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Resveratrol synthase transgene expression and accumulation of resveratrol glycoside in *Rehmannia glutinosa*

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Abstract

Rehmannia glutinosa L. is an important medicinal crop in Asian countries and contains trace amount of resveratrol compounds. To increase production of the compounds, we attempted ectopic expression of peanut resveratrol synthase gene (*AhRS3*) in *R. glutinosa*. The *AhRS3* sequence that encompassed the open reading frame, including a 312 bp-long intron present between the 59th and 60th codon, was driven by the CaMV35S promoter and introduced into *R. glutinosa* via *Agrobacterium*-mediated transformation of leaf explants. The transgenic plants with one to three copies of *AhRS3* transgene showed normal growth and development. The transgene was expressed constitutively in the leaf, root and flower at similar levels. Transgene expression in the leaf resulted in the production of new compounds identified as resveratrol and 3′-H-resveratrol-3-*O*-β-D-glucoside (R-gluc) through nuclear magnetic resonance spectroscopy and mass spectrometry. R-gluc accumulated predominantly and its content in the leaf of the 11 transgenic lines ranged from 22 to 116 μg/gFW. The contents of resveratrol compounds in the transgenic plants were further increased by cold, UV, ethylene, and paraquat treatments, and were positively associated with the levels of *AhRS3* mRNA levels. The R-gluc isolated from the transgenic plants exhibited antioxidant activity equivalent to one-third of resveratrol. Transgenic plants were highly resistant to *Fusarium oxysporum* infection. The results indicate that the ectopic expression of *AhRS3* in *R. glutinosa* results in the production of R-gluc and resveratrol at hundreds of times higher levels than in peanut seed. The increased production of resveratrol compounds from *R. glutinosa*, which show diverse benefits for human and plant health, could provide a new opportunity for the improvement of *R. glutinosa* products.

Introduction

Rehmannia glutinosa L. is an important medicinal crop in Asian countries. *Rehmanniae radix* is commonly used in oriental clinics as a haemostatic, cardiotonic, and diuretic agent (Oshio et al. 1981; Hasegawa et al. 1982). Findings of antibacterial, anti-inflammatory and immunity-promoting properties of *Rehmanniae radix* (Huang 1993; Kubo et al. 1994; Tomoda et al. 1994; Kim et al. 1999) provide a scientific background for its traditional clinical uses. Many components have been

isolated from both the *Rehmanniae radix* and from the fresh plant, including stigmasterol, campesterol, catalpol, rehmannin, and vitamin A (Chang and But 1986; Ni et al. 1992). Though no reports are available regarding the occurrence of resveratrol in *Rehmanniae radix* and fresh leaves, we obtained analytical evidence on the presence of a trace amount of resveratrol in the root of *R. glutinosa* (Lim et al. 2004).

Resveratrol (3,5,4′-trihydroxylstilbene) is a naturally occurring stilbene found in a limited number of unrelated plant species such as grapevine, peanut, and pine (Ingham 1976; Hart 1981; Jeandet et al. 2002). Due to its antifungal activity and induction in response to pathogen infection, resveratrol has been considered a phytoalexin (Ingham 1976; Keen and Ingham 1976; Hart 1981; Hain et al. 1993; Jeandet et al. 2002), and has drawn considerable attention as a phytochemical which supports disease resistance in plants.

Moreover, resveratrol is well known for its potent antioxidant activity and health-promoting effects. Various studies have demonstrated the effects have of resveratrol in cardioprotection (Ignatowicz and Baer-Dubowska 2001). A demonstrated association of resveratrol with reduced cancer risk has also been observed (Jang et al. 1997; Cal et al. 2003). New evidence also indicates that resveratrol can exert neuroprotective effects by increasing heme oxygenase activity in the brain (Zhuang et al. 2003). Therefore, resveratrol has received considerable attention as a beneficial phytochemical in food and heath-related industries.

Resveratrol is synthesized by resveratrol synthase (RS; EC 2.3.1.95), also known as stilbene synthase, using one molecule of coumaroyl-CoA and three molecules of malonyl-CoA. RS genes have been differentiated in peanut (Chung et al. 2001, 2003; Schroder et al. 1988; Lanz et al. 1990), grape (Wiese et al. 1994) and pine (Preisig-Muller et al. 1999; Kodan et al. 2002).

The rare occurrence of resveratrol and its beneficial effects on plant and human health have driven attempts to produce resveratrol through transgenic RS expression. Contrary to the rare occurrence of resveratrol, the substrates for resveratrol synthesis, coumaroyl-CoA and malonyl-CoA, are abundant in most plant species, providing a practical background for the transgenic approach. The expression of *RS* transcripts has been associated with an increased resistance to various fungal pathogens in transgenic tobacco (Hain et al. 1993), tomato (Thomzik et al. 1997), rice (Stark-Lorenzen et al. 1997), and wheat (Fettig and Hess 1999). In transgenic alfalfa, ectopic expression of RS resulted in the accumulation of a resveratrol-3-*O*-*β*-*d*-glucoside (R-gluc) (Hipskind and Paiva 2000). However, no attempt has been reported regarding the production of resveratrol in medicinal herbs that produce little or no amount of the compound. In this report, we demonstrate accumulation of R-gluc by the expression of *AhRS3* transgene in *R. glutinosa*, a strong antioxidant activity of the R-gluc isolated from transgenic *R. glutinosa* plants, and also increased resistance of the transgenic plants to *Fusarium oxysporum* infection.

Materials and methods

Plant materials

The plants of *R. glutinosa* were raised from tubers harvested in the previous growing season, and grown in a glasshouse at Kangwon National University, Korea. Young healthy leaves of *R. glutinosa* were used as explants for transformation experiments. The transgenic plants were maintained in growth chambers kept at 22 °C under 16 h light/8 h dark. Uniform plants of the non-transformed and RgAhRS3-1 transgenic T_0 line at the 5–6th leaf stage were subjected to various abiotic stresses. Cold treatment was imposed through the placement of the plants in a cold chamber at 4 ± 0.5 °C with dim light for 0, 2, 5, 10, and 24 h, respectively (Yamaguchi-Shinozaki and Shinozaki 1994). For UV light treatment, plants were irradiated under high pressure mercury vapor lamps (30 W, 60 mm YOE Incorporated, USA) with a fixed spectrum of 306 nm. For paraquat and ethephon treatments, plants were sprayed with 0.1 mM paraquat or 5 mM ethephon solutions. Leaf and root samples were collected after 0, 2, 5, 10, and 24 h after treatments, respectively. All tissue samples were then frozen in liquid nitrogen and stored at −80 °C until utilization.

RS expression vector construction and Agrobacterium-mediated *transformation of* R. glutinosa

The peanut RS genomic DNA sequence, *AhRS3* (GenBank Accession number, AF227963) encompassing the complete coding sequence for a polypeptide of 389 amino acid residues, was cloned into the *Xba* I/*Cla* I sites of binary expression vector pGA643 (provided by Dr G. Ahn Pohang University of Science and Technology, Korea) under the CaMV35S promoter. This produced a recombinant *AhRS3* expression plasmid, pMG-AhRS3 (Figure 1). The *AhRS3* sequence contains a single 312 bp-long intron between 198 and 511, which resides between the 59th and 60th codons of the complete open reading frame of *AhRS3*. In the intron, two silencer binding factor (SBF-1) elements are located at 272 to 285 and 328 to 338, respectively. SBF-1 is involved in organ-specific expression, in development and functions as a transcriptional silencer (Lawton et al. 1991). SBF-1 elements are found in phenylpropanoid/flavonoid biosynthetic genes such as chalcone synthase, stilbene synthase, cytochrome P450 (An et al. 1993; Bilodeau et al. 1999. The plasmid pMG-AhRS3 was introduced into *Agrobacterium tumerfacians* strain LBA4404 by the freeze-thaw method, An 1987).

Young healthy leaves were surface-sterilized by subsequent immersion in 70% (v/v) ethanol for 10 s and in 1% (v/v) $NaClO_3$ solution containing a few drops of Tween 20 for 10 min. After thorough rinsing in sterilized water, leaves were cut aseptically into 7×7 mm^2 sections. These sections were used as explants. Inoculation of explants with *Agrobacterium* harboring the plasmid pMG-AhRS was performed following the protocol of Park et al. (2002). The leaf explants were cultured for 2–3 days on MS medium containing 1 mg l^{-1} 6-benzylamino-purine (BAP), 2 mg·l^{-1} thidiazuron (TDZ, *N*-phenyl-*N*′-1,2,3-thiadiazol-5-yl urea), 0.2 mg l^{-1} naphthalene acetic acid (NAA), Murashige and Skoog (MS) vitamin, 3% sucrose, and 0.8% agar (pH 5.2). Pretreated explants were dipped into the *Agrobacterium* suspension in liquid inoculation medium for 10–15 min, blotted dry on sterile filter paper and incubated in a shoot induction medium (MS medium containing 2 mg l^{-1} BAP, 1 mg·l^{-1} TDZ, 0.2 mg l^{-1} NAA, MS vitamin, 3% sucrose, and 0.8% agar at pH 5.2.) in the dark at 23 ± 1 °C. After cocultivation for 2 days, the explants were transferred to shoot induction medium containing 50 mg l^{-1} kanamycin and 200 mg l^{-1} timentin (mixture of ticarcillin disodium and clavulanate potassium) and were transferred to fresh selection medium every 2 weeks. Putative transgenic shoots were regenerated 6–8 weeks after the first sub-culture. Shoots larger than 3 cm in length were subcultured on MS or 1/2 MS media which had been supplemented with kanamycin and timentin, for root induction. Putative transgenic shoots were incubated in a growth chamber with a 16 h photoperiod under standard cool white fluorescent tubes (35 μmol s^{-1} m^{-2}) at 23 ± 1 °C for 30 days. Putative transgenic plantlets were then transferred to pots containing autoclaved vermiculite, covered with polyethylene bags to maintain high humidity, and kept at 23 ± 1 °C in a growth chamber for 7 days. After 7 days, the bags were perforated and the plants were grown in the glasshouse.

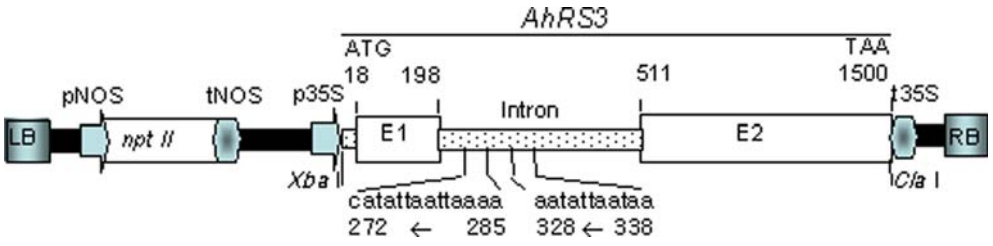

Figure 1. Partial structure of the binary vector, pMG-AhRS3, containing peanut resveratrol synthase 3 (*AhRS3*) expression cassette. The 1500 bp *AhRS3* genomic sequence contains the sequence encompassing the complete open reading frame sequence in the exon 1 (E1) and 2 (E2), including the 312 bp intron located between the two exons. The intron sequence contains duplicate elements similar to the SBF-1 binding site in the positions as indicated. Abbreviations: *nptII*, neomycin phosphotransferase II; pNOS and tNOS, promoter and polyadenylation signal of the nopaline synthase gene, respectively; p35S and t35S, 35S RNA promoter and polyadenylate signal of Cauliflower Mosaic Virus, respectively; BR, T-DNA right border; BL, T-DNA left border.

PCR analysis

Genomic DNA was extracted from the leaves of non-transformed and transgenic plants by the cetyltrimethyl ammonium bromide (CTAB) method (Rogers and Bendich 1988). This genomic DNA was used to screen transformed plants using polymerase chain reaction (PCR) analysis. The selection marker *npt* II gene was detected using the primer pair, pN-1 (5′-GAAGCTATTCGGCTA TGACTG-3′) and pN-2 (5′-ATCGGGAGCGGC GATACCCTA-3′). The AhRS3-specific primer pair, RS3-1 (5′-AGGCACCGTCGTTGGATGC AAGG-3′) and RS3-2 (5′-GGCCACACTGCGG AGAACAACGG), was used for *AhRS* transgene detection. PCR reactions were performed with the pre-made reaction mixture (PCR-Express, Hybaid, Middlesex, UK). For *npt* II gene amplification, each cycle consisted of denaturation (1 min at 94 °C), annealing (1 min at 60 °C), and extension (1 min 30 s at 72 °C) steps. For *AhRS3* sequence amplification, annealing was performed at 59 °C. After 35 cycles, a final extension step was performed at 72 °C for 5 min. The amplification products were then separated on 1% (w/v) agarose gels, stained with ethidium bromide and visualized with UV light.

Southern and northern blot analysis

Southern blot analysis was carried out with genomic DNA (Sambrook and Russell 2001). Genomic DNA (10 μg) that had been digested to completion with a single restriction enzyme, *BamH* I, was separated by agarose gel electrophoresis. It was then transferred onto a nylon membrane (Hybond-N^+; Amersham, UK). The membrane was probed with DIG-labeled antisense *AhRS3* RNA, which had been prepared by *in vitro* transcription using an RNA transcription Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. The nylon membranes were washed twice with a mixture of 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) and 0.1% sodium dodecyl sulphate (SDS) for 5 min at room temperature, then twice with a mixture of 0.1×SSC and 0.1% SDS for 15 min at 68 °C, and subjected to detection with DIG-labeled RNA probes using the DIG Chemiluminescent Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany).

Total RNA was prepared from the non-transformed and transgenic *R. glutinosa* plants using the phenol method (Sambrook and Russell 2001). The total RNA (10 μg) was denatured with a mixture of 2.15 M formaldehyde and 50% formamide, fractionated by electrophoresis on a 1.0% agarose gel containing 2.2 M formaldehyde, and subsequently transferred to a nylon membrane. The membrane was probed with DIG-labeled antisense *AhRS3* RNA prepared as in Southern blot analysis.

Purification and identification of unknown compounds from transgenic R. glutinosa *plants*

Only the compounds which appeared as major peaks in the transgenic plants were isolated and their chemical structures were identified using nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry. Whole plants were harvested, dried (1 kg) and homogenized with 70% acetone (500 ml × 2). The extract was concentrated at 40 °C *in vacuo* and the resulting aqueous extract (150 ml) was extracted successively with *n*-haxane (150 ml × 2), methylene chrolide (150 ml × 2), and ethyl acetate (EtOAc, 150 ml × 2). The EtOAc fraction (1 g) was dissolved in MeOH–H_2O (1:1, 1 ml), chromatographed on a Sephadex LH-20 column (1.5×60 cm), and eluted using MeOH–H_2O (7:13) at a flow rate of 1 ml min^{-1}. The compounds appearing as new peaks in the transgenic plants were included in the EtOAc fraction. Recrystallization was performed for the final fraction and the crystal compound was separated on TLC plates (25DC-Plastic-folien Cellulose F, Merck), developed in solvent A (BuOH-HOAc-H_2O; 3:1:1, v/v) and solvent B (6% HOAc), and observed under a UV lamp (254, 365 nm) after being sprayed with vanillin–HCl–ethanol (4.8 g:12 ml:480 ml) reagent (Sarkar and Howarth 1976). The purity of the isolated compounds was confirmed by two-dimensional TLC using solvents A and B.

The chemical structures of the purified compounds were identified by ^{13}C-NMR, ^{1}H-NMR and mass spectrometry. NMR studies were performed with Brucker Avance DPX-400 NMR spectroscopy (400 MHz, Brucker, Germany).

TMS was used as the internal standard. The structures of the purified compounds were identified using NMR spectra of ^{1}H-NMR (400 MHz), ^{13}C-NMR (100 MHz), COSY, and HECTOR. The molecular weights of the purified compounds were measured by electron ionization mass spectrometer and fast atom bombardment (FAB) mass spectrometer using Micromass Autospec M363 (Micromass, Euroscience, Manchester U.K).

Detection and quantification of resveratrol and resveratrol-3-O-β-D-glucoside

Resveratrol and resveratrol-3-*O*-*β*-D-glucoside (R-gluc) were quantified by HPLC (LC-10A, Shimadzu Co., Kyoto, Japan) equipped with a spectrophotometer (Shimadzu SPD-10A, Japan) and operated at the wavelength of 280 nm. The separation of resveratrol-forming stilbene was performed on a fractionation column (S-4 μm 80A, 250×10 mm, J'sphere ODS-H80, YMC, Japan) with a flow rate of 1 ml min^{-1}. Gradient elution was performed using two solvents. Solvent A consisted of 2% 0.018 M aqueous ammonium acetate and solvent B comprising 70% solvent A and 30% of organic solvent. The organic solvent was composed of 82% MeOH, 16% *n*-butanol, and 2% 0.018 M ammonium acetate. After sample injection, gradient elution was performed as follows: 0–1 min, wash with 90% solvent A; 1–21 min, linear gradient from 90 to 75% solvent A; 21–36 min, linear gradient from 75 to 55% solvent A; 36–56 min, linear gradient 100% solvent B; 56–82 min, wash with 100% solvent B. Identification and quantification of compounds were carried out by comparing retention times and peak areas with standard values. Spike tests were also used.

Antioxidant activity assay

Antioxidant activity of resveratrol and resveratrol-3-*O*-*β*-D-glucoside, isolated from transgenic *R. glutinosa* L. and the standard compounds trans-resveratrol and astringin, (Sigma, USA) were determined through measurement of the inhibition of lipid peroxidation induced by Cu^{2+} in fresh mouse low density lipoproteins (LDL) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. The standard compounds were purchased from Sigma (USA) and Polyphenol laboratories (USA). Inhibition of lipid peroxidation was determined by measuring thiobarbituric acid-reactive substance production (Buege and Aust 1978). Radical scavenging activity of stilbene compounds was measured according to Yoshida et al. (1989). The radical scavenging activity of each compound was expressed by the percent ratio of the absorption of DPPH in the presence and in the absence of the compound.

Disease response to Fusarium oxysporum

Fusarium oxysporum was isolated from infected plants and confirmed through an identification service by CBS (Fungal Biodiversity Center-Utrecht, The Netherlands). The isolated pathogen was grown in a 250 ml flask containing 100 ml of potato dextrose broth (PDB medium, Duchefa, The Netherlands). It was shaken (150 rpm) at 26 °C under constant light. Fungal cultures were filtered through four layer of cheese cloth after 7 days. The final cell density was maintained at 10^{6} CFU/ml. The root systems of non-transgenic and transgenic plant were dipped into the spore suspension of *F. oxysporum* for 30 min and were transplanted to the soil in pods. Disease incidence was evaluated 4 weeks after inoculation by a count of the number of diseased plants (Killebrew et al. 1988)

Results

Transformation of R. glutinosa *with peanut RS gene*

Thirty putative transgenic lines were established from the separately transformed shoot explants. Most of these transgenic plants exhibited normal growth and development under routine glasshouse conditions. Eleven transgene-positive T_0 lines were selected by PCR screening. The lines containing *AhRS3* and *npt II* transgene sequences were chosen for further characterization (Figure 2). Results of Southern blot analysis confirmed the presence of the *AhRS3* transgene sequence in PCR-selected transgenic plants (Figure 3). The copy number of the *AhRS3* transgene was approximated based on

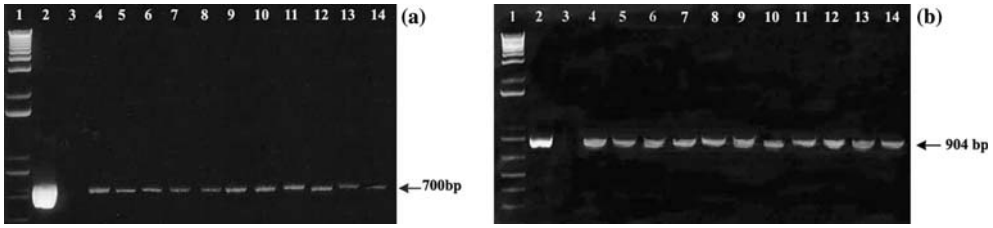

Figure 2. PCR detection of the selection marker *npt II* (a) and *AhRS3* transgene (b) sequences in the non-transgenic and T_0 transgenic *R. glutinosa* plants. Lane 1, 1 kb DNA marker; lane 2, positive control (*AhRS3* expression vector DNA); lane 3, non-transformed *R. glutinosa* plant; lanes 4–14, transgenic *R. glutinosa* plants. The size of the amplified DNA fragments for *npt II* and *AhRS3* are 700 and 904 bp, respectively.

the intensity and pattern of the bands. Eight lines were estimated to contain one, two lines two, and one line three copies of the transgene, respectively.

Expression of AhRS3 *transgene*

The expression of the transgene was examined in the tissue culture-propagated RgAhRS3-1 plants. The *AhRS3* transgene was expressed constitutively in the leaf, root, and flower tissues of *R. glutinosa* (Figure 4a). Transgene expression remained similar in the root and flower but increased in the leaf by cold treatment. In the non-transgenic plants, a faint band was detected only in the root, indicating the presence of mRNA sequence showing a significant homology to *AhRS3* in the tissue. No reports are available as to the occurrences of resveratol and resveratrol synthase gene in *R. glutinosa*. However, our recent results indicate the presence of limited amounts of resveratrol in the root of *R. glutinosa* (Lim et al. 2004). Thus, the faint band detected in the root suggests its presence and its root-specific expression of RS gene in *R. glutinosa*. However, the putative endogenous RS sequence was not expressed in the leaf or flower after cold treatment (Figure 4a). Transgene expression in the leaf was also increased by UV light, ethephon, and paraquat (Figure 4b). In peanut, AhRS3 expression was also induced by UV, paraquat and ethephon treatments in a time-dependent manner (Chung et al. 2003). Since no signals were detected in the leaves of stress-treated non-transgenic plants (Figure 4b), the results indicate that *AhRS3* transgene expression was further increased by stress treatments. Regarding the increased expression of *AhRS3* transgene under the control of the CaMV35S promoter, it is noted that the *AhRS3* transgene sequence contains an intron where two transcriptional silencer SBF-1 elements are located (Lawton et al. 1991).

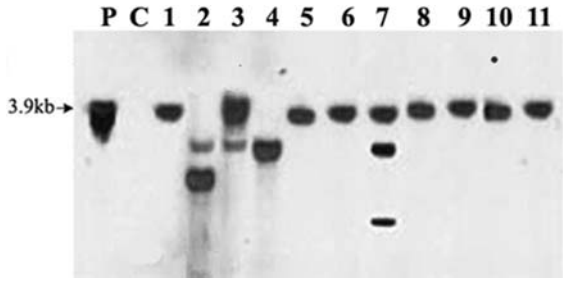

Figure 3. Southern blot analysis of genomic DNAs of *AhRS3* transgenic T_0 lines. Lane P, *AhRS3* expression vector DNA digested with *BamH* I. The insert size is about 1.5 kb. Lane C, non-transformed control plant, Lane 1–11, genomic DNA from the transgenic lines 1–11 digested with *BamH* I. The 1.5 kb DNA fragment is indicated by an arrow head.

Verification of resveratrol and resveratrol glucoside production

Northern blot analysis strongly indicated that the *AhRS3* transgene was expressed in the transgenic *R. glutinosa*. To estimate the functional consequence of the expressed transgenic, we analyzed the anticipated reaction products of the enzyme by HPLC in the control and transgenic plants. From HPLC analysis, one or two new peaks were detected in the leaf and root of transgenic plants. Also, the peaks coincided with those of R-gluc and resveratrol, respectively (Figure 5), indicating the successful production of functional RS proteins.

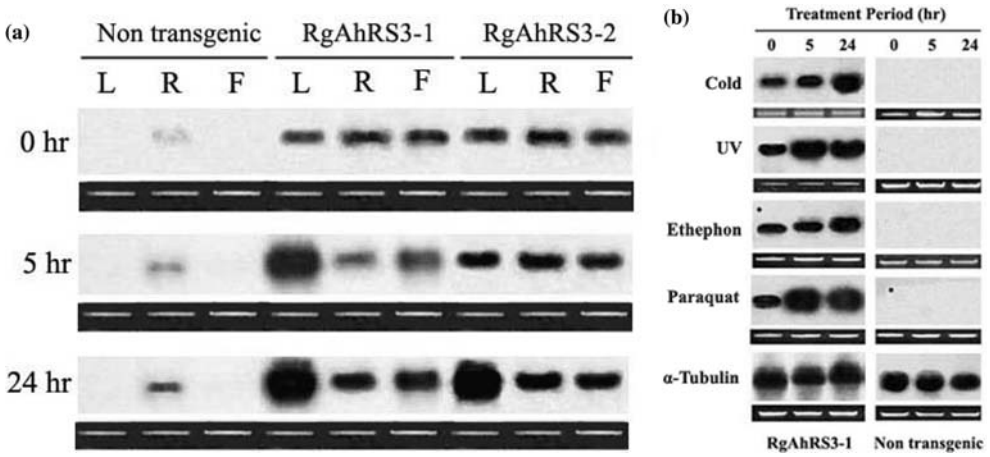

Figure 4. Northern blot analysis of *AhRS3* transgene expression in the transgenic line RgAhRS3-1. (a) Expression of the *AhRS3* transgene in the leaf (L), root (R), and flower (F) after cold treatment for 0, 5, and 24 h, respectively. (b) Increased expression of the *AhRS3* transgene by treatments with various stresses. Non-transformed and RgAhRS3-1 transgenic plants were treated by cold (4 °C), UV light, ethephon, or paraquat for 0, 5, and 24 h, respectively.

For physicochemical identification of the new compounds, the compounds were purified and analyzed via thin layer chromatography (TLC), NMR and mass spectrometry. TLC results showed that the unknown compounds were predominantly present in the EtOAc extract of the initial aqueous residue. The white crystalline powder obtained for the unknown compound produced a violet coloration on cellulose 2D-TLC, and appeared as a dark brown spot under UV light (Rf, 0.29/solvent A, 0.28/solvent B, data not shown). The ^{1}H-NMR spectrum (Figure 6a) showed the presence of two sets of signals. The former, between δ3 and δ5, was assigned to glycosyl protons. This is consistent with the ^{13}C-NMR spectrum (Figure 6b), which showed six characteristic signals of β glucose unit. The latter set, between δ6.3 and 7.5, was represented by three systems of olefinic and aromatic protons. The two doublets ($J = 8.62$ Hz) at 7.33 and 6.26 were designated to an AA′XX′ system of a 1,4 disubstituted aromatic ring; and the three broad singlets at δ6.44, 6.61, 6.78 to the three meta related protons of a 1,3,5 trisubstituted aromatic ring. The two doublets at δ6.83, and 7.00 with a significant coupling constant ($J = 16.2$ Hz) showed a *trans* olefinic proton system. These signals are consistent with a *trans* stilbene system substituted by a glucoside. Moreover, the coupling constant ($J = 7.3$ Hz) and the chemical shift (δ4.89 H-1″) were indicative of the β configuration of the glucosyl bond. Therefore, a *trans* resveratrol glucoside structure was proposed for this compound. Due to precise positioning of the glucosyl unit, bonding to 4′ could not occur because the long range coupling cross peak at H-1″/C-3 in HMBC confirmed that the glucose unit was attached to the aglycone at the 3-OH between the anomeric proton and C-3. All meta aromatic protons were non-equivalent. As a result, only one position was in proper agreement with the above data. This compound was confirmed as *trans*-3′-H-resveratrol-3-*O*-β-glucoside (Figure 6d). Also, the spectroscopic data for the compound of the new major peak detected in the transgenic plant were consistent with those for piceid (Mattivi et al. 1995). Likewise, the spectroscopic data for the compound of the new minor peak detected in the transgenic plant were identical to those of resveratrol (data not shown). It is recognized that resveratrol is often present in conjugated forms such as resveratrol glucosides (Hipskind and Paiva 2000). It is also well-known that piceid is more abundant in grape skin than is resveratrol (Roggero and Archier 1994).

In plants, the conjugation of glucose to small hydrophobic molecules and compounds can lead to the formation of glucose esters or glucosides. The former are high energy compounds and have long been regarded as biosynthetic intermediates (Mock and Strack 1993), whereas glucosides are generally considered representative of the storage forms of aglycons (Hostel 1981).

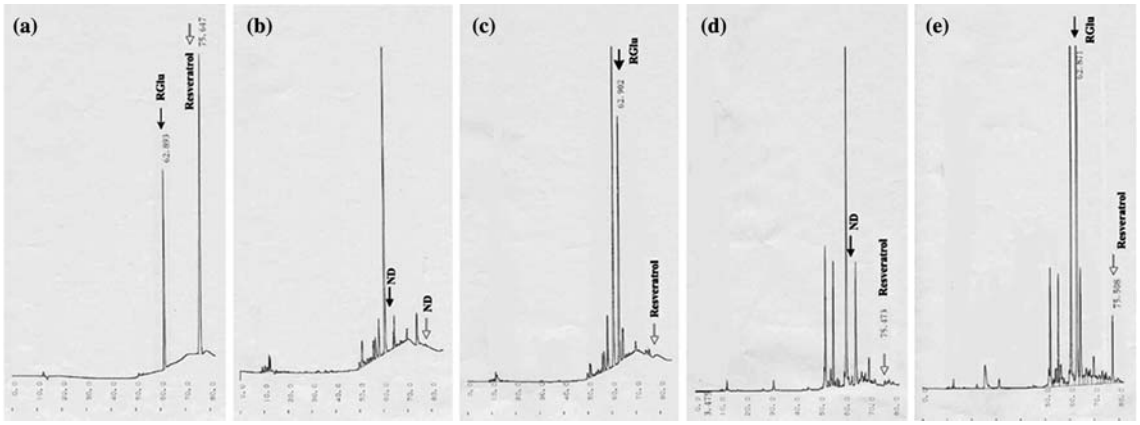

Figure 5. Detection of resveratrol and R-gluc by HPLC in the leaf and root of RgAhRS3-1 transgenic plants. (a) R-gluc and resveratrol standard compounds were separated as the peaks with retention time of 62.3 (closed vertical arrow) and 75.6 min (open vertical arrow), respectively. (b and c) HPLC chromatograms of leaf from control (b) and from transgenic plants (c). (d and e) HPLC chromatograms of root from control (d) and from transgenic plants (e). The plants were treated at 4 °C for 24 h. ND: not detected.

Predominant accumulation of resveratrol glucoside in transgenic plants

Since the production of resveratrol and R-gluc in the transgenic plants was confirmed through rigorous physicochemical analyses, we were able to quantify the amount of the compounds in the transgenic plants. R-gluc accumulated in the leaves of 11 T_0 transgenic lines ranged from 22 to 116 μg/gFW with trace amounts of resveratrol. The R-gluc content was generally lower in the lines with more transgene copies (Table 1). The R-gluc contents found in the transgenic *R. glutinosa* were similar or up to 4-fold higher than those in transgenic alfalfa (Hipskind and Paiva 2000) and peanut (1.2–56 μg/g FW, Chung et al. 2003) but lower than those in grape (50–400 μg/g FW, Langcake and Pryce, 1976) and transgenic Kiwi (182 ug/g FW, Kobayashi et al. 2000).

As the levels of transgene *AhRS3* mRNA expression were increased by the various oxidative stresses and hormones, the accumulation kinetics of the compounds was examined in the cold-stressed RgAhRA3-1 transgenic plants. Resveratrol and R-gluc were not detected in the leaf of the non-transgenic plant, but resveratrol was, in fact, present at about 2 μg/gFW and R-gluc at about 115 μg/gFW in the transgenic plants. The content of resveratrol and R-gluc in the transgenic leaves cold-treated for 5 h was further increased over 2-fold, to 4 and 270 μg/gFW, respectively (Figure 7). Cold treatment longer than 5 h resulted in no further increase in R-gluc content in the leaf of transgenic plants. R-gluc in the transgenic leaf was also increased to 341.8, 345.6, and 172.8 μg/gFW by UV, ethephon, and paraquat treatments for 24 h, respectively. In the root of the non-transgenic plant, very little resveratrol (about 5 μg/gFW) was present, and no R-gluc was detected. The amount of resveratrol and R-gluc increased to 35 and 650 μg/gFW, respectively, in the cold-stressed transgenic roots (Figure 7).

Antioxidant activity of resveratrol glucoside isolated from transgenic R. glutinosa

When the production of resveratrol and preferential accumulation of R-gluc was confirmed, we examined the biological activity of resveratrol and R-gluc isolated from the transgenic plants. The antioxidant potency of the compounds was investigated by measuring the inhibition of lipid peroxidation induced by Cu^{2+}. The resveratrol compounds from the transgenic plant and commercial source showed similar levels of LDL peroxidation inhibition activity. However, the R-gluc (3′-H-R-gluc) from the transgenic plant required about 6-times more than that of resveratrol or 3′-OH-trans-R-gluc to achieve the same level of inhibition activity against LDL peroxidation (Table 2). The resveratrols from the transgenic plant and commercial source showed similar levels of radical scavenging activity. Again, the R-gluc (3′-H-R-

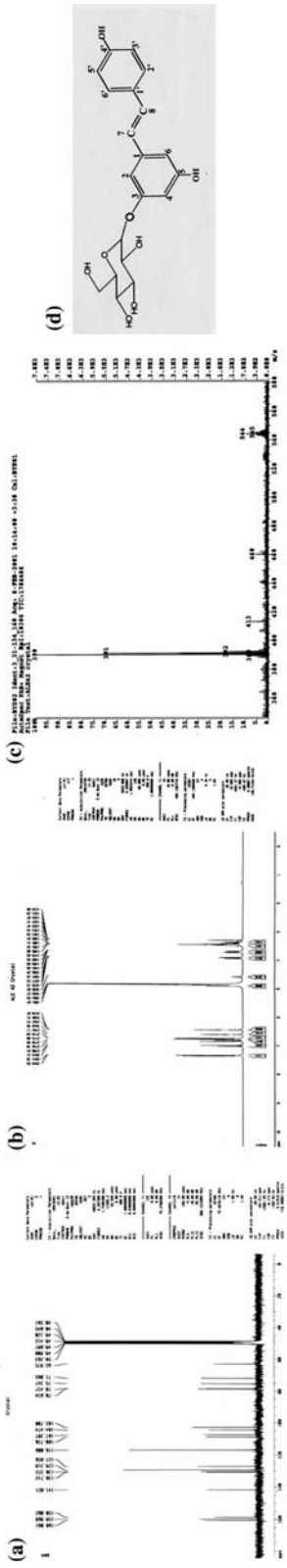

Figure 6. Identification of compound isolated from transgenic plants via NMR and mass spectroscopy. (a) ^{13}C-NMR spectrum of the compound. ^{13}C-NMR (100.6 MHz, δ, MeOH-*d*4): 62.97(C-6″), 71.86(C-4″), 75.34(C-2″), 78.43(C-3″), 78.63(C-5″), 102.78(C-1″), 104.47(C-4), 107.39(C-2), 108.73(C-6), 116.88(C-3′,5′), 127.05(C-7), 129.31(C-2′,6′), 130.37(C-8), 130.71(C-1'), 141.82(C-1), 158.86(C-4′), 159.96(C-5), 160.86(C-3). (b) ^{1}H-NMR spectrum of the compound. ^{1}H-NMR(400 MHz, δ, MeOH-*d*4): 3.33–3.49 (^{4}H, *m*, H-2″,3″,4″,5″), 3.70 (^{1}H, *dd*, $J = 5.75$ Hz, and $J = 12.01$ Hz, H-6″b), 3.92(^{1}H, *dd*, $J = 2.01$ Hz, and $J = 12.08$ Hz, H-6″a), 4.89(^{1}H, *d*, $J = 7.3$ Hz, H-1″), 6.44(^{1}H, *t*, $J = 2.14$ Hz, H-4), 6.61(^{1}H, *br s*, H-6), 6.26(2H, *d*, $J = 8.62$ Hz H-3′,5′), 6.78(^{1}H, *br s*, H-2), 6.83(^{1}H, *d*, $J = 16.29$ Hz, H-7), 7.0(^{1}H, *d*, $J = 16.27$ Hz, H-8), 7.33(^{2}H, *d*, $J = 8.62$ Hz, H-2′,6′). (c) FAB-MASS spectrum. FAB-MS: $[M + H] + m/z$ 391. (d) The structure of resveratrol-3-*O*-β-D-glucoside (piceid).

gluc) from the transgenic plant required about 3-times that of resveratrol or 3′-OH-trans-R-gluc to achieve the same level of scavenging activity (Table 2). Compared with the antioxidant activity of Trolox® (6–Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a cell-permeable, water-soluble derivative of vitamin E with potent antioxidant properties, peroxidation inhibition activity and radical scavenging activity of R-gluc (3′-H-R-gluc) were about one fifth and one 19th of those of Trolox. According to the results, the conjugation of an OH group with a sugar decreased the antioxidant potential of resveratrol. Moreover, the significantly lower antioxidant activity of 3′-H-trans-R-gluc compared to 3′-OH-trans-R-gluc suggests the importance of catechol structure in the antioxidant effect of resveratrol and its derivatives (Goldberg 1995; Norren et al. 1997).

Disease response of RgAh3 transgenic plants to Fusarium oxysporum

F. oxysporum is a common soilborn fungus, well-represented in every soil type throughout the world (Burgess 1981). It is a serious pathogen which causes root rot disease in *R. glutinosa*. Disease response of the transgenic and non-transgenic plants to *F. oxysporum* was examined after inoculation of the root with spore suspension. Disease symptoms had fully developed 4 weeks after infection. Mortality rate due to the disease was as low as 2% in transgenic plants, but as high as 57% in non-transgenic plants (Figure 8

Table 1. Accumulation of resveratrol-3-*O*-*β*-D-glucoside (R-gluc) in the leaves of 11 T_0 transgenic lines with variable *AhRS3* transgene copy numbers.

	R-gluc (μ/gFW)	Estimated transgene copy number
Non-transgenic ontrol	Not detected*	0
RgAhRS3-1	115.2 ± 3.4	1
RgAhRS3-2	42.1 ± 8.5	2
RgAhRS3-3	31.9 ± 5.2	2
RgAhRS3-4	100.1 ± 5.4	1
RgAhRS3-5	89.2 ± 3.5	1
RgAhRS3-6	40.9 ± 4.7	1
RgAhRS3-7	22.2 ± 6.4	3
RgAhRS3-8	116.8 ± 11.3	1
RgAhRS3-9	95.3 ± 4.3	1
RgAhRS3-10	64.7 ± 2.8	1
RgAhRS3-11	52.9 ± 9.7	1

*No or trace amount ($< 0.001\,\mu$/gFW) of resveratrol was detected.

and Table 3). Only 12% of the transgenic plants had disease rating over 3 compared to 67% of the non-transgenic plants (Table 3). In general, the transgenic lines with higher R-gluc had fewer disease symptoms than those with lower R-gluc, indicating a weak reverse association ($r = -0.67$) between R-gluc content and disease severity. Resveratrol and R-gluc have a similar antifungal activity regarding *Phoma medicaginis* and hyphal growth was inhibited over 50% at 50 μg/ml (Hipskind and Paiva 2000). *AhRS* transgenic alfalfa plants accumulating R-gluc at similar or lower levels than transgenic *R. glutinosa* are highly

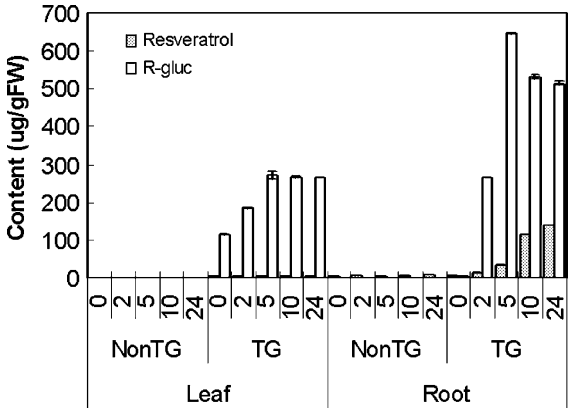

Figure 7. Accumulation of resveratrol and resveratrol-3-*O*-*β*-D-glucoside (R-gluc) in the leaf and root of non-transgenic control (Non-TG) and transgenic RgAhRS3-1 (TG) plants treated at 4 °C for 0, 2, 5, 10, and 24 h, respectively.

resistant to *Phoma medicaginis*. Tobacco and wheat transgenic plants producing resveratrol are also resistant to *Botrytis cinerea* (Hain et al. 1993; Leckband and Lorz 1998).

Discussion

The peanut *AhRS3* genomic DNA sequence was successfully introduced into the *R. glutinosa* genome via *Agrobacterium*-mediated transformation of leaf explants. The transgene copy number was estimated to be variable from 1 to 3 copies for the selected eleven transgenic lines based on Southern blot analysis. The occurrence of multiple bands on Southern blots with genomic DNAs digested with a cloning site enzyme with no recognition sites in the insert DNA, indicates that some sequence modifications have occurred on the inserted DNA in some transgenic lines. It is not rare that the transgenes are inserted into chromosomal DNA as repeated, multiple copies (Pawlowski and Somers 1996; Laufs et al. 1999). Several rearrangements of the transgene construct can also occur which result in inversion and translocation (Castle et al. 1993; Laufs et al. 1999).

The constitutive expression of the transgene was detected in the leaf, root, and flower tissues. Interestingly, the expression of the transgene was increased in the leaf by low temperature, UV light, ethylene, and paraquat. The variable expression of the *AhRS* transgene driven by the CaMV35S promoter was also observed in alfalfa (Hipskind and Paiva 2000). No data were presented regarding the variability of transgene expression. Variable expression levels of a transgene have often been related to the complex integration patterns (Fladung 1999) and effects of surrounding regions (Gelvin 1998). Epigenetic modifications of promoter sequences affect promoter activity and result in altered transcription rates of transgenes (Fojtova et al. 2003). However, the increased expression of the *AhRS* transgene by stresses is not likely related to the integration patterns and locations or promoter modifications, since the plants used for the expression analysis were vegetatively propagated from the transgenic line with one copy transgene. Alternatively, the SBF-1 silencer binding elements in the intron sequence of the transgene could be associated with the increased transgene expression under stress. In

Table 2. The antioxidant activities of resvetratrol and R-gluc isolated from transgenic *R. glutinosa.*

Compounds		IC_{50} values (μM)*	
		DPPH	LDL
Standard	*trans*-Resveratrol	68.0 ± 4.5^{b}	2.6 ± 0.4^{b}
	trans-3′-OH-Rgluc	67.0 ± 3.7^{b}	3.3 ± 1.0^{bc}
Stilbenes compound from transgenic plant	*trans*-Resveratrol	72.0 ± 4.5^{b}	2.4 ± 0.2^{b}
	trans-3′-H-Rgluc	198.0 ± 16.8^{a}	19.1 ± 0.8^{a}
Trolox		10.1 ± 0.5^{c}	4.7 ± 0.4^{c}

*Concentration for 50% inhibition. Each value is the mean of at least three independent experiments ± SD. The values with the same letter within the column are not significantly different each other. Statistical analysis was performed by using Student's *t*-test ($p < 0.05$).

peanut, the expression of *AhRS3* is not detected in the leaf free of biotic or abiotic stresses. *AhRS3* expression, however, is strongly induced by abiotic and biotic stresses, including UV light, wounding, ethylene, paraquat, and a general elicitor, yeast extract. The difference in the gene expression kinetics could be affected by many factors, including affinity of *cis*- and *trans*-acting elements. The *P. vulgaris* chalcone synthase silencer sequence also functions in tobacco (Hotter et al. 1995). In *Phaseolus vulgaris*, SBF-1 is constitutively expressed and is not induced by wounding or ethylene (Harrison et al. 1991). Therefore, it is postulated that the collective effects of the silencer element binding sequences in the *AhRS3* transgene and the binding proteins similar to SBF-1 of *R. glutinosa* could contribute, at least in part, to the increased expression of *AhRS3* transgene by the stresses. The role of the SBF-1 silencer binding elements in the intron sequence of the transgene could be elucidated by a comparative expression analysis with cDNA *AhRS3* lacking the silencer elements.

Figure 8. A typical response of non-transgenic and transgenic plants (RgAhRS3-1) to *Fusarium oxysporum* infection. The arrow indicates symptom in the root caused by pathogen.

Confirming the production of resveratrol and accumulation of R-gluc in the transgenic plants were the chemical characterizations of the purified compounds representing the new peaks detected in the transgenic plants via NMR and MS (Figure 6). Ectopic expression of the *AhRS* coding sequence driven by the CaMV35S promoter in alfalfa resulted in constitutive accumulation of R-gluc with no detectable levels of resveratrol (Hipskin and Paiva 2000). The expression of the grape *RS* transgene in apple also resulted in the accumulation of R-gluc (Szankowski et al. 2003). In contrast, the major accumulation product in transgenic wheat and tobacco overexpressing the grapevine *RS* gene was resveratrol, (Fischer et al. 1997; Sebastian and Dieter 1999). In peanut, different forms of resveratrol compounds accumulate in different tissues. The free resveratrol is predominantly detected in the leaf, whereas equal amounts of conjugated forms are also detected in the pod (Chung et al. 2003). Resveratrol derivatives are produced following resveratrol synthesis via conjugation of sugars and methylation, prenylation or condensation reactions (Sotheeswaran and Pasupathy 1993). The predominant accumulation of R-gluc indicated that the reaction product resveratrol is readily conjugated by a glucose moiety presumably through the involvement of glycosyltransferases.

In the leaf and roots of transgenic plants, the amount of resveratrol and R-gluc, and *AhRS3*

Table 3. Disease responses of the transgenic RgAhRS3 lines and non-transgenic plants to *Fusarium oxysporum* infection.

	Disease rating[a]				
	1	2	3	4	5
Non-transgenic plants	–	–	1	12	17
RgAhRS3-1	13[b]	11	6	0	–
−2	4	10	9	5	1
−3	6	12	8	4	–
−4	10	12	7	1	–
−5	11	13	6	–	–
−6	9	13	5	3	–
−7	6	8	5	8	3
−8	9	10	10	1	0
−9	7	8	9	4	2
−10	5	8	10	6	1
−11	7	8	8	7	–

[a]Disease ratings (1–5 scale: 1, no symptoms; 2, topical root rot; 3, topical root rot without apparent leaf symptoms; 4, vascular discoloration with leaf wilt symptoms; 5, dead plant).
[b]The number of plants showing the disease ratings indicated out of 30 total plants tested.

mRNA levels were increased by cold treatments (Figures 4 and 7), indicating that *AhRS3* transgene expression is regulated at the transcriptional level. Similarly, RS expression is controlled at the transcription step and a high correlation exists between *AhRS3* mRNA levels and resveratrol contents in peanut (Chung et al. 2003). Transcriptional control of *RS* transgenes has also been observed in alfalfa (Hipskin and Paiva 2000) and wheat (Fettig and Hess 1999). After cold treatment longer than 5 h, however, R-gluc content was significantly higher in the root even with lower *AhRS3* expression levels. Leaf-specific uncoupling of *AhRS3* mRNA from AhRS3 protein could be a possible reason. By the constitutive expression of nitrate reductase (NR) coding region with a CaMV35S promoter in tobacco, NR protein levels drop 4- to 5-fold after 72 h of dark treatment, even though NR mRNA levels remain high (Vincentz and Caboche 1991). Another possible reason could be a relatively lower availability of the substrates for resveratrol synthesis, malonyl-CoA and coumaroyl-CoA, in the leaf. In transgenic tomato expressing grape stilbene synthase, R-gluc content was affected by the availability of substrates (Giovinazzo et al. 2005).

The contents of resveratrol and R-gluc in the unstressed leaf and root of transgenic *R. glutinosa* were one or two orders of magnitude higher than in peanut (Chung et al. 2003), and similar to or higher than those of the *AhRS3* transgenic alfalfa (Hipskin and Paiva 2000). This difference might be the resultant of many factors, including the promoter and transgene structure used in the *R. glutinosa* transformation and the availability of substrates. The strong activity of the CaMV35S promoter might have contributed to the higher levels accumulation in *R. glutinosa* than in the peanut.

Since the primary use of *R. glutinosa* is medicinal, increased amounts of resveratrol and its derivatives could add values to *R. glutinosa* products. R-gluc, the major form accumulated in the transgenic plants, showed high levels of antioxidant activity, though the activity was lower than that of resveratrol (Table 2). The resveratrol glucosides can be hydrolyzed by β-glucosidase in the human gastro-intestinal tract. These also act to liberate the resveratrol aglycon, as in flavonoid absorption (Nemeth et al. 2003). So, the high levels of antioxidant activities of the resveratrol and R-gluc produced in the transgenic plants provides promising evidence for the use of resveratrol compounds as phytochemicals, a value-adding trait. Antioxidants prevent the formation and oppose action of reactive oxygen and nitrogen species, which damage DNA, lipids, proteins and other biomolecules (Fang et al. 2002). Diet-derived antioxidants, including resveratrol, also have beneficial effects in human health (Halliwell 1996; Ignatowicz and Baer-Dubowska 2001). The benefits of resveratrol to human heath have been revealed by the quantity and diversity of data from epidemiological, *in vitro* and *in vivo* models, and human dietary applications. Resveratrol has been shown to exert cardioprotective (Ingnatowicz and Baer-Dubowska 2001), neuroprotective (Zhuang et al. 2003), and cancer-protective activities (Jang et al. 1997; Cal et al. 2003).

Furthermore, the transgenic *R. glutinosa* plants were highly resistant to *Fusarium oxysporum*. Resveratrol compounds are well known phytoalexin-possessing antimicrobial effects (Pezet and Pont 1995). The ectopic production of resveratrol and R-gluc provides improved broad spectrum resistance in a wide range of plant species, including monocots and dicots (Hain et al. 1993; Stark-Lorenzen et al. 1997; Thomzik et al. 1997; Fettig and Hess 1999). Therefore, resveratrol and R-gluc could also prove to be beneficial for plant health.

In conclusion, production of R-gluc and resveratrol at several hundred-fold higher levels than in peanut seed was successfully achieved via the ectopic expression of *AhRS3* in *R. glutinosa*. Increased production of resveratrol compounds showing beneficial pleiotropic effects on human and plant health in *R. glutinosa*, while using a limited amount of the compounds, could provide a new opportunity for the improvement of *R. glutinosa* products.

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