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Note

A Tolerance Gene for Prenylated Flavonoid Encodes a 26S Proteasome Regulatory Subunit in *Sophora flavescens*

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Yeast functional screening with a *Sophora flavescens* cDNA library was performed to identify the genes involved in the tolerant mechanism to the self-producing prenylated flavonoid sophoraflavanone G (SFG). One cDNA, which conferred SFG tolerance, encoded a regulatory particle triple-A ATPase 2 (SfRPT2), a member of the 26S proteasome subunit. The yeast transformant of SfRPT2 showed reduced SFG accumulation in the cells.

Key words: prenylated flavonoids; *Sophora flavescens*; functional screening; proteasome subunit; 26S proteasome

Plants produce a large variety of natural products categorized as secondary metabolites, which have diverse chemical structures and biological activities. These organic compounds play important roles in plants as endogenous chemical barriers protecting plants against pathogens or herbivores due to their strong cytotoxicity or antimicrobial activities. These active secondary metabolites are potentially toxic to the plants, but the plants producing them appear to be insensitive to their own metabolites. This suggests that they have a species-specific tolerance mechanism,[1)] but understanding of this mechanism is very limited.

Prenylated flavonoids have been identified as active components of various medicinal plants. They show a variety of biological activities, including antimicrobial, antitumor, anti-leushmania, and anti-NO production.[2,3)] *Sophora flavescens*, a leguminous plant, produces diverse prenylated flavonoids, including sophoraflavanone G (SFG) and kurarinone. Since 2.5 μg/mL of SFG reduced the root growth of *Lotus japonicus* to 64% as compared to control (our unpublished data), this compound is potentially toxic to a SFG-non-producing plant. Cultured cells of this plant produce SFG as a major flavonoid (0.3 mg/20 mL medium), and appear to be insensitive to this metabolite.[4,5)]

In order to clarify the mechanism of tolerance for prenylated flavonoids in this plant species, we performed yeast functional screening with the *S. flavescens* cDNA library, which was constructed using a yeast expression vector.[6)] To screen for tolerant genes, the plasmid library was introduced into *Saccharomyces cerevisiae* strain AD12345678 (*yor1*Δ, *snq2*Δ, *pdr5*Δ, *pdr10*Δ, *pdr11*Δ, *ycf1*Δ, *pdr3*Δ, *pdr15*Δ),[7)] which is sensitive to various drugs due to its lack of many pleiotropic drug-resistance (PDR) genes. Screening for the tolerance-genes was performed with SFG-containing plates at a concentration toxic to this yeast strain. Colonies that grew on one-half strength (1/2) synthetic dextrose (SD) medium containing 8 μg/mL of SFG were chosen, and in a second screening, each colony selected was preincubated in SD medium again and then spotted onto 1/2 SD medium to which several concentrations of SFG were added. These SFG-tolerant colonies are shown in Fig. 1A. Screening of approximately 2×10^5 primary transformants allowed us to isolate 20 clones that showed tolerance to SFG. For further confirmation (third screening), the plasmids were recovered from those yeast transformants and re-introduced into the same host strain. By using these re-introduced transformants, serial dilution spot tests were done on SFG-containing plates. As for the results, one clone showed distinct SFG tolerance, as shown in Fig. 1B. This cDNA encoded a putative polypeptide composed of 446 amino acids and showed strong similarity with regulatory particle triple-A ATPase 2 (RPT2), a member of the 26S proteasome subunit. Hence we designated this gene *Sfrpt2* (*Sophora flavescens* Regulatory Particle triple-A ATPase 2) (accession no. AB583750).

The 26S proteasome plays an essential role in protein degradation and turnover in eukaryotes. It is a multisubunit ATP-dependent protease complex assembled from two particles: a 20S core particle (CP) and a 19S regulatory particle (RP).[8)] RPT2 is one of the triple-A ATPase subunits of 19S RP, which catalyzes ATP hydrolysis in the protein degradation process. A BLAST search showed that SfRPT2 has relatively moderate sequence identity to RPT2s of non-plant organisms, *viz.*, *Homo sapiens* (75%), *Drosophila melanogaster* (76%), and *Saccharomyces cerevisiae* (65%). SfRPT2 has high amino acid sequence identity (>90%) with other RPT2s of higher plant species (Fig. 2). It contains a characteristic triple-A ATPase motif: Walker A, Walker B, and

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Abbreviations: PDR, pleiotropic drug-resistance; RPT, regulatory particle triple-A ATPase; SFG, sophoraflavanone G; SD, synthetic dextrose

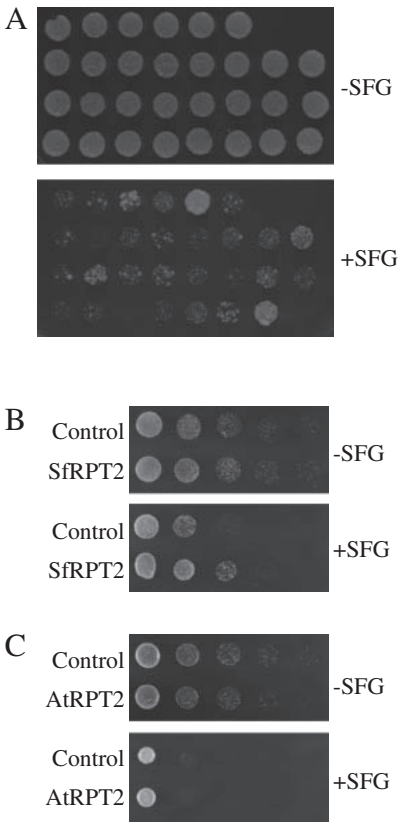

Fig. 1. Screening of the SFG-Tolerant Gene by Yeast Functional Screening.

A, Representative yeast functional screening on an SFG-containing plate (second screening). Colonies showing better growth on 8 μg/mL of SFG-containing 1/2 SD medium (-uracil) at the first screening were chosen and transferred into liquid SD (-uracil), and grown overnight for pre-culture. Cell densities were adjusted to $OD_{600} = 0.5$, cells (5 μL each) were spotted on the same SFG-containing 1/2 SD medium (-uracil), and SFG tolerance was re-evaluated. B, A yeast transformant of pDR196 (vector control) or SfRPT2 was pre-cultured, diluted to a given density, $A_{600} = 0.5$, and serially diluted 3-fold. A 5-μL aliquot was spotted onto an 1/2 SD medium (-uracil) plate containing 0 or 9.5 μg/mL SFG, and growth was monitored after incubation of the cells for 45 h (without SFG) or 70 h (SFG-containing plate) at 30 °C. C, A yeast transformant of pDR196 (vector control) or AtRPT2 was pre-cultured, diluted to a given density, $A_{600} = 0.5$, and serially diluted 3-fold. A 5-μL aliquot was spotted onto an 1/2 SD medium (-uracil) plate containing 0 or 9.0 μg/mL SFG, and growth was monitored after incubating the cells for 45 h (without SFG) or 50 h (SFG-containing plate) at 30 °C.

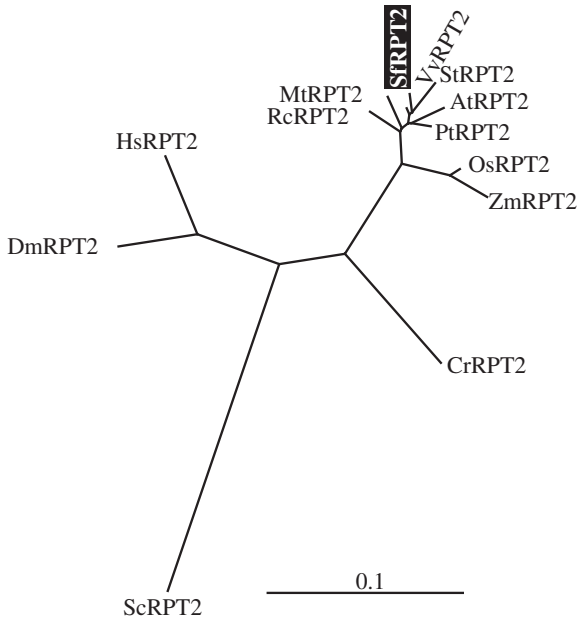

Fig. 2. Phylogenetic Tree of RPT2 Proteins.

The tree is based on multiple alignment of the peptide sequences of RPT2s from *S. flavescens* and other species using the CLUSTAL W program (http://clustalw.ddbj.nig.ac.jp/top-j.html). The resulting tree was produced using TREEVIEW. The accession no.: VvRPT2 (*Vitis vinifera*, XP_002263334), PtRPT2 (*Populus trichocarpa*, XP_002301829), MtRPT2 (*Medicago truncatula*, ABD32889), AtRPT2 (*Arabidopsis thaliana*, NP_194633), StRPT2 (*Solanum tuberosum*, ABB02638), RcRPT2 (*Ricinus communis*, XP_002526219), OsRPT2 (*Oryza sativa*, NP_001049843), ZmRPT2 (*Zea mays*, NP_001146268), CrRPT2 (*Chlamydomonas reinhardtii*, XP_001690446), DmRPT2 (*Drosophila melanogaster*, NP_524469), HsRPT2 (*Homo sapiens*, BAD96388), ScRPT2 (*Saccharomyces cerevisiae*, CAY78502).

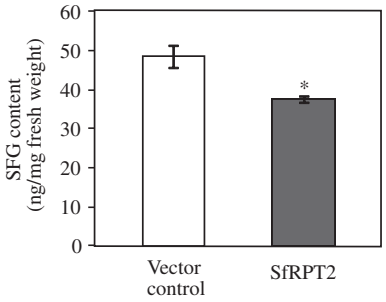

Fig. 3. SFG Content of SfRPT2-Expressing Yeast Cells.

A yeast transformant of SfRPT2 was pre-cultured in SD medium (-uracil), and suspended by 15 mL of 1/2 SD medium (-uracil) containing 4 μg/mL of SFG at $A_{600} = 0.5$. The cells were incubated at 30 °C with shaking at 200 rpm, harvested at 6.5 h by centrifugation, and washed twice with deionized cold water. Next, they were disrupted with acid-washed glass beads in extraction buffer (50% methanol). Samples were centrifuged, and the supernatant was subjected to LC-MS analysis. The LC-ESI-MS system used for the assay was a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) coupled to an API3000 LC-MS system (Applied Biosystems, Foster City, CA) equipped with an ESI interface. SFG was separated on a Cosmosil $5C_{18}$ MS-II column (5 μm, 4.6 mm × 150 mm, Nacalai Tesque, Kyoto, Japan). The mobile phase was flow rate, 0.2 mL/min, and solvent system (A) water containing 0.3% v/v formic acid and (B) acetonitrile: a 10 min linear gradient from 50 to 85% B, followed by 30 min 85% B. Asterisks indicate statistically significant difference as compared to control (Student's *t*-test; $^{*}p < 0.01$).

arginine finger. It has been reported that the plant 26S proteasome is involved in several physiological functions including plant-pathogen interaction[9] and control of trichome endoreduplication,[10] but to our knowledge, there is no finding that plant RPT2 is involved in tolerance of secondary metabolites. AtRPT2, an Arabidopsis ortholog with 97% amino acid identity to SfRPT2, did not show SFG tolerance (Fig. 1C). This suggests that SfRPT2-mediated tolerance is species-specific, although the details of the mechanism are unclear.

To determine the basis of the SFG tolerance conferred by SfRPT2, the SFG content in SfRPT2-expressing yeast cells was measured by LC/ESI-MS. The cellular level of SFG in SfRPT2-expressing yeast grown in SFG-containing medium was significantly lower than that in the empty vector transformant (Fig. 3). This suggests that heterologous expression of SfRPT2 prevents cellu-

lar accumulation of this toxic compound, although the molecular mechanism by which SfRPT2 reduced SFG content in the cells remains to be determined. In *S. flavescens*-cultured cells, endogenously produced SFG is secreted from the cytosol to the apoplastic space and accumulates in cell-wall fraction, which suggests that a decrease in the cellular concentration of SFG might be one tolerance mechanism. Since the addition of purified RPT to proteasome accelerated protein degradation *in vitro*,[11] SfRPT2 might enhance the degradation of proteins damaged by SFG toxicity. This should be beneficial in recycling and synthesis of functional proteins, such as putative SFG exporter, and it should help in maintaining cellular homeostasis. Thus yeast SfRPT2 transformant might show better growth and reduced cellular SFG contents. In conclusion, SfRPT2 is one possible way to confer SFG tolerance in the complicated tolerant mechanism in *S. flavescens*-cultured cells.

Prenylated flavonoids have beneficial effects on human health,[3] and hence they attract substantial interest as lead compounds for new drugs and for application in functional foods, but most of them occur at low levels in nature. The prenyl moieties are crucial to these biological activities, and the first plant flavonoid prenyltransferase, a naringenin 8-dimethylallyltransfesrase cDNA (SfN8DT), was isolated from *S. flavescens*.[6] Our recent attempt at biotransformation using a transgenic yeast expressing SfN8DT successfully produced 8-dimethylallylnaringenin, a prenylated flavonoid showing a potent phytoestrogen, and indicates the possibility of generating prenylated flavonoids that occur only rarely in nature by biotransformation.[12] However, probably due to the toxicity of the prenylated product, the yeast growth and production rates decreased under a high concentration of substrate, and a solution to this problem aiming to the increase in the productivity is desirable. The introduction of SfRPT2, isolated in this study as a gene tolerant of prenylated flavonoid, to the yeast SfN8DT transformant might be useful method to increase the productivity of prenylated flavonoids.

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