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Heterologous expression of furostanol glycoside 26-*O*-β-glucosidase of *Costus speciosus* in *Nicotiana tabacum*

Koji Ichinose[a], Song You[b], Noriaki Kawano[a], Kaori Hayashi[a], Xin-Sheng Yao[b], Yutaka Ebizuka[a,*]

[a]*Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[b]*Department of Natural Product, Shenyang Pharmaceutical University, Wen-hua Road, Shenyang 110015, People's Republic of China*

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Abstract

Furostanol glycoside 26-*O*-β-glucosidase (F26G) is a specific β-glucosidase converting a furostanol glycoside (FG) to its corresponding spirostanol glycoside (SG). A cDNA encoding F26G from *Costus speciosus* was introduced into a heterologous plant, *Nicotiana tabacum* via *Agrobacterium tumefaciens* using a binary vector method. Successful integration of the cDNA into tobacco chromosomal DNA was confirmed by PCR analysis. F26G activity was also detected in cell-free extracts of the transgenic plantlets.

Keywords: Costus speciosus; Zingiberaceae; β-glucosidase; Furostanol glycoside; Steroid saponin; Spirostanol glycoside; *Agrobacterium tumefaciens*; *Nicotiana tabacum*

1. Introduction

Steroidal saponins (SSs) are one of the major classes of saponins, mostly occurring as furostanol glycosides (FGs) as well as spirostanol glycosides (SGs) (Mahato, Ganguly, & Sahu, 1982; Marston & Hostettmann, 1991). SG is biosynthesized from its corresponding FG by a β-glucosidase (F26G) cleaving the 26-*O*-β-glucosidic bond to form the E/F spiro rings. Monocotyledonous (monocot) families such as the Liliaceae, Dioscoreaceae and Amaryllidaceae are known to be rich sources of SSs (Mahato et al., 1982). Our recent studies on *Costus speciosus* (Koenig.) SM. (Zingiberaceae) identified a number of FGs and SSs in the rhizome (Inoue, Kobayashi, Noguchi, Sankawa, & Ebizuka, 1995). It was also demonstrated that a specific F26G (CSF26G) is involved (Inoue, Shimomura, Kobayashi, Sankawa, & Ebizuka, 1996) in the formation of SGs in this species by its purification and enzymatic characterization (Inoue & Ebizuka, 1996a, 1996b). Subsequent molecular cloning of a cDNA encoding CSF26G followed by functional expression in *E. coli* (Inoue, Shibuya, Yamamoto, & Ebizuka, 1996) afforded possibilities of detailed biosynthetic and physiological investigations of SS. In the present study, the CSF26G was subjected to heterologous plant expression.

2. Results and discussion

F26G from *C. speciosus* shows a high substrate specificity for its native FG such as protodioscin and protogracillin (Inoue & Ebizuka, 1996a, 1996b), indicating its specific role in the conversion of FG to SG. One of the interesting applications of this gene is its use in metabolic engineering of other SS-producing plants. The fact that *N. tabacum* accumulates FGs in its seeds (Grünweller, Schröder, & Kesselmeier, 1990; Shvets, Kintia, & Gutsu, 1994, 1995; Shvets, Gutsu, & Kintia, 1996) and the readily available transformation techniques using *Agrobacterium*, led us to use tobacco

* Corresponding author. Tel./fax: +81-3-5689-2962.
E-mail address: yebiz@mol.f.u-tokyo.ac.jp (Y. Ebizuka)

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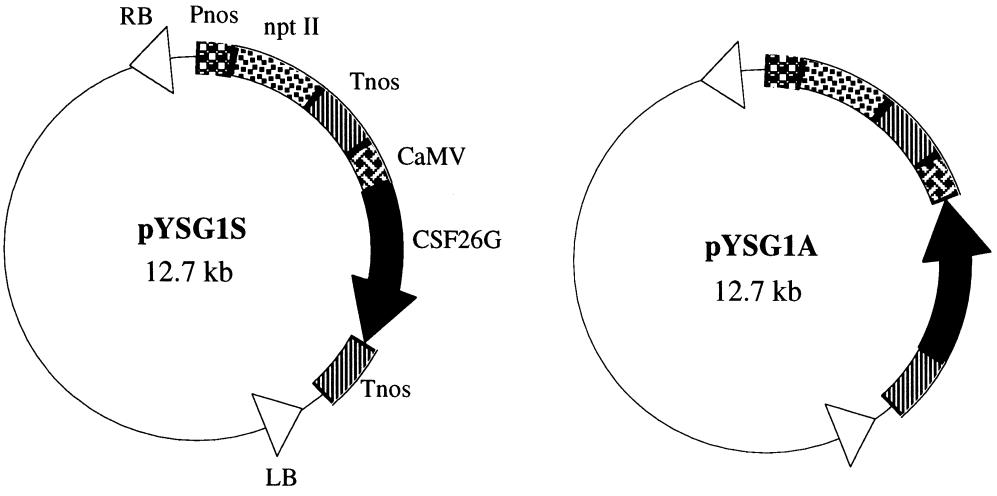

Fig. 1. Schematic representation of binary vectors, pYSG1S and pYSG1A, containing right border sequence (RB), nopaline synthase promoter (Pnos), neomycin phosphotransferase II gene (npt II), nopaline synthase terminator (Tnos), cauliflower mosaic virus 35*S* promoter (CaMV), CSF26G, and left border sequence (LB).

as plant material for this study. pBI121 (Jefferson, Kavanagh, & Bevan, 1987) was used as a vector for delivery as well as expression of the F26G cDNA from *C. speciosus*. Engineering was performed by replacement of the GUS gene with the F26G cDNA fragment derived from a derivative of the bacterial expression vector, pT7-CSF26G1 (Inoue et al., 1996a). The resulting plasmids (Fig. 1) harbored the fragment in the

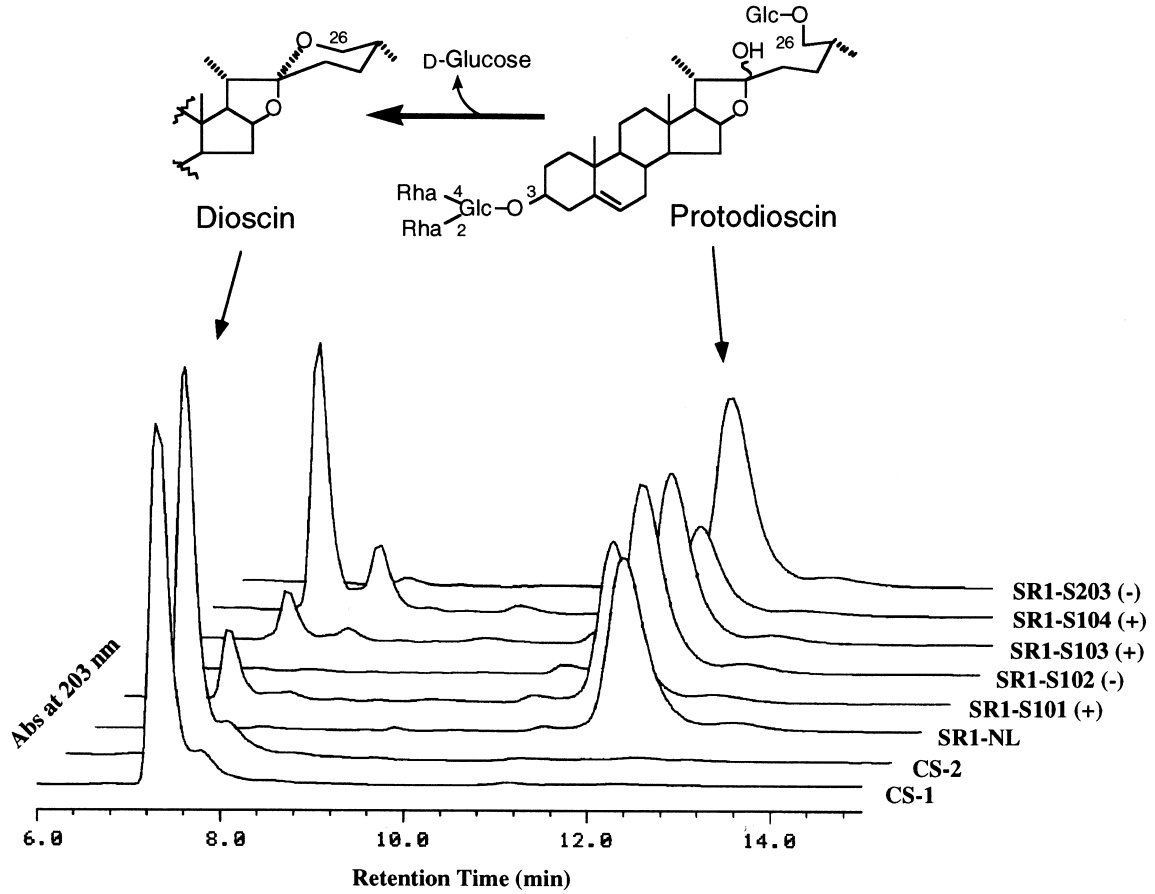

Fig. 2. HPLC profile in F26G assay using protodioscin as a substrate. The SR1 group is shown as an example. See Inoue et al. (1996b) for the HPLC conditions. NL (normal plant); CS-1 and 2 (in vitro cultured plantlet); (−) PCR check negative; (+) PCR check positive.

sense (pYSG1S) or antisense (pYSG1A) directions with respect to the control of cauliflower mosaic virus (CaMV) *35S* promoter. Transformation of tobacco was then carried out using the standard leaf disc method (Rogers, Horsch, & Fraley, 1986) with *A. tumefaciens* LBA4404 (Ooms et al., 1982) carrying either pYSG1S or pYSG1A. The cultivars of *N. tabacum* used were Petite Havana SR1 (SR1), which is most extensively studied in plant molecular biology, and Xanthi whose seeds contain steroid saponins (Grünweller et al., 1990). The experimental combinations between the plant cultivars and the plasmids are: SR1-pYSG1S (SR1-S); SR1-pYSG1A (SR1-A); Xanthi-pYSG1S (Xt-S); Xanthi-pYSG1A (Xt-A).

Eight clones, each of the regenerated plantlets (for each combination, an individual clone was assigned three digits, e.g. SR1-S101) were subjected to PCR analysis with the CSF26G-specific primers (see Section 3) to check for intact integration of the cDNA into the plant genome. For the sense-groups, three each of the SR1 and Xanthi clones gave a single amplified fragment of ca. 1.7 kb, suggesting that the whole CSF26G cDNA is present in the plant genome. A similar integration ratio was observed for the antisense groups: SR1-A (four were positive); Xt-A (three were positive), indicating that the transformation proceeded equally well regardless of the plant cultivar used or the orientation of CSF26G cDNA on the binary vector.

Using the sense groups, cell-free homogenates were prepared from the above PCR-positive clones as well as from some control plantlets including *C. speciosus*, untransformed (normal) tobacco, and PCR-negative clones, and subsequently used for F26G assays (Inoue & Ebizuka, 1996a, 1996b). HPLC analysis (Fig. 2) of the n-BuOH extracts of the incubation mixture from the PCR-positive groups clearly showed the production of dioscin (SG) from protodioscin (FG). No detectable activity was observed in the groups of untransformed SR1 and PCR-negative clones. It is concluded that F26G activities detected in the PCR-positive groups are derived from the functional expression of the introduced F26G cDNA. A summary of the PCR screenings and F26G assays is shown in Table 1. Consistent results concerning PCR analysis and F26G assay were obtained for all the clones but one (Xt-S202). Processing of F26G cDNA was presumably not appropriate at the level of transcription or posttranslation in the case of Xt-S202. Other activities were also noted with the clones of both cultivars (SR1-S104 and Xt-S102) using *p*-nitrophenyl-β-D-glucoside (pNPG) as a substrate. It was found that their activities against pNPG were significantly higher than that derived from untransformed (normal) tobacco, albeit ca. 20–30 times lower (data not shown) than the corresponding F26G activities i.e. suggesting that the heterologously expressed F26Gs possess a similar high substrate specificity shown by native F26G (Inoue et al., 1996a). Thus, successful heterologous plant expression of F26G was demonstrated although its level was significantly low (one 50th) compared with that in *C. speciosus* Table 1.

Monodesmosidic SGs show typical surface-active (saponin) properties including antifungal and hemolytic activities as well as inhibitory effects on platelet aggregation, whereas the FGs having an additional glucose at C-26 (bisdesmosidic) are biologically inactive (Mahato et al., 1982; Roddick, 1987; Yang & Li, 1996; Peng & Yao, 1996). Postharvest treatment and storage of plant materials are known to result in the conversion of FGs to their corresponding SGs (Kawasaki et al., 1974), thereby providing an intriguing possibility that this conversion is involved in plant defense mechanisms (Shvets, Kintia, & Naibi, 1996). The work described here is the foundation for genetic engineering in a plant to provide a novel metabolic capability leading to a desired defense response.

In addition, the *Arabidopsis thaliana* genome project recently identified a homologue (EMBL accession number AL022140) of CSF26G (54% similarity for the deduced product). To the best of our knowledge, no spirostanol saponins have been identified from this species. Therefore, it will be quite interesting to know its physiological function. Very little is known about the biosynthetic mechanism of steroid saponins whose aglycones are presumed to be derived from cycloartenol, the first cyclic intermediate in the pathway. Recent success in cDNA cloning of plant cycloartenol synthase (Corey, Matsuda, & Bartel, 1993; Morita,

Table 1
Summary of the screening results of transformed *N. tabacum*, and F26G activity in cell-free homogenates of *C. speciosus* and *N. tabacum*

Sample[a]	PCR check[b]	Specific activity[c] (nkat × mg^{-1} protein)
CS-1	n.t.	9.90
CS-2	n.t.	12.1
SR1-WT	−	n.d.
SR1-S101	+	0.16
SR1-S102	−	n.d.
SR1-S103	+	0.11
SR1-S104	+	0.16
SR1-S203	−	n.d.
Xt-WT	−	n.d.
Xt-S102	+	0.24
Xt-S102 (boiled)	+	n.d.
Xt-S202	+	n.d.
Xt-S203	+	0.18

[a] WT (wild type); CS-1 and 2 are derived from in vitro cultured plantlets.

[b] Presence of CSF26G cDNA at chromosomal DNA was checked by PCR (see Section 3). n.t. means not tested), + positive and − negative.

[c] Kat is expressed as dioscin produced (mol) for 1 min under the assay conditions used (for details see Inoue & Ebizuka, 1996a, 1996b).

Shibuya, Lee, Sankawa, & Ebizuka, 1997; You et al., 1998), together with our characterization of a specific F26G (Inoue & Ebizuka, 1996a, 1996b; Inoue et al., 1996a; You et al., 1998), would lead to a better understanding of the whole biosynthetic pathway of steroid saponins.

3. Experimental

3.1. Materials and general methods

Seeds of *Nicotiana tabacum cv*. Petite Havana SR1 and Xanthi were obtained from JT (Japan Tobacco) Co. Ltd, Japan. Germination of tobacco seeds and maintenance of transgenic tobacco plantlets were performed on Murashige–Skoog (MS) (Murashige & Skoog, 1962) basal medium containing 30 g l^{-1} sucrose and 0.8% agar in a plastic culture box at 25°C under 16 h light per day. In vitro cultured *Costus speciosus* plantlets used have been as described previously (Inoue et al., 1996b). *Agrobacterium tumefaciens* LBA4404 (Ooms et al., 1982) and plasmid, pBI121 (Jefferson et al., 1987) were obtained from Clontech (Palo Alto, CA). DNA manipulations were according to standard procedures (Sambrook, Fritsch, & Maniatis, 1989). Electroporation was performed on a Electro Cell Manipulator 600 (BTX Inc, San Diego, CA) following the manufacturer's protocol.

3.2. Construction of CSF26G expression vectors

CSF26G was introduced in place of the β-glucuronidase (GUS) gene in the binary vector, pBI 121, as follows: (1) digestion with *Sma*I and *Sac*I followed by self-ligation, (2) treatment with *Xba*I and Klenow fragment to afford a dephosphorylated vector (3) blunt-end ligation with CSF26G cDNA fragment (1689 bp) derived from pT7-CSF26G (Inoue et al., 1996a) by digestion with *Xba*I and *Kpn*I followed by Klenow treatment.

3.3. Transformation of tobacco

Mobilization of the vectors (pYSG1S or pYSG1A) into *A. tumefaciens* LBA4404 was by electroporation. Tobacco leaf discs were transformed using the *Agrobacterium* binary vector method described by Rogers et al. (1986). Transgenic plants were selected on media containing kanamycin 100 mg l^{-1}.

3.4. Genomic DNA extraction and PCR analysis

Aerial parts (ca. 0.2 g fr. wt) of the regenerated kanamycin-resistant plantlet were used for DNA extraction as described previously (Murray & Thompson, 1980) to afford ca. 50 μg of DNA in a volume of 25 μl. Part (1 μl) was used as a template for PCR with the CSF26G-specific primers (1 μg each), CSFG-1S (5′–ATG GCC GCT CAG TTA GGG CTT C–3′) and CSFG1689A (5′–TCA CGT CCT CAG GAA CTT GCT–G–3′) in a final volume of 100 μl for 25 cycles of amplification using a step program (1 min at 94°C, 2 min at 68°C and 3 min at 72°C).

3.5. Enzyme extraction and assay

Cell-free protein extracts were obtained from transgenic tobacco or *C. speciosus* plantlets as previously described (Inoue et al., 1996b). Protein was quantified by the method of Bradford (1976) using BSA as a standard. F26G assay was performed according to the method described (Inoue & Ebizuka, 1996a, 1996b). β-Glucosidase activity using *p*-nitrophenyl β-D-glucoside (pNPG) as a substrate was determined by the absorbance at 400 nm of released *p*-nitrophenol under the same assay conditions used for F26G.

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