NUECIN was confirmed by Northern blot analysis. Total RNAs were isolated from the five transformants containing pTubnuecin. In preliminary experiments, only three transformants showed a band in Northern blot analysis. These three transformants were analyzed further. By Northern blot analysis, two transformants, strains 40 and 46, showed strong expression of *NUECIN cDNA*. Transformant strain 27 showed a faint band in Northern blot analysis (Fig. 3).

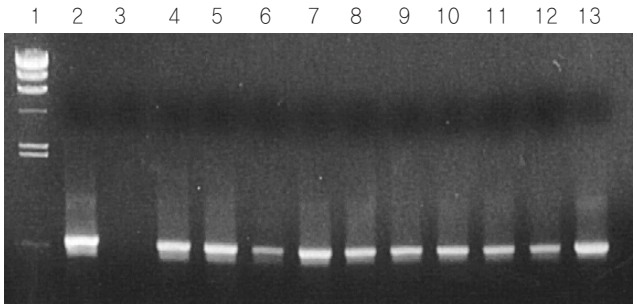

Fig. 1. PCR amplification of transformants containing the *NUECIN* cDNA. Lanes 1 Marker, Lane 2: positive control (pTubnuecin), Lane 3: negative control, Lanes 4-13: transformants nuet10, 14, 17, 22, 27, 30, 40, 47, and 56.

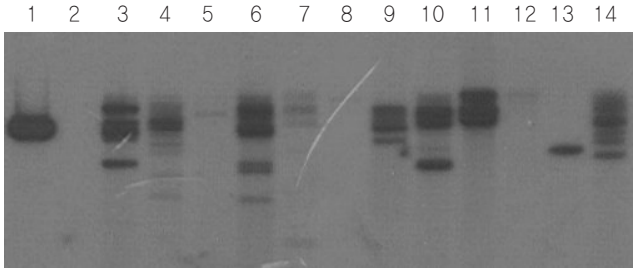

Fig. 2. Southern blot analysis of the transformants *EcoR*I-digested DNA hybridized with *NUECIN* cDNA amplified with PCR. Lane 1: positive control (*EcoR*I-digested pTubNuecin), Lane 2: negative control (u-13 strain), Lanes 3 – 14: transformants (nuet10, 14, 16, 21, 22, 23, 27, 40, 46, 56, 59, and 8).

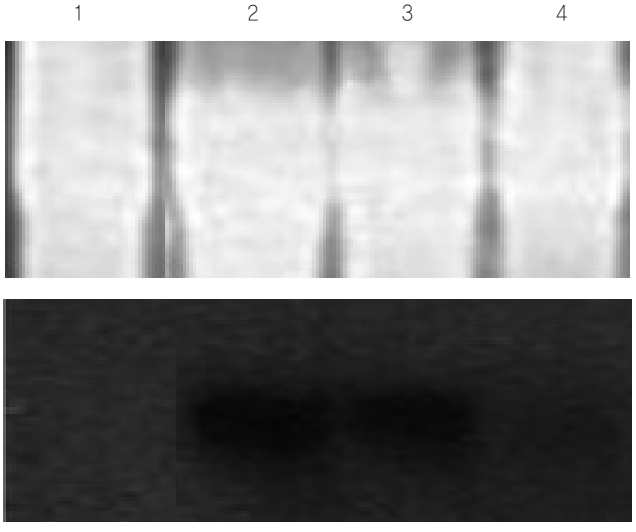

Fig. 3. Northern hybridization analysis of *NUECIN* transformants Total RNA was hybridized with nuecin cDNA labeled with α-^{32}P-dCTP. Lane 1: negative control, Lanes 2 – 4: nuecin transformants (46, 40, and 27).

Transformants did not show antibacterial activity against *P. tolaasii*

To confirm the antibacterial activity of the transformants, two simple experiments were performed. Firstly, the paper disk method was used. Liquid media in which the mycelia of transformants were cultured were added to a paper disk on the media in which *P. tolaasii* was plated. After overnight culture, an inhibition zone is shown if the transformant has antibacterial activity. However, no antibacterial activity was shown by the transformants. Therefore, in the broth media of transformants, antibacterial activity was not shown. Secondly, when *P. tolaasii* was directly spotted on the mycelia of transformants, the mycelia were lyzed and showed a red color around the infection area 5 days after the infection. All of the transformants tested did not show any antibacterial activity (data not shown).

NUECIN protein was not detected in the transformants

To confirm the expression of NUECIN protein at the translational level, total protein was isolated from the transformants, and using the NUECIN antibody, Western blot analysis was performed. The signal occurred at about 28kD in the positive control, but it was not shown in the transformants. NUECIN protein might be expressed in very small quantities or not expressed at all based on this Western blot analysis (Fig. 4).

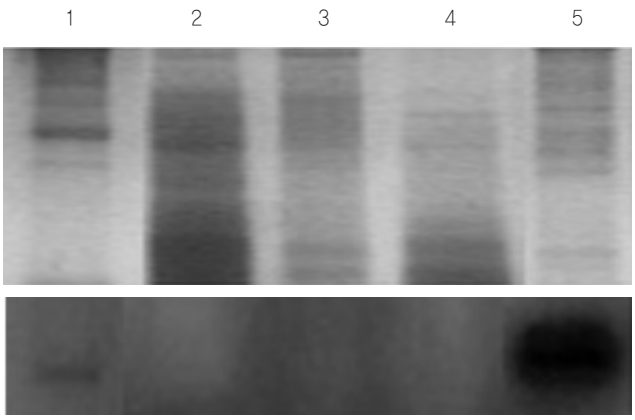

Fig. 4. Western blot analysis of transformants that expressed the *NUECIN* gene in *P. ostreatus* Lane 1: positive control (media of *B. mori* cell culture infected with vaculovirus expressing NUECIN), Lanes 2 – 4: transformants (27, 40, and 46), Lane 5: positive control (*B. mori* cell infected with vaculovirus expressing NUECIN).

Discussion

This experiment was performed toward the development of transgenic oyster mushroom that expresses the antibacterial protein of *B. mori*, which could prevent bacterial blotch disease. Many kinds of antibacterial proteins have been cloned from insects and transformed into plant proteins (Mourgues et al., 1998). The ATTACIN E gene cloned from giant silk moth has been introduced into apple plants, and several transgenic plants have shown a reduced susceptibility to *Erwinia amylovora* (Mourgues et al., 1998). We also applied the same strategies to the edible mushroom *P. ostreatus*. In these experiments, we showed the transcriptional expression of the NUECIN gene of *B. mori* using the *β-TUBULIN* promoter of *P. sajor-caju* in *P. ostreatus*. However, translational expression and antibacterial activity were not detected. Northern blot analysis showed that the NUECIN gene was highly expressed at the transcriptional level. However, no antibacterial activity was shown. Transgenic tobacco expressing the *CECROPIN B* gene, an antibacterial protein cloned from giant silk moth, did not show resistance to bacterial disease. This was found in transgenic tobacco expressing the *CECROPIN B* gene as a result of the degradation of *CECROPIN B*y plant proteases (Mourgues et al., 1998). The finding suggests one reason why the transformants could not show any antibacterial activity even if the gene was expressed. NUECIN protein might also be degraded and thus could not show antibacterial activity.

NUECIN is a secretion protein and has a signal peptide that is cleaved during secretion. The exact cleavage site of the signal peptide produces the functional antibacterial protein. This can also explain the absence of antibacterial activity in the transformants. During the translation of the NUECIN transcript or the secretion of the protein, functional NUECIN cannot be synthesized or secreted.

This study used the co-transformation method, which has been shown to be useful in the development of commercial transgenic mushroom because it can eliminate the selection marker and overcome problems related to selection marker safety in a GMO (genetically modified organism).

적 요

느타리버섯은 가장 중요한 식용버섯 중 하나이다. 느타리버섯은 *Pseudomonas tolaasii*에 의한 세균성 갈변병에 매우 감수성이므로, 저항성 품종을 만들기 위한 노력의 하나로 누에에서 분리된 항 세균성 단백질인 누에신을 느타리버섯에서 과발현시키고자 하였다. 누에신 cDNA는 여름 느타리버섯의 β -tubulin 프로모터에 결합되어 pTRura3-2 vector와 함께 우라실 영양요구성 돌연변이 균주에 형질전환되었다. 누에신 cDNA가 형질전환된 느타리버섯을 genomic PCR과 Southern blot을 통하여 분리할 수가 있었으며, 이들 중 3개의 형질전환체가 누에신 유전자를 발현시킴을 확인하였다. 그러나 이들 형질전환체들에서 누에신 단백질을 검출할 수 없었으며, 또한 항 세균 효과도 확인할 수 없었다. 이들 결과는 형질전환기술을 이용한 병 저항성 개발 가능성을 보여주고 있다.

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