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Antifungal Gene (Rs-AFP) Introduction into *Rehmannia glutinosa* and Gene Expression Mediated by *Agrobacterium tumefaciens*

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***Rehmannia glutinosa* is one of the most important medicinal crops in Korea. However, various plant pathogens, including *Fusarium* spp., cause great damage on *R. glutinosa* and result in enormous economic losses. This study was conducted to breed *Fusarium*-resistant plants by using *Agrobacterium tumefaciences* and AFP (anti-fungal protein) gene. The plant material used was a native accession of *R. glutinosa*. The PCR analysis was conducted to verify transgenicity. Based on the PCR analysis, *npt*II band was observed in transgenic plant genome. Southern blot and AFP protein analyses also showed the expression of this gene in transgenic plants. Expression of AFP in transgenic plants offers the possibility of developing resistance to fungal infection.**

Keywords : *Agrobacterium tumefaciens*, *Rehmannia glutinosa*, Radish seed antifungal protein.

Rehmannia glutinosa is a perennial medicinal plant belonging to the family *Scrophulariaceae*, with an estimated 300 species known in the world, especially in the temperate regions. Its root has long been widely used in Korea for medicinal purposes. *R. glutinosa* contains iridoid, catalpol, leonuride, stachyose, sucrose, mannitol, and amino acid (Hasegawa et al., 1982), and is used for hematic, robustness, cardiotonic drug, diabetes treatment, antifebriel, and detoxification purposes (Choi et al., 1995).

R. glutinosa shows weak fertility and mainly proliferates through root branching and rootlet growth. However, roots for proliferation are usually infected with various pathogens during storage, and these infections cause great damages to the roots and impede the intensive farming of the crop. *R. glutinosa* can be infected with various pathogens in the field and shows various symptoms including leaf spot by *Phoma* sp., root rot by *R. solani* and *F. oxysporum*, *Fusarium* wilt by *F. solani*, and *Corticium* rot by *C. rolfsii*. Mosaic symptoms caused by various viruses can also be observed. Hence, it is important to breed disease resistance against these pathogens. In recent years, research works on pathogenesis-related (PR) proteins related with disease resistance have been extensively published (Linthorst, 1991). Various PR-proteins show anti-fungal activities (Hejgaard et al., 1992; Mauch et al., 1988; Niderman et al., 1993; Woloshuk et al., 1991), and these PR-proteins expressed in some transformed plants show disease resistance (De Wit, 1992; Mauch et al., 1988; Woloshuk et al., 1991).

Recently, 5-KD cystein-rich anti-fungal protein (AFP) with new characteristics was identified from radish seeds (Franky et al., 1992). Rs (radish seed)-AFPs showed wide spectrum of anti-fungal activities and they were less sensitive to cations compared with other plant origin anti-fungal proteins. They also showed high specificity against fungi. 2S storage albuminose from radish is known as an anti-microbial protein and its isoforms are believed to inhibit the growth of bacteria and fungi. However, its anti-microbial reactions are different from that of Rs-AFPs in that they are sensitive to cations. In recent years, the induction of Rs-AFPs genes in plants during the germination of radish upon infection was reported (Franky et al., 1992). Also, the role of Rs-AFPs was verified through gene expression of Rs-AFP2 in disease resistance of tobacco (Franky et al., 1992). Therefore, in this study, disease resistance breeding of *R. glutinosa* was performed with selection marker *npt*II and anti-microbial protein gene Rs-AFP1 using *Agrobacterium* as a vector. The gene expression in transformed plantlets was verified through PCR, SDS-PAGE and Southern blot analyses.

Materials and Methods

Plant material. Leaf and stem explants obtained from *R. glutinosa* grown *in vitro* were used for this study. *In vitro* regeneration and growth of the plant materials were done on MS base medium (Murashige and Skoog, 1962) and growth regulators 2,4-D, NAA, and TDZ were used at a concentration of 0.01, 0.1, and 2 mg/l, respectively.

Pathogen. Pathogenic isolates of *Fusarium oxysporum* was isolated

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  1 gtttattagt  gatcatggct aagtttgcgt ccatcatcgc acttcttttt gctgctcttg
 61 ttctttttgc  tgctttcgaa gcaccaacaa tggtggaagc acagaagttg tgcgaaaggc
121 caagtgggac atggtcagga gtctgtggaa acaataacgc atgcaagaat cagtgcatta
181 accttgagaa agcacgacat ggatcttgca actatgtctt cccagctcac aagtgtatct
241 gctactttcc ttgttaattt atcgcaaact ctttggtgaa tagtttttat gtaatttaca
301 caaaataagt cagtgtcact atccatgagt gattttaaga catgtaccag atatgttatg
361 ttggttcggt tatacaaata aagttttatt cacca
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Fig. 1. Nucleotide sequence of Rs-AFP1.

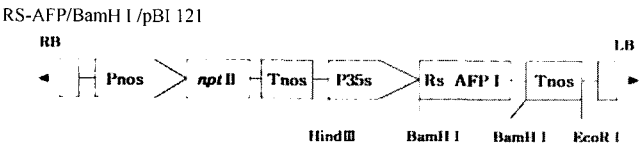

Fig. 2. Diagram of the T-DNA region of pFRG1.
LB, left boder; RB, right boder; Pnos, nopaline synthase promoter; Tnos, nopaline synthase terminator; *npt*II, neomycine phosphotransferase II; p35s, CaMV 35S promoter and Rs-AFPI, coding region of Rs-AFPI.

from roots of infected *R. glutinosa* and their pathogenicities were tested with a method described previously (Singleton et al., 1992).

Expression and transformation vectors. A partial AFP cDNA fragment Rs-AFP1 (U18557) was identified at Dr. HIlde's laboratory in a screen of radish seed specific cDNA library (Terras et al., 1995) Fig. 1 shows that the 395bp nucleotide of Rs-AFP 1 cDNA Rs-AFP1 cDNA clone of Rs-AFPs was obtained through the construction of radish cDNA library at λZAPII. Four nucleotide sequence inserts and six other inserts were determined. All the 400bp cDNA of full-length Rs-AFP1 transcripts contained open reading frame of 240bp. Nucleotide and amino acid sequences were analyzed for one of these pFRG1 clones. Inserts from positive clones were cut with *Eco*RI and the sizes were determined. Coding sequence of Rs-AFP1 (Fig. 2) was cloned between the cauliflower mosaic virus (CaMV) 35S promoter and the CaMV 35S terminator of pFAJ3002. Rs-AFP1 expression cassette with *Hin*dIII fragments was cloned with *Hin*dIII segment of T-DNA of plant transformation vector pBin19Ri, and original chimeric neomycine phosphotransferase II (*npt*II) gene was inserted into T-DNA border of pBin19Ri. Then, pFRG1 was inserted into *Agrobacterium tumefeciens* LBA4404 using eletrophoration (Franky et al., 1995). Transformation vector was cultured at 28°C for 48 hours in 3 ml of LB liquid medium (Bacto-tryptone, 10 g; Bacto-yeast, 5 g; NaCl, 10 g; and 100 mg/Km, pH 7.0) under dark condition until the O.D. value reaches A_{600}=1.0. Culture broth of *Agrobacterium* strain was diluted at 10^{-1} for inoculation.

Transformation of *R. glutinosa*. *In vitro*-cultured *R. glutinosa* was cut into auxiliary buds, stem, and leaf tissues, and cultured for 2-3 days in a pre-treatment MS medium containing 1 mg/l BAP, 2 mg/l TDZ, 0.2 mg/l NAA, MS vitamin, 3% sucrose, and 0.8% agar at pH 5.2. Pre-treated plant materials were washed in sterile distilled water, and in MS liquid medium with 250 mg/l cefataxime, twice and thrice, respectively, to remove the *Agrobacterium*. Washed plant materials were finally blotted with sterile paper towel. Eight washed and blotted plant materials per plate were placed in a MS selection medium supplemented with 2 mg/l TDZ, 1 mg/l BAP, 0.2 mg/l NAA, kanamycin 0, 50, 80, 100, 150, and 200 mg/l, and 500 mg/l cabenicillin. Plant materials were sub-cultured at an interval of 7-14 days. Shoot regeneration was observed at 6-8 weeks after the first sub-culture. Shoots longer than 3 cm were sub-cultured in a MS and 1/2 MS media supplemented with kanamycin for root induction.

PCR analysis. In order to determine whether the Rs-AFP1 gene was introduced, DNA of kanamycin-resistant plantlets was extracted with CTAB (cetyltrimethyl ammonium bromide) method (Murray and Thompson, 1980). Selection marker *npt*II gene was detected with N-1 (5'-GAAGCTATTCG GCTATGAC TG-3') and N-2 (5'-ATCGGGAGC GGCGATACCCTA-3'). PCR reactions were performed with TOUCHDOWNTM (HYBRID), and amplification conditions were: 45 cycles of pre-denature (at 94°C, 5 minutes), denaturation (at 94°C, 1 minute), annealing (at 35°C, 1 minute), and extension (at 72°C, 2 minutes), 40 cycle of post-elongation (at 72°C, 10 minutes), denaturation (at 94°C, 1 minute), annealing (at 60°C, 1 minute), and extension (at 72°C, 1 minute 30 seconds), followed by post-elongation (at 72°C, 5 minutes). Amplified DNA was fractionated on 1.5% agarose gel and stained with EtBr.

Southern blot analysis. Selection marker gene *npt*II in pFRG1 was amplified with PCR and observed through electrophoresis. Marker gene was eluted with Hoffer eluter (Hoffer, USA) and, using DIG-Label method, *npt*II gene was labeled with digoxigenine (DIG)-label. PCR products on 1.5% agarose gel was isolated, vacuum transferred to nylon membrane, and fixed at 80°C for 2 hours. The DNA was incubated for 30 minutes at pre-hybridization solution (5 SSC, 0.1% lauroylsacosine 0.02% SDS, and 1% blocking reagent). DIG-labeled probe was added after the pre-hybridization. Hybridization was performed for 16 hours. After the hybridization, washing was performed twice with 50 ml of 2 SSC, 0.1% SDS for 5 minutes at room temperature, twice with 50 ml of 0.1 SSC, 0.1% SDS solution for 5 minutes at 68°C, and once with washing buffer (0.1M maleic acid, 0.15M NaCl (pH 7.5)+ 3% tween 20). After washing, incubation at 1% blocking reagent for 30 minutes was followed by incubation at anti DIG-AP conjugate for 30 minutes. The remaining antibody was washed with washing buffer and treated with detection buffer (0.1M Tris-HCl, 0.1M NaCl, 50 mM MgCl2, pH 9.5). Finally, the band was detected after the treatment with color solution: nitroblue tetrazolium (NBT) and bromochloroindolyl (BCIP), the reaction at dark conditions for 10-15 minutes.

SDS-PAGE. Roots of AFP transformed *R. glutinosa* were inoculated with *F. oxysporum* for 30 minutes, and the crude protein was isolated according to the methods of Paul and Robert (1999) after symptom appearance was confirmed by the naked eye. Isolation of protein was performed in a cold room at 0-4°C. Leaves were harvested and cooled to 4°C prior to the addition of liquid nitrogen. The powdered leaf tissues were transferred to pre-chilled extraction buffer (100 mM Tris, 100 mM ascorbic acid, 20 mM EDTA, 2.5% w/v polyvinyl polypyrrolidone, 150 mM NaCl, 0.1% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride) at 1 ml per gram of leaves. After vigorous shaking, the extract was centrifuged at 4°C by 10,000 rpm for 10 minutes. The concentration of protein in the supernatant was measured by Bradford

method. Protein was separated by SDS-PAGE minigel in four-fold concentrated sample buffer containing 200 mM Tris-HCl, 1% (w/v) SDS, 1 mM EDTA, 0.005% (w/v) bromophenol blue, and 1% (w/v) dithioerythritol. Silver staining of separated proteins was done using a mixture of EtOH/glacial acetic acid/distilled water (30:10:60) or 12.5% glutaraldehyde as fixative. Diffusion blotting of proteins (on Hybond C super nitrocellulose blotting paper) followed by silver staining of the blots was performed. Precast immobiline Dry stripes (Pharmcia) rehydrated in 8M urea were used to perform isoelectric focusing.

***Fusarium oxysporum* pathogenicity tests for AFP transformed *R. glutinosa*.** For the pathogenicity tests, AFP transformed *R. glutinosa* was inoculated with *F. oxysporum* isolates R-3 and R-10 isolated from field-infected roots of *R. glutinosa* by following previously described test method (Singleton et al., 1992).

Antifungal activity. Antifungal activity was measured by microspectrophotometry. Spores were used to inoculate the cultures, except for *Asperigillus awamori* and *Cladosporium herbarum*, for which the inoculum consisted of mycelial fragments. Tests were performed in spore germination inhibitory test broth (0.2% glucose, 0.1% yeast extract, 0.1% citric acid, 0.37% $Na_2HPO_4 \cdot 12H_2O$, 100 μl) in 96-well microassay plate. Five μg/100 μl of Rs-AFP1 protein extracted from transgenic plants was added in spore germination inhibitory test broth, followed by culture for 24 hours (dark, 27°C).

Results and Discussion

Transformation with *Agrobacterium*. Four to eight weeks old explant tissues were pre-treated for 2 days before they were co-cultured with *Agrobacterium* for 2 days and incubated for 8 weeks followed by selection of regenerated plantlets. Roots were induced after the regenerated plantlets were transferred to MS basal medium supplemented with kanamycin.

It has been reported earlier that pre-treatment does not affect transformation of *Daucus, Nicotiana,* and *Petunia*, and that it enhances transformation rate for *Arabidopsis* and *Datura* (Pawlick et al., 1992). In *R. glutinosa*, pre-treatment seemed to enhance the transformation rate. Two days of co-culture for *R. glutinosa* were found to be acceptable. GUS activities reached the highest point at 6th day for *Kalanchoe laciniata* (Jia et al., 1989), whereas, the transformation rate for carrots (*Daucus carota* L.) reached the highest point between the 2nd and 3rd day and showed declined rate after the 7th day, and the removal of *Agrobacterium* was difficult thereafter (Pawlick et al., 1992). These results indicate that 2 or 3 days of co-culture would be enough for transformation (Jia et al., 1989). In general, kanamycin-resistant line selection takes 4 weeks for various crops. However, *R. glutinosa* took 8 weeks for the selection. Plant materials such as lower abaxial parts, new shoots, and roots were known to be used for the transformation. However, leaf explants, the most widely used materials, having large surface area, absorb nutrients and hormones efficiently, and were less stressed compared with the other materials resulting in higher regeneration rates (Barbier and Dulieu, 1883). Various parts of *R. glutinosa* were used for transformation experiments, and, as a result, auxiliary bud culture resulted in 100% regeneration rate in transformation at each kanamycin concentration. Whereas, the shoot explants culture resulted in failed regeneration, growth retardation, and no root formation. These results indicate that auxiliary buds were not suitable for the selection of kanamycin resistance. PCR analysis also showed that the plantlets which regenerated from the auxiliary buds were not successfully transformed.

There were no changes in the number of calli at each growth stage (data not shown), and the number of new shoots increased 8 weeks after the inoculation and resulted in increased regeneration rate after 8 weeks. Regeneration rate was greater at lower concentrations of kanamycin.

Growth regulators used in this experiment included hormones that directly regenerate the explant tissues. 2,4-D, commonly used for callus induction, can cause morphological and genetic variations in plantlet regenerations, and, thus, was excluded. Two kinds of cytokinins were used for effective regenerations.

PCR analysis of transformed plantlets. In the PCR analysis of transformation with primers F-1 and F-2, no bands were observed both in plantlets regenerated on a medium with 500 mg/l of cabemicillin only, and in control plantlets. Bands, 1 kb in size, were observed in selected

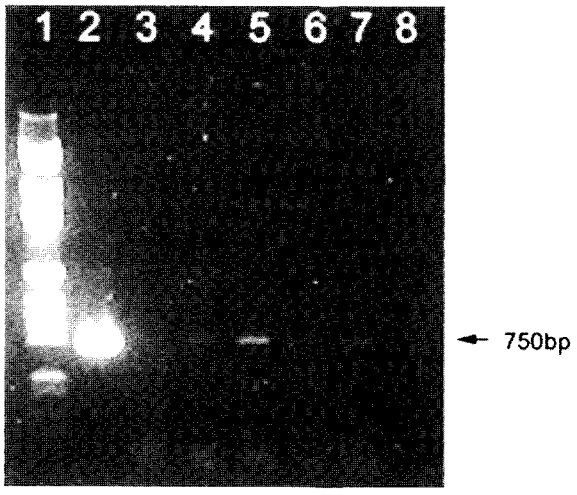

Fig. 3. Detection of *npt*II gene in transgenic plant by using N-1 and N-2 primer.
Lane 1, DNA ladder maker; lane 2, PCR product of *npt*II gene fragment; lane 3, PCR amplification of normal plant (wild type, control); lane 4-7, PCR amplification of transgenic plants selected in medium containing Km 50 mg/l (stem disk); and lane 8, PCR amplification of transgenic plant selected in medium containing Km 10 mg/l (leaf disk).

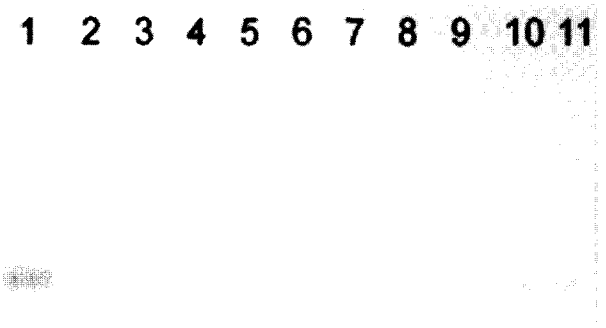

Fig. 4. Southern blot analysis of transgenic plants by *Agrobacterium* using a *npt*II probe.
Lane 1, *npt*II fragment (signal); lane 2, L10-4 transgenic plant; lane 3, L10-2 transgenic plant; lane 4, S10-14 transgenic plant; lane 5, S10-2 transgenic plant; lane 6, S10-1 transgenic plant; lane 7, S10-13 transgenic plant; lane 8, S10-5 transgenic plant; lane 9, L10-1 transgenic plant; lane 10, L30-1 transgenic plant and lane 11, L30-2 transgenic plant.

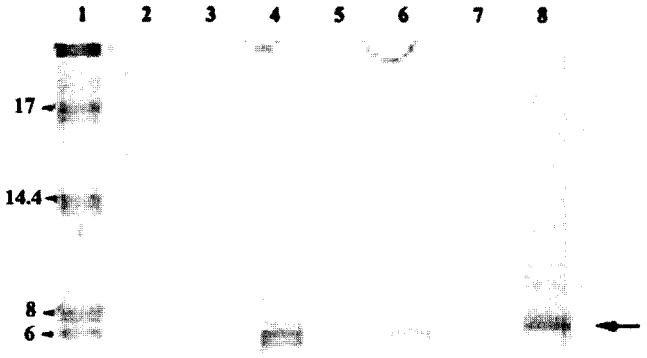

Fig. 5. Comparison of protein pattern against *Fusarium oxysporum* infection through SDS-PAGE in control (non-transgenic) and transgenic (AFP) *Rehmannia glutinosa.*
Lane 1, protein marker; lane 2, non-infected control plant; lanes 3, 5 and 7, infected control plants; and lanes 4, 6 and 8, infected AFP-inserted transgenic plants.

kanamycin. Weak bands were observed in plantlets transformed and regenerated from stem explants, and strong and weak band each was observed in plantlets transformed and regenerated from leaf explants.

In the analysis of transformed plantlets, DNA fragments, 750bp in size, were observed in all the transformed plantlets (Fig. 3). Among the plantlets, only in plantlets regenerated from stem explants and cultured with 30 mg/l and 50 mg/l of kanamycin concentrations where the *npt*II gene was found inserted into the genome. Transformation was also confirmed with plantlets cultured only at 10 mg/l of kanamycin concentration and regenerated from leaf explants.

Based on the results, it can be concluded that 50 mg/l and 10 mg/l of kanamycin concentrations were suitable for stem and leaf explant culture, respectively. In transformation of *R. glutinosa*, stem explant culture on a medium with 50 mg/l of kanamycin would be useful to increase the regeneration rates, and leaf explant culture on a medium with 10 mg/l of kanamycin would be useful to increase the successful transformation rates.

Southern analysis with *npt*II probe confirmed that the targeted gene was introduced into the plant genome (Fig. 4). Some of the transformed plantlet lines selected on the medium supplemented with kanamycin did not show any positive band in PCR and Southern blot. This was possible because, as Socristan and Melchers reported previously (1987), hormones produced from kanamycin-resistant cells caused the neighboring untransformed cells to regenerate into plantlets. As a result of this study, transformation of Rs-AFP gene by means of *Agrobacterium* and transplanting in the field was successfully established in *R. glutinosa*. These transformed *R. glutinosa* plantlets would be tested successfully for their resistance in the field and used for disease resistance breeding program.

Analysis of AFP protein expression in transgenic plants. The AFP protein was detected in leaf tissues of transgenic plants infected with *F. oxysporum*. A single band of 5 kD in SDS-PAGE gel, which corresponded to the AFP protein, was detected in the leaf tissue of transgenic plants as shown in Fig. 5. These results showed that the unreduced RS-AFP yields a single band with apparent molecular mass of 20 kDa, whereas, reduced and S-pyridylethylated derivatives of Rs-AFP migrated as single bands with a molecular mass of approximately 5 kDa. As previously reported (Franky et al., 1992), this study confirmed that Rs-AFP appeared to be an oligomer protein build-up of 5 kDa protomers and the 20 kDa of RS-AFP represented tetrameric form. Also, intact disulfide bridge seemed to be necessary for stabilization of the oligomeric structure.

***Fusarium oxysporum* pathogenicity tests with AFP transformed *R. glutinosa*.** AFP gene, which was obtained from turnip and characterized as protein gene showing

Table 1. Results of pathogenicity test of transformed *Rehmamnia glutinosa*

Genotype/ Pathogen		Shoot height (cm)	No. of leaf	Sub-astral weight (g)	Sub-terral weight (g)	Root rot rating mean[a]
Control	R-3	12.32	10	3.58	8.32	4.2[ab]
	R-10	14.28	11	4.82	9.20	4.5[a]
AFP	R-3	13.00	12	6.28	15.41	1.0[b]
	R-10	19.18	15	9.07	15.56	1.5[b]

[a] Extent of root rot symptom was rated on a scale of 1-5 where 1=normal and healthy, and 5=severe symptoms.
[b] Significant at P=0.05.

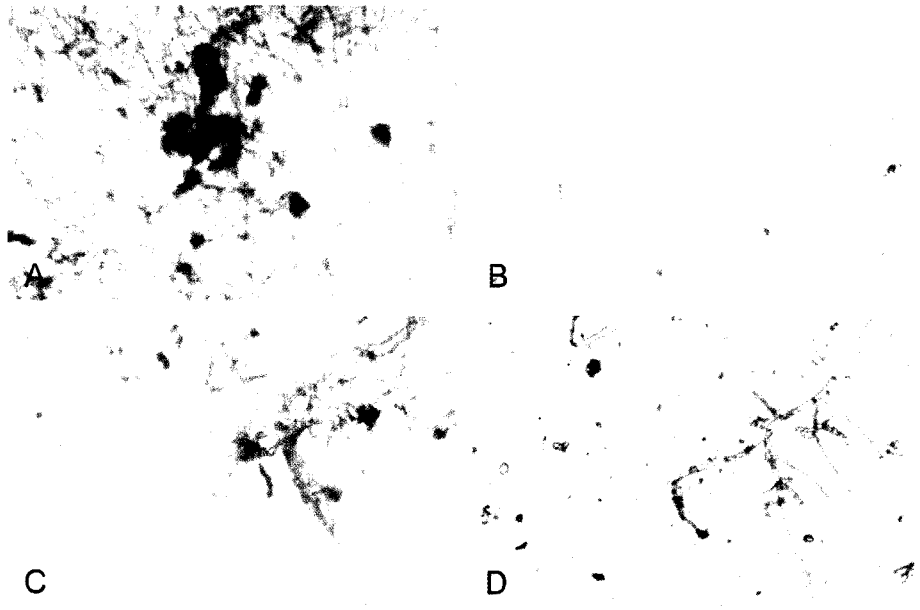

Fig. 6. Difference in morphology of inhibited hyphae. Photomicrograph was taken after 24 hours of incubation of *Asperigillus awamori* and *Cladosporium herbarum* spore suspension in 96-well microassay plate.
A: *Asperigillus awamori* (control), **C**: *Cladosporium herbarum* (control), and **B**, **D**: AFP transgenic plant.

antifungal activity, especially in the inhibition of secondary mycelial growth, was transformed into *R. glutinosa*. Transformed *R. glutinosa* was then tested for its resistance against *F. oxysporum* in the pathogenicity tests. As a result, even if shoot height and number of leaves formed did not show much difference between the transformed and non-transformed (control) plantlets, sub-astral and sub-terral weights of the transformed and non-transformed (control) plantlets showed significant difference. In disease ratings, transformed plantlets showed resistance against *F. oxysporum* infections as compared with those of non-transformed (control) plantlets (Table 1). This result indicates that the transformation was successful and the gene was expressed in the transformed host plantlets.

Antifungal activity. Fungal germination inhibition was routinely checked microscopically to confirm the micro-spectrophotometric data (Fig. 6) Microcultures to which extracted Rs-AFP1 protein from transgenic *R. glutinosa* were added revealed complete growth inhibitions. However, at high concentrations of extracted Rs-AFP1 protein from transgenic *R. glutinosa*, no spore germination occurred (data not shown).

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