RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Journal Name

RSCPublishing

ARTICLE

Resveratrol Improves Fungal Ribosylation Capacity through a Unique Mechanism

Cite this: DOI: 10.1039/x0xx00000x

Guo-Yan Zhao[1,2], Jing-Yang Fan[1], Cheng-Pin Hua[1], Wei Yan[1], Chao-Jun Chen[1], Yan-Hua Lu[2], Rui-Hua Jiao[1*], Ren-Xiang Tan[1*]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Ribosylation is a significant modification conferring new complex and broadening cellular roles of compounds, but small organic molecules are rarely ribosylated. Here, we report the 3-*O*- and 4'-*O*-α-ribosylation of resveratrol, a phytoalexin that is exogenous to the fungus *Daldinia eschscholzii* IFB-TL01. The ribosylation is mechanistically due to the resveratrol activated expression of the silent or less active fungal genes governing the ribosylation of the non-fungal phytophenol. The resveratrol-induced ribosylation is also characterized by the increased expression of ribosyltransferases in concert with a rare ribosylating reaction using nicotinamide mononucleotide (NMN) as a ribose donor. The ribosylation-reduced toxicity of resveratrol to *D. eschscholzii*, along with the involvement of at least two p-glycoproteins in the glycosylation, suggests that such a glycosylation process may be a general strategy for the fungal detoxification of phenolic chemicals. The findings present an updated view of ribosylation on small molecules, and provide direct chemogenetic evidence helping understand how phytophenols such as resveratrol function differently in plant, microbial and animal kingdoms.

Introduction

Ribosylation plays key roles in a variety of biological processes such as gene regulation, actin-clustering, defects in phagocytosis and cell death.[1] Some functional proteins tend to be readily ribosylated in response to the insulting factors such as bacterial toxin exposures, and in this case covalent modifications lead to the enzyme dysfunction owing to the incorporation of the ribose motif derived from nicotinamide adenine dinucleotide (NAD^+).[2] However, the ribosylation of small molecule organic compounds including natural products (secondary metabolites) is rarely detected in nature,[3] and little information has been documented concerning the mechanism of the ribosylation of the natural molecules. In contrast, glycosylation and xylosylation have been found to decorate an array of natural products categories such terpenes, coumarins, flavonoids and macrolides through the glycosyltransferase catalyzed nucleophilic attack of nucleoside diphosphate (NDP)-sugars.[4] Distinct from the widely occurring glycosylations, the mechanism underlying the ribosylation of small organic molecules remains largely elusive.

Daldinia eschscholzii IFB-TL01, a fungus belonging to saprophytic and endophytic Xylariaceae family,[5] is a versatile producer of phenolic polyketides.[6] However, our continuous efforts failed to detect any glycosylated metabolite from the fungal cultivations accomplished in several laboratory cultivation conditions. Surprisingly, this observation disagrees with the presence of glycosyltransferase genes in the fungal genome.[6] In the light of the genomic plasticity,[7] these genes are not evolutional excrescences, but their activation may depend on a particular combination of external factors such as nitrogen, carbon, pH and temperature.[8] Resveratrol (3,5,4'-trihydroxystilbene, Figure 1) is a phenolic phytochemical that accumulates in many plants such as peanuts, grapes, blueberries, mulberries and cranberries.[9] The natural phenol has been addressed to interfere with a variety of signaling pathways in mammal cells to exhibit anti-carcinogenic, anti-aging, anti-inflammatory, anti-diabetic and antioxidant actions.[10] Attention to the metabolic fate of resveratrol has clarified its glycosylatability into glucuronides in human,[11] and glucopyranosides in plant cells[12] or diglucosides by cyclodextrin glucanotransferase and sucrose phosphorylase from *Thermoanaerobacter* sp.[13] In addition, resveratrol functions as a phytoalexin in response to microbial stresses.[14] Engineering of resveratrol synthase-encoding genes in heterogenous plants such as *Solanum lycopersicum* and *Oryza sativa* can increase their resistance against fungal pathogens.[15] These observations, along with the reported fungal glycosylation of stilbenoids,[16] prompted us to use resveratrol as a xenochemical tool to investigate the glycosylating capacity of *D. eschscholzii* with an intention to obtain unprecedented fungal glycosides. Here, resveratrol is demonstrated to multiply the ribosylation capacity of *D. eschscholzii* through its activation (or up-regulation) of silent (or less active) ribosyltransferase and p-glycoprotein genes, and the rare fungal ribosylation takes place uniquely through an undescribed mechanism using nicotinamide mononucleotide (NMN) as the ribosyl donor. As detailed below, the work clarifies a conventionally undetectable ribosylation process that provides direct evidences for fungal detoxification of phenolic chemicals and new access toward new ribosides with biological and/or biomedical significance.

1.State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, P. R. China
2. State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, 200000, P. R. China
E-mail: rxtan@nju.edu.cn; rhjiao@nju.edu.cn
† Electronic supplementary information (ESI) available: See DOI: 10.1039/x0xx00000x

Results and Discussion

Ribosylation of resveratrol and fungal phenols in *D. schscholzii* culture

To study the xenobiotic response of *D. eschscholzii* to resveratrol, the fungus was grown for seven days, and the afforded healthy mycelia were inoculated subsequently into the media with resveratrol supplemented at varying concentrations. As expected, the fungal growth in petri dishes was substantially attenuated in a dose-dependent manner by its exposure to resveratrol at 10, 25, 50 and 100 mg/L (Figure 1A (a, b)), implying that this strain was sensitive to resveratrol. Re-challenge of the strain in liquid culture exposed to resveratrol showed that the colour of the fermentation extracts turned to red from the black of the control (free of resveratrol, Figure 1A (c)), showing an induced change resulting from the resveratrol supplementation in the *D. eschscholzii* culture.

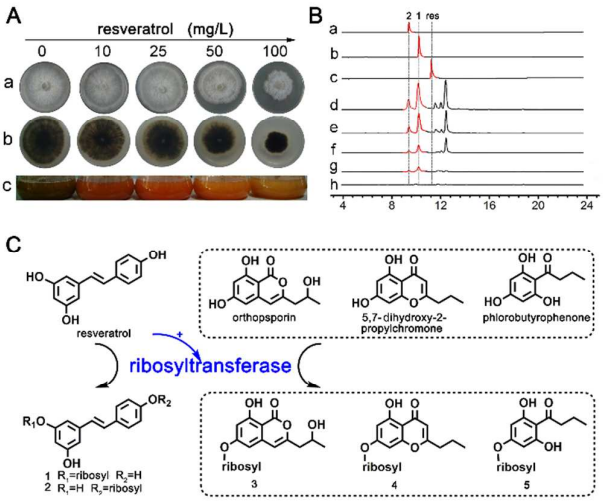

Figure 1. Ribosylation of resveratrol by *D. eschscholzii*. (A) Dose-dependent changes in the phenotype of the fungal strain exposed to resveratrol. The top (a) and bottom (b) views of petri dish and liquid cultivations (c) at 28 °C for 7 days. (B) LC-MS chromatograms of extracts derived from liquid fungal cultures with resveratrol (res) supplemented at 100 (d), 50 (e), 25 (f), 10 (g) and 0 mg/L (h), using an extracted ion at *m/z* 383.11 arising from e.g. $[M+Na]^+$ ion of 3- and 4'-*O*-α-D-ribosyl-resveratrol (**1** and **2**). (C) Ribosyltransferase gene activation by resveratrol enabled/up-regulated ribosylations of orthosporin, 5,7-dihydroxy-2-propylchromone and phlorobutyrophenone, there fungal products failed to be detected as glycosides in our previous laboratory culture of the fungus.

To assess the deviation of fungal metabolites in the xenochemical exposed cultures, the extracts derived from the culture broth at varied resveratrol concentrations were separately collected, and analyzed by the LC-UV-MS (Figure 1B). Encouragingly, a new peak on the chromatograph, corresponding to a UV curve resembling that of resveratrol was found, and gave a quasimolecular ion at *m/z* 383.1106 requiring an elemental composition of $C_{19}H_{20}O_7Na$ (calcd. weight, 383.1101). To elucidate the structure of the "newly appeared" metabolite, the resveratrol-supplemented fermentation was scaled up, and the afforded extract was fractionated by column chromatographies over silica gel and Sephadex LH-20, followed if necessary by semi-preparative HPLC to yield a new metabolite 3-*O*-α-D-ribosyl-resveratrol (**1**, Figure 1B), whose structure was elucidated by subsequent interpretations of its MS, UV and NMR spectra (^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, NOESY, HSQC and HMBC) as detailed in Supporting Information.

Resveratrol possesses 3,5- and 4'-hydroxy groups, the former two being chemically equivalent. The characterization of 3-*O*-α-D-ribosyl-resveratrol reminded the possible co-generation of its 4'-*O*-ribosylated counterpart. To address the probability, more scaled-up growth of the fungal strain was performed, followed by the LC-MS tracing analysis for the presence of the supposed 4'-*O*-ribosyl derivative of resveratrol. As expected, 4'-*O*-α-D-ribosyl-resveratrol (**2**) was characterized as another ribosylated metabolite of resveratrol as worked up for **1** (see above and Supporting Information). Further ribosylation of **1** or **2** was negligible since no di- and tri-ribosyl derivatives of resveratrol were detected in the original extract and the mother liquor resulted from the fractionation of the two resveratrol ribosides.

Prior to this work, no glycosidic metabolite could be isolated during our continuous chemical investigation on *D. eschscholzii*, capable of producing non-glycosidic phenolic polyketides.[6] In the fermentation of the strain with resveratrol at 50 mg/L, surprisingly, more novel ribosylated phenols, 6-*O*-α-D-ribosyl-orthosporin (**3**, $C_{17}H_{20}O_9$), 7-*O*-α-D-ribosyl-5-hydroxy-2-propylchromone (**4**, $C_{17}H_{20}O_8$) and 4-*O*-α-D-ribosyl-phlorobutyrophenone (**5**, $C_{15}H_{20}O_8$), were detected by LC-MS, and were further purified by the semi-preparative HPLC (Figure 1C). The aglycone motifs of the ribosides are derived from orthosporin, 5,7-dihydroxy-2-propylchromone and phlorobutyrophenone, which are all fungal metabolites.[6] Thus, resveratrol can facilitate *in vivo* ribosylation of some phenolic metabolites of the strain. In conclusion, the findings suggested that the genes responsible for ribosylation in *D. eschscholzii* are likely silent or poorly activated, but might be activated by the resveratrol exposure to lead to the regioselective mono-ribosylation of itself and some phenolic metabolites of the fungus.

Mechanism of the fungal ribosylation

Glycotransferases catalyse the endogenous glycosylation of small molecule substrates using sugar nucleotides as glycosyl donors.[4] The genome of *D. eschscholzii* (BioProject ID: PRJNA157267) contains as many as sixteen ribosylation-related genes governing potentially the expression of six ADP-ribosyltransferases (AT) and ten phosphoribosyltransferases (PT). Thus, a ribosyl transferation mechanism could be proposed for the fungal ribosylation. To confirm the assumption, we performed the resveratrol-exposed fungal cultivation supplemented simultaneously with sodium dodecyl sulfate (SDS), a glycosyltransferase inhibitor[16] that was tested in the study to affect negligibly the growth of *D. eschscholzii*. As anticipated, resveratrol 3-*O*- and 4'-*O*-α-D-ribosides **1** and **2** became undetectable by the LC-UV-MS analysis in the fungal culture exposed to SDS at 0.5 mM (Figure 2). This reinforced that the ribosyltransferases might have contributed to the resveratrol ribosylation, and as ascertained above, their encoding genes might be conventionally inactive.

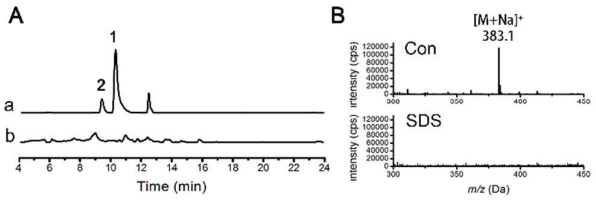

Figure 2. LC-MS assessment of resveratrol ribosylation in absence (a) and presence (b) of ribosyltransferase inhibitor SDS at 0.5 mM. All traces were monitored at EIC=*m/z* 383.1 (A) and ESI/MS at 10 min (B).

To clarify the regulative effect of resveratrol to the ribosyl-transferase gene expression of the fungus, the RNAs isolated from the five-day cultures with (50 mg/L) and without resveratrol were reversely transcripted to obtain the corre-sponding cDNAs with the mRNA transcriptional level of each ribosyltransferase assessed by real-time PCR approach (Figure 3). Among the sixteen ribosylation-related genes recognized from the genomic analysis, the expression of one AT- and five PT-encoding ones was increased by at least 5-fold in the presence of resveratrol compared with that of the untreated group. However, the other five AT-encoding genes were negligibly affected by the addition of resveratrol. This suggests that resveratrol can increase the expression level of endogenous ribosyltransferases with phosphoribosyl-transferases more sensitive to resveratrol than ADP-ribosyltransferases. Therefore, the ribosylation of resveratrol was most probably catalyzed by the phosphoribosyltransferase in *D. eschscholzii* although the particular enzyme failed to be identified through the gene knock-out approach because of the inevitable interference from the compensatory ribosylation catalyzed by undeprivable isoenzymes.

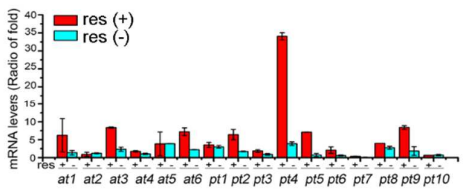

Figure 3. qRT-PCR comparison of the transcriptional levels of ADP-ribosyltransferases (at1~at6) and phosphoribosyltransferases (pt1~pt10) genes in *D.eschscholzii*

with [res(+), 50 mg/L] or without [res(−)] resveratrol supplementation.

Glycosyl donor for resveratrol ribosylation

We were curious about the glycosyl origin of the resveratrol ribosides, which is also of value in understanding the mechanism about the biological function of resveratrol. Exogenous D-ribose from culture or carbohydrate trans-formations[17] was first presumed to be a glycosyl donor for the resveratrol ribosylation. However, resveratrol failed to be ribosylated upon its co-incubation with D-ribose (150 mg/L) in the presence of laccase (10 U) prepared from the fungus as described.[18] We subsequently motivated to test the possible ribosylation by primary metabolic intermediates such as ribosyl-containing NDP-sugars, nucleotides and coenzymes (*e.g.*, NAD(H)s), both being active ribosylating agents.[19] In the fresh fungal homogenates (ultrasonicated thoroughly to liberate endogenous enzymes), resveratrol (2 mM) was allowed to react independently with the four potential ribosyl donors UDP, NAD^+, AMP and ADP, respectively. The LC-UV-MS monitoring demonstrated that ribosides **1** and **2** were formed after incubation with NAD^+ at 28 °C (Figure 4). But, no resveratrol riboside could be detected in the reaction mixture even after 24 hour incubation with UDP, AMP and ADP. In view that NAD^+ is relatively well described to ADP-ribosylate, but not monoribosylate, it should not be proposed as the direct donor for the resveratrol ribosylation. Further study showed that nicotinamide mononucleotide (NMN), a key intermediate produced in the NAD^+ biosynthetic pathway, could prominently increase the yields of **1** and **2** under the same conditions as tested with NAD^+, and the production of **1** was much higher that the NAD^+-treated one (Figure 4). Thus, in *D. eschscholzii*, NMN functions as an effective ribosyl donor for phenol ribosylations, which is catalyzed by phosphor-ribosyltransferase whose expression can be substantially activated by resveratrol exposure. Moreover, NAD^+ is probably cleaved by nucleotide pyrophosphatases to produce NMN for the ribosylation.[20]

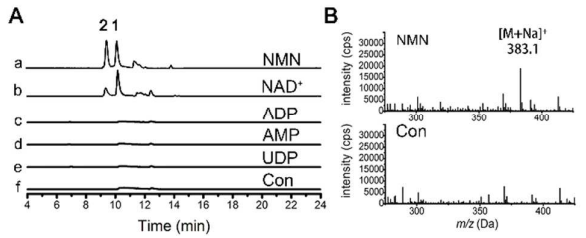

Figure 4. The ribosyl donor for resveratrol glycosylation. LC-MS monitorings of fungal homogenate treatments of resveratrol without (f) and with addition of NMN (a), NAD+ (b), ADP (c), AMP (d) and UDP (e). All traces were monitored at EIC=*m/z* 383.1 (A) and ESI/MS at 10 min (B).

Previous investigations demonstrated that ribosylation of peptides is generally associated with ADP-ribosyltransferases using NAD^+ as the source of ribose, and glycosylation of small molecule aglycones occurs by using NDP-sugars as donors. Distinct from the observation, our results suggests a novel mechanism for the phosphor-ribosyltransferase catalyzed ribosylation of small organic molecules with NMN as the ribose donor.

Ribosylation as a detoxifying mechanism of *D. eschscholzii* upon exposure to resveratrol

The nematode (*Caenorhabditis elegans*) has been reported to detoxify alkaloidal and phenolic toxins through glucosylation.[21] The glucosylation-based detoxification mechanism is also adopted by the bacterial pathogen *Helicobacter pylori* for the deactivation of 7-dehydrocholesterol,[22] by the phytopathogenic fungus *Sclerotinia sclerotiorum* for metabolizing cruciferous phytoalexins camalexin, brassinin, cyclobrassinin and brassilexin,[23] and by plants *Nicotiana tabacum* for the naphthol inactivation[24] and *Arabidopsis thaliana* for detoxifying xenobiotic chemicals such as benzoxazolin-2(3H)-one[25] and 3,4-dichloroaniline.[26] Resveratrol is a phytoalexin[15] and toxic to *D. eschscholzii* (see above). We hypothesized that the ribosylation of resveratrol could contribute to the fungal detoxification of the phytoalexin. Accordingly, resveratrol and its ribosides were tested at 0.5 mM in 96-well plates using the fungus as a target microbe. As hypothesized, the fungal germination appeared in the plates treated with **1** and **2** was much stronger than that discerned in resveratrol-treated group (Figure 5A). Thus, the fungicidal effect of resveratrol to *D. eschscholzii* is diminished significantly by the fungal ribosylation of the phytoalexin.

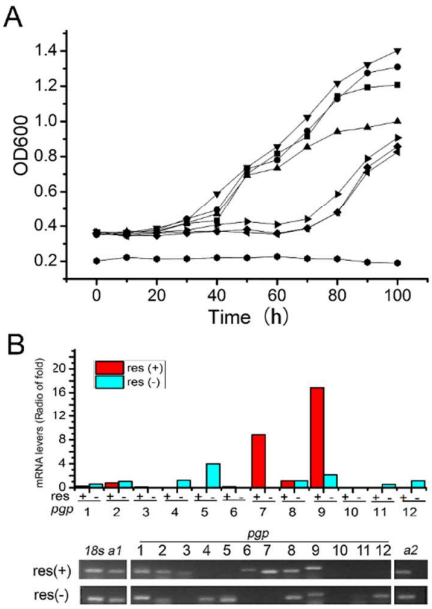

Figure 5. Detoxification of *D. eschscholzii* to resveratrol. A. The susceptibility of *D. eschscholzii* toward resveratrol and its ribosides. Spores of *D. eschscholzii* were incubated separately in malt-extract culture at 28 °C with exposure to resveratrol, ribosides **1** and **2** (0.5 mM). The germination rates of these spores were determined according to the absorption at OD600. ▼: Wild type strain without addition of any test compound. ▲, ■ and ●: Wild type strain supplemented with resveratrol, **1** and **2**, respectively. ♦, ◄ and ►: Resveratrol-exposed

cultures of *Δpgp3*,*Δpgp6* and *Δpgp11* strains, respectively. ◊: The malt-extract medium taken as a control. The data are the mean of triplicates with S.D. ≤5%. B. qRT-PCR comparison of the transcriptional levels and gel electrophoresis for qRT-PCR products of p-glycoprotein genes (*pgp1*−*pgp12*) from the strain with [res(+), 50 mg/L] or without [res(–)] exposure to resveratrol, using 18S rRNA (*18s*) and actin-encoding genes (*a1* and *a2*) as controls.

To get more information about the detoxification machinery of *D. eschscholzii* to resveratrol, we scrutinized the genomic information of the fungus, indicating that the fungus carries a total of forty-three ATP-binding cassette (ABC) transporter genes with twelve predicted to encode p-glycoproteins (pgps). Previous studies have showed that the ABC transporters were functional proteins capable of transporting secondary metabolites,[27] and one of its subfamily, pgp, is involved in the drug resistance in mammals.[28] However, the functions of ABC proteins have been largely unknown for fungi although increased expression of an ABC transporter gene was discerned earlier in *Aspergillus fumigatus*.[29] The real-time PCR analysis was therefore performed to examine the presumed regulation of pgp genes in *D. eschscholzii* by resveratrol (50 mg/L, 5 day exposure) with 18S rRNA and two actin encoding genes allotted as internal references. As a result, three pgp genes *pgp*3, *pgp*6 and *pgp*7 were substantially up-regulated (Figure 5B). In particular, these three pgp genes are usually inactive in the intact strain (Figure 5B), but can be activated by the fungal exposure to resveratrol.

As shown by phylogenetic analysis, these pgp genes are significantly diverse in sequence, and can be classified into three different subfamilies of ABC transporter proteins (Figure S1),[30] The disruption of the above activated pgp genes was carried out using modified method of split-PCR (Figure S2).[31] Toxicity assays showed that upon treatment with resveratrol at 0.5 mM, the *D. eschscholzii pgp*-3 and *pgp*-6 mutants were much more susceptible to resveratrol than the wild strain (Figure 5A). *Pgp*3 belongs to the full-size superfamily B involved in the oxidative stress response, and is required for the host penetration, an initial key step for the microbial colonization in host tissues.[32] The *pgp*3 knockout strain of *D. eschscholzii* showed reduced production of resveratrol riboside **2**, a "detoxification" product of the phytoalexin (Figure 6). The *pgp*6, possessing an excessive C-terminal relative to other pgps, is unique with low (46%) identity to the most similar ABC transporters described to date and belongs to the superfamily G whose biochemical function remains elusive.[25] Thus, the *pgp*6 represents a new-type ABC transporter. Our data showed that the pgp6 knockout strain of *D. eschscholzii* was unable to transform resveratrol into its riboside **1** or **2** (Figure 6). In addition, a contrapositive gene-deletion experiment was also carried out with the gene *pgp*11, a lipid transporter under the superfamily A[33] and showed no transcriptional change between the resveratrol-treated and -untreated strains (Figure 5B). As expected, the deletion of *pgp*11 did not affect the generation of ribosides **1** and **2** (Figure 6). The data collectively suggest that resveratrol can be detoxified by the resistance machinery in the fungus, and the fungal p-glycoproteins *pgp*3 and *pgp*6 are involved in the detoxification process.

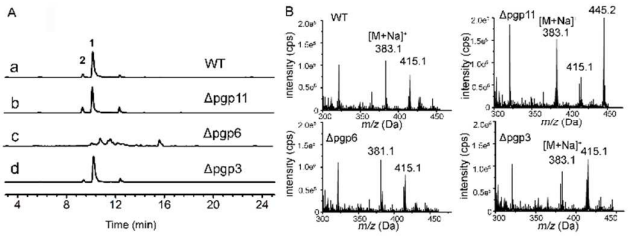

Figure 6.LC-MS evaluated transformation of resveratrol into **1** and **2** by wild-type (a), *pgp*11- (b), *pgp*6- (c) and *pgp*3-deleted (d) strains of *D. eschschozii*. All traces were monitored at EIC=*m/z* 383.1 (A) and ESI/MS at 10 min (B).

Experimental Section

Strain, cultivation, and metabolite fractionation

D. eschscholzii IFB-TL01 was described earlier.[6] The fresh mycelium taken from the fungal colony in the petri dish was inoculated in malt-extract medium (20 g/L malt extract, 20 g/L sucrose, 1 g/L peptone), followed by resveratrol (150 mg/L)-supplemented cultivation for 7 days at 28 °C and 200 rpm/min. The broth was extracted with EtOAc, and *in vauo* evaporations of the solvent gave a residue which was subjected to column chromatography over silica gel (100 g, 200–300 mesh, 50 × 3 cm) eluted with CH_2Cl_2/MeOH mixtures (v/v 100:0, 100:1, 100:2, 100:4, 100:8, 100:16, 100:32, 0:100). Further purification of the "100:16" eluate was accomplished by gel filtration over Sephadex LH-20 in MeOH, followed by semi-preparative HPLC (MeOH/H_2O, 3:7) to give **1** (R_t= 30.9 min) and **2** (R_t= 29.7 min). Purification of the "100:4" eluate by Sephadex LH-20 and by HPLC (MeOH/H_2O, 53:47) gave **3** (R_t= 14.0 min). Purification of the "100:4" eluate by Sephadex LH-20 and by HPLC (MeOH/H_2O, 45:55) gave **4** (R_t= 20.0 min) and **5** (R_t= 24.0 min).

LC-UV-MS analysis

The filtrates of the fungal fermentation was extracted with EtOAc, and *in vacuo* evaporation of solvent from the extract gave a residue which was dissolved in MeOH and filtrated using a 0.45 μm membrane filter. The LC-MS measurements were performed on an Agilent 1200 series LC system hyphenated with an Agilent 6210 TOF mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) and with an Agilent ZORBAX Eclipse Plus C18 column (3.5 μm, 100 mm×4.6 mm) using MeOH as mobile phase.

Glycosyltransferase inhibition by SDS

To test the effect of glycosyltransferase inhibitor on the ribosylation of resveratrol, the fungus strain was grown with resveratrol at 50 mg/L to the logarithmic phase (28 °C, 48 h), SDS at 0.5 mM was added into the culture and incubated at 28 °C for another 7 days. The filtrate of the fermentation was extracted with EtOAc, and analyzed by LC-MS.

Reaction of resveratrol with potential ribosyl donors

The *D. eschscholzii* cells afforded after grown at 28 °C for 7 days in presence of resveratrol (50 mg/L) were suspended in Tris-HCl buffer (20 mM, pH 7.5) and ultrasonicated to liberate the enzyme. The presumed sugar donors (UDP, NAD^+, NMN, AMP, and ADP) were separately added at 4 mM to test tubes preloaded with 2 ml of the enzyme mixture containing resveratrol at 2 mM, followed by incubation at 28 °C. The filtrates of reaction mixtures taken from the tubes were analyzed by LC–MS to monitor the production of resveratrol ribosides **1** and **2**. Equally processed enzyme mixture without exposure to resveratrol and any saccharide donor was taken as a control.

Real-Time PCR analysis

Total RNA of the fungus with or without treatment of resveratrol for 4 days was isolated by using the RNAiso Plus (Takara). cDNA was generated from equal amounts of RNAs of the assayed strains by using an PrimeScript II 1st Strand cDNA Synthesis Kit (Takara). Quantitative real-time PCR (qRT-PCR) was performed with One Step SYBR PrimeScript RT-PCR Kit (Takara) using the C1000 Thermal Cycler (Bio-Rad) and quantified using CFX96 Real-Time System (Bio-Rad). The primers used for qRT-PCR are tabulated in Supporting Information. The expression of assayed genes was normalized to the endogenous 18S rRNA gene and actin-encoding genes for variation in RNA quantity and quality.

Susceptibility Test

Modified broth-based method was used to examine the growth rate of *D. eschscholzii* and its mutants. Strains were grown on PDA plates for 10 days, the spores were collected and were diluted to a starting inoculum of 1×10^4 CFU/mL (OD_{600}=0.4) in malt-extract medium. They were further incubated with resveratrol or its ribosides at 0.5 mM in 96-well microdilution plates (200 μl) and were cultivated at 28 °C for 0-100 h. The germinate rates of the stains were determined with the absorption at OD_{600}.

Knockout of pgp genes

The fungus was grown on potato dextrose agar plates (200 g/L boiled potato, 20 g/L sucrose, 15 g/L agar) for 10 days at 28 °C. The spores were collected and treated with 10 mg/L glucanex in order to harvest the protoplasts. To knockout a pgp gene of interest, two split-marker cassettes were constructed. Each cassette contains sequences flanking the target gene (*pgp*U for upstream sequence and *pgp*D as downstream one) fused to an incomplete gene fragment of hygromycin as a selection marker. Both of the cassettes were electroporated (1.4 kV, 25 mF, 800 Ω) into the above protoplasts. Mutated colonies were selected in PDA medium containing hygromycin (250 μg/mL), and identified by diagnostic PCR. Primers used in this work were listed in Supporting Information.

Accession Codes

The nucleotide sequences of *pt*4, *pgp*3, *pgp*6, *pgp*7 and *pgp*11 have been deposited at the GenBank under accession numbers KJ462507, KF030722, KF030723, KF030724 and KF030725.

Conclusions

In summary, resveratrol up-regulates the expression of the ribosylation-related genes of *D. eschscholzii*, and the activated ribosyltransferase gene expressions enable the ribosylation of the phytophenol and four fungal phenols. The resveratrol ribosylation requires a sugar donor of nicotinamide mononucleotide (NMN) which is likely attacked by resveratrol oxygen atoms on a nucleophilic substitution basis to generate the α-configuration of the anomeric carbon. Biochemically, at least two p-glycoproteins associated with the drug resistance, are involved in the resveratrol ribosylation. More biological implication lies in the substantial toxicity reduction of resveratrol to *D. eschscholzii*, suggesting a general ribosylation-based detoxification mechanism concerning the fungal endurance to the phytoalexin. Providing an access towards the new phenolic riboside library, the work adds the direct chemogenetic evidence helping to understand the molecular mechanism of resveratrol which is active in a broad range of prokaryotic and eukaryotic cells. The investigation suggests as well that the silent or less active fungal genes may be a "reserved arsenal" ready to cope with challenges and hostilities. The research methods in this study are significant to the basic and applied sciences of natural product drug discovery and promote the development of research on the technology of interdisciplinary subjects. Additionally, with the discovery and modification of new drug molecules, it paves a new way for drug discovery.

Acknowledgements

This work was co-financed by the NSFC (91413120, 21132004, 81421091 & 21302091) and MOST grants (2013AA092901), in addition to supports from Natural Science Foundation of Jiangsu Province (BK2012308) and State Key Laboratory of Bioreactor Engineering.

Keywords: resveratrol • ribosylation • nicotinamide mononucleotide • detoxification.

Corresponding Authors

*rxtan@nju.edu.cn, *rhjiao@nju.edu.cn

Author Contributions

Guo-Yan Zhao and Jing-Yang Fan contributed equally to the work.

1 C. Gatsogiannis, A. E. Lang, D. Meusch, V. Pfaumann, O. Hofnagel, R. Benz, K. Aktories, S. Raunser, *Nature,*2013, **495**, 520-523； A. A. Sauve, *BBA-Proteins Proteom.,*2010, **1804**, 1591-1603.

2 T. Jank, K. Aktories, *Proc. Natl. Acad. Sci. U.S.A,***2013**, *110*, 4163-4164.； K. Aktories, *Nat. Rev. Microbiol.,*2011, **9**, 487-498.

3 S. Quan, T. Imai, Y. Mikami, K. Yazawa, E. R. Dabbs, N. Morisaki, S. Iwasaki, Y. Hashimoto, K. Furihata, *Antimicrob. Agents Ch,.* 1999, **43**, 181-184.

4 G. J. Williams, C. Zhang, J. S. Thorson, *Nat. Chem. Biol.,*2007, **3**, 657-662；A. D. Harper, M. Bar-Peled, *Plant Physiol.,*2002, **130**, 2188-2198； C. J. Thibodeaux, C. E. Melancon, H. W. Liu, *Nature,***2007**, *446*, 1008-1016.

5J. Chen, L. C. Zhang, Y. M. Xing, Y. Q. Wang, X. K. Xing, D. W. Zhang, H. Q. Liang, S. X. Guo, *Plos One,*2003, **8** e58268.

8 C. P. Woloshuk, W. B. Shim, *FEMS Microbiol. Rev.,*2013, **37**, 94-109.

9 G. J. Soleas, E. P. Diamandis, D. M. Goldberg, *Clin. Biochem.,*1997, **30**, 91-113.

10 S. J. Park, F. Ahmad, A. Philp, K. Baar, T. Williams, H. Luo, H. Ke, H. Rehmann, R. Taussig, A. L. Brown, M. K. Kim, M. A. Beaven, A. B. Burgin, V. Manganiello, J. H. Chung, *Cell,*2012, **148**, 421-433; S. Bae, E. M. Lee, H. J. Cha, K. Kim, Y. Yoon, H. Lee, J. Kim, Y. J. Kim, H. G. Lee, H. K. Jeung, Y. H. Min, S. An, *Mol. Cells,*2011, **32**, 243-249.

11 L. X. Wang, A. Heredia, H. J. Song, Z. J. Zhang, B. Yu, C. Davis, R. Redfield, *J. Pharm. Sci.,*2004, **93**, 2448-2457.

12 H. Imai, M. Kitagawa, K. Ishihara, N. Masuoka, K. Shimoda, N. Nakajima, H. Hamada, *Biosci. Biotech. Bioch.,*2012, **76**, 1552-1554.

13 T. Desmet, W. Soetaert, P. Bojarova, V. Kren, L. Dijkhuizen, V. Eastwick-Field, A. Schiller, *Chem.-Eur. J.,*2012, **18**, 10786-10801.

14 I. Ahuja, R. Kissen, A. M. Bones, *Trends Plant Sci.,*2012, **17**, 73-90; A. Hammerbacher, S. G. Ralph, J. Bohlmann, T. M. Fenning, J. Gershenzon, A. Schmidt, *Plant Physiol.,*2011, **157**, 876-890; P. Jeandet, B. Delaunois, A. Conreux, D. Donnez, V. Nuzzo, S. Cordelier, C. Clement, E. Courot, *BioFactors,*2010, **36**, 331-341.

15 I. Ahuja, R. Kissen, A. M. Bones, *Trends Plant Sci.,*2012, **17**, 73-90. b) B. Delaunois, S. Cordelier, A. Conreux, C. Clement, P. Jeandet, *Plant Biotechnol., J.* 2009, **7**, 2-12.

16 H. X. Ge, J. Zhang, C. Kai, J. H. Liu, B. Y. Yu, *Appl. Microbiol. Biotechnol.,*2012, **93**, 2357-2364.

17 Z. Ahmed, *EJB Electron. J. Biotechn.,*2011, **4**, 1-9.

18 W. Fang, S. Ji, N. Jiang, W. Wang, G. Y. Zhao, S. Zhang, H. M. Ge, Q. Xu, A. H. Zhang, Y. L. Zhang, Y. C. Song, J. Zhang, R. X. Tan, *Nat. Commun.,*2012,**3**, DOI: 10.1038/ncomms2031;M. Marzorati, B. Danieli, D. Haltrich, S. Riva, *Green Chemistry* 2005, **7**, 310-315.

19 R. W. Gantt, P. Peltier-Pain, S. Singh, M. Zhou, J. S. Thorson, *Proc. Natl. Acad. Sci. U.S.A,*2013, **110**, 7648-7653; H. Lin, *Org. Biomol. Chem.,*2007, 5, 2541-2554.

20 R. K. Gholson, *Nature,*1966, **212**, 933-935.

21 G. S. Stupp, S. H. von Reuss, Y. Izrayelit, R. Ajredini, F. C. Schroeder, A. S. Edison, *ACS Chem. Biol.,*2013, **8**, 309-313.

22 H. Shimomura, K. Hosoda, D. J. McGee, S. Hayashi, K. Yokota, Y. Hirai, *J. Bacteriol.,*2013, **195**, 359-367.

23 M. S. C. Pedras, S. Hossain, R. B. Snitynsky, *Phytochemistry,*2011, **72**, 199-206; A. C. Sexton, Z. Minic, A. J. Cozijnsen, M. S. C. Pedras, B. J. Howlett, *Fungal Genet. Biol.,*2009, **46**, 201-209.

24 G. Taguchi, M. Nakamura, N. Hayashida, M. Okazaki, *Plant Sci.,*2003, **164**, 231-240.

25 S. R. Baerson, A. Sanchez-Moreiras, N. Pedrol-Bonjoch, M. Schulz, I. A. Kagan, A. K. Agarwal, M. J. Reigosa, S. O. Duke, *J. Biol. Chem.,*2005, **280**, 21867-21881.

26 C. Loutre, D. P. Dixon, M. Brazier, M. Slater, D. J. Cole, R. Edwards, *Plant J.,*2003, **34**, 485-493.

6 Y. L. Zhang, H. M. Ge, W. Zhao, H. Dong, Q. Xu, S. H. Li, J. Li, J. Zhang, Y. C. Song, R. X. Tan, *Angew. Chem.-Int. Edit.,*2008, **47**, 5823-5826；Y. L. Zhang, J. Zhang, N. Jiang, Y. H. Lu, L. Wang, S. H. Xu, W. Wang, G. F. Zhang, Q. Xu, H. M. Ge, J. Ma, Y. C. Song, R. X. Tan, *J. Am. Chem. Soc.,*2011, **133**, 5931-5940；W. Fang, S. Ji, N. Jiang, W. Wang, G. Y. Zhao, S. Zhang, H. M. Ge, Q. Xu, A. H. Zhang, Y. L. Zhang, Y. C. Song, J. Zhang, R. X. Tan, *Nat. Commun.,*2012, **3**, DOI: 10.1038/ncomms2031.

7 S. Raffaele, S. Kamoun, *Nat. Rev. Microbiol.,*2012, **10**, 417-430; J. P. McCutcheon, N. A. Moran, *Nat. Rev. Microbiol.,*2012, **10**, 13-26.

27 L. Cuthbertson, V. Kos, C. Whitfield, *Microbiol. Mol. Biol. Rev.,*2010, **74**, 341-362.

28 C. F. Higgins, *Nature,*2007, **446**, 749-757;M. S. Jin, M. L. Oldham, Q. Zhang, J. Chen, *Nature,*2012, **490**, 566-569.

29 J. W. Slaven, M. J. Anderson, D. Sanglard, G. K. Dixon, J. Bille, I. S. Roberts, D. W. Denning, *Fungal Genet. Biol.,*2002, **36**, 199-206.

30 A. Kovalchuk, A. J. M. Driessen, *BMC Genomics,*2010, **11**, 177.

Journal Name

RSCPublishing

ARTICLE

A table of contents entry.

Conventionally undetectable fungal ribosylations of phenols is addressed to provide evidence for microbial detoxification mechanisms and access to new ribosides.