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Modification of Limonoid Metabolism in Suspension Cell Culture of *Citrus*

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Abstract

For the metabolic engineering of bio-functional substances in *Citrus*, metabolic changes of limonoids were investigated in embryogenic cell cultures of *Citrus* and *Fortunella*. When embryogenic cells of *Citrus* and *Fortunella* were cultured for four weeks in the liquid subculture medium containing nomilin, most species converted nomilin to obacunone and limonin. In addition, calamin group limonoids were produced in *Fortunella* and Calamondin cells. Deacetylnomilin was produced from nomilin in the cells of *C. ichangensis* and its related species. Exogenously added limonin into medium was not metabolized. The embryoid formation in *C.aurantium* did not affect the limonoid metabolism. All cells cultured without the presence of nomilin or limonin did not generate any limonoids. Furthermore, transgenic *C. sinensis* cell harboring a chimeric limonoid UDP-glucosyltransferase (limonoid GTase) gene was generated. The conversion of limonin into limonin 17-β-D-glucopyranoside (LG) demonstrated an efficient assay system for the transgene.

Key words: bitterness, *Citrus*, limonoid, limonoid GTase, metabolic engineering.

Abbreviations

DMSO, dimethylsulfoxide; GTase, UDP-glucosyltransferase; LG, limonin 17-β-D-glucopyranoside.

Introduction

Recently, it has been shown that citrus fruit is rich in bio-functional and health-promoting substances such as polymethoxylated flavones (Kawai *et al.*, 2000), auraptene (Ogawa *et al.*, 2000), β-cryptoxanthin (Ikoma *et al.*, 2001). Among such chemical constituents, there is a group of triterpenoid present in Rutaceae known as limonoids. Limonoids are the cause of delayed-bitterness in juice because intensely bitter limonoids, such as limonin, are gradually developed from non-bitter precursors during the process of juice production. In addition, they have been shown to possess biological functions such as anti-insect feeding (Bentley *et al.*, 1990) and anti-cancer activities (Lam and Hasegawa, 1989; Tanaka *et al.*, 2000). Metabolic engineering of citrus limonoids is of great interest in agricultural, food processing and medical fields.There are wide derivatives in limonoids among Rutaceae family plants. Major citrus species accumulate limonin (**Fig. 1III**), obacunone (II), nomilin (I) and deacetylnomilin (IV) (Hasegawa and Herman, 1985, 1986; Herman and Hasegawa, 1985). *Citrus ichangensis* and the relatives accumulate ichangensin (IX, keto and X, ketal) (Benett *et al.*, 1988; Herman *et al.*, 1989; Berhow *et al.*, 1994). *Fortunella* and related species accumulate calamin group limonids such as calamin (VII) and cyclocalamin (VIII) (Benett and Hasegawa, 1981; Herman *et al.*, 1987).

Radioactive tracer work demonstrated the biosynthesis of limonoids from acetate or mevalonate in the phloem region of stem (Hasegawa *et al.*, 1984, 1986; Ou *et al.*, 1988). Seeds and fruit tissues are capable of biosynthesizing other limonoids starting from nomilin. Limonoid aglycones are endogenously converted to tasteless limonoid glucosides during fruit maturation (Hasegawa *et al.*, 1991; Fong *et al.*, 1992, 1993). This natural debittering process is catalyzed by the enzyme UDP-D-gluco-

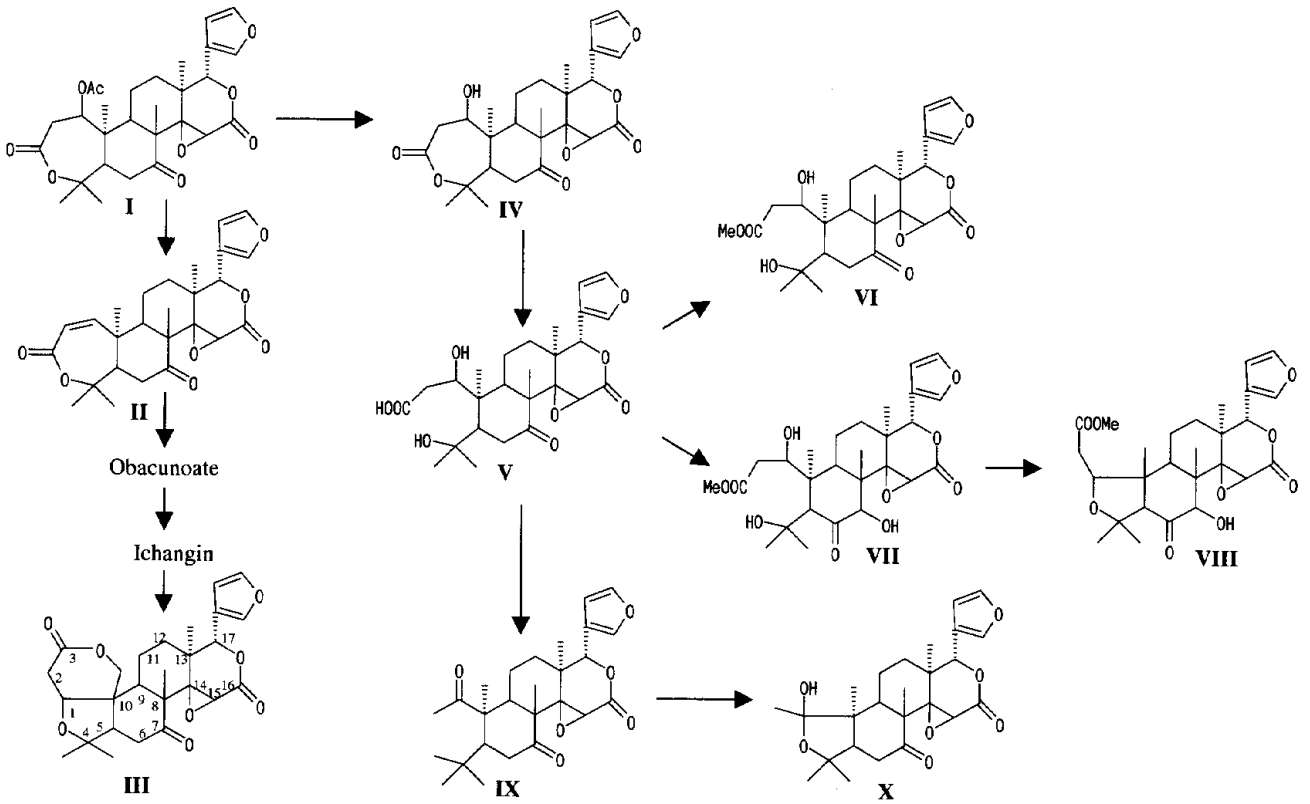

Fig. 1 Limonoids in citrus.
I: Nomilin, II: Obacunone, III: Limonin, IV: Deacetylnomilin, V: Deacetylnomilinate, VI: Methyl deacetylnomilinate, VII: Calamin, VIII: Cyclocalamin, IX: Ichangensin (keto), and X: Ichangensin (ketal).

side: limonoid glucosyltransferase (limonoid GTase) (Hasegawa *et al.*, 1997). Recently, *CitLGT*, a gene encoding for a limonoid GTase, was isolated from *C. unshiu* Marc. (Kita *et al.*, 2000) and could be used as an important tool to manipulate citrus limonoid metabolism (Omura *et al.*, 2000). A plant regeneration system has also been developed for the purpose of genetic engineering. Embryos can be obtained from embryogenic callus by change of culture medium (Hidaka and Omura, 1989). Embryogenic calli can be transformed by an *Agrobactrium* -mediated method (Hidaka *et al.*, 1990).

Genetic engineering of *Citrus* to maximize the formation of limonoid glucosides, thereby reducing limonoid bitterness, could be a target for *Citrus* breeding (Omura *et al.*, 2000). However, it takes long years to evaluate whether such transgenic *Citrus* actually altered the content of limonoid glucosides in fruits because of the long juvenile duration in woody plants. Therefore, an adequate assay system, such as in calli or young seedlings to evaluate such metabolism has been desired.

However, there has been limited information on limonoid metabolism in such callus cultures in *Citrus*. Here, we investigated the conversion of limonoids added into medium by citrus embryogenic cells. By using this system in cultured cells, metabolic alteration by constitutively expressed *CitLGT* was investigated.

Materials and Methods

Callus culture

Embryogenic calli were produced from young or immature nucellar embryos of *Citrus aurantium* L., *C. tangerina* (cv. Dancy tangerin), *C. reticulata* Blanco (cv. Ohta ponkan), *C. sinensis* Osbeck (cvs. Trovita orange, Washington navel, Valencia orange), *C. ichangensis* Swingle, *C. junos* hort. Ex Tanaka, *C. hystrix* DC., *C. madurensis* Lour., *Fortunella crassifolia* Swingle, and *F. hindsii* Swingle. They were induced on the Gelrite-solidified medium consisted with Murashige and Skoog's basal components (Murashige and Skoog, 1962) supplemented with 0.2 M sucrose and 50 μM kinetin (pH 5.6). Cultures were maintained by subculturing at 2 -month intervals under 16 h illumination regime at 25 ℃ for 5 to 10 years as previously described (Hidaka and Omura, 1989). Prior to experiments, calli were suspended in the liquid medium with the same components unless otherwise mentioned.

Addition of limonoids into culture medium

For the assay of limonoid conversion, approximately 1 g calli were suspended in the 200 ml liquid medium. Limonin and nomilin were dissolved with dimethylsulfoxide (DMSO) and added into liquid medium to final concentration of 0.01% (w/v). Equal amount of DMSO was added to the medium of the controls. Suspension cultures were orvitary shaken at 120 rpm under 16 h illumination regime at 25 ℃ for 4 weeks. To investigate the limonoid metabolism in callus during embryogenesis, suspension cells of *C. aurantium* were placed on the nylon membrane filter (22 μm pore size), under which 3 sheets of filter paper were layered and supplied culture medium to the cells. The medium for embryogenesis was consisted of MS basal components with 0.1 M sorbitol and 0.1 M galactose (Hidaka and Omura, 1989). The medium for callus proliferation containing 0.2 M sucrose and 50 μM kinetin was also examined. Both cultures were under 16 h illumination regime at 25 ℃ for 4 weeks.

Analysis of limonoids

After culturing for 4 weeks, the cells were collected on nylon mesh by filtration and washed with water 3 times to remove the culture medium. They were dried in the oven at 65 ℃ for 3 days and used for analysis. One gram of dried sample was grounded in 5 ml of 0.5 M Tris-HCl buffer (pH 8.0) with a Polytron homogenizer. The mixture was then filtered through celite and the filtrate was treated with a C-18 Sep-Pak. Limonoids were analyzed by TLC and HPLC (Herman *et al.*, 1989) or LC-MS (Manners *et al.*, 2000). In the representative sample from *C. sinensis* (cv. Trovita orange), the quantities of limonoids were determined on the chromatographic areas with two independently replicated analyses.

Transformation of callus with CitLGT

Plasmid for transformation was generated as follows. Full length of *CitLGT* cDNA (Kita *et al.*, 2000) was excised with *Xba*I and *Kpn*I. A binary vector pBE2113 (Mitsuhara *et al.*, 1996) was digested with *Xba*I and *Sac*I, and ligated *Xba*I-*Sac*I linker fragment from pUC18 (Yanisch-Perron *et al.*, 1985). Resultant vector plasmid was digested with *Xba*I and *Kpn*I, and ligated with the *CitLGT* fragment. This construct was incorporated into *Agrobacterium tumefaciens* strain LBA4404 by triparent mating. *Agrobacterium* infection of Washington navel callus was carried out according to the method of Hidaka *et al.* (1990) with a slight modification. About 1 g of callus was suspended in 40 ml liquid medium for transformation. During 3 days co-cultivation, calli were plated on the nylon membrane filter, same as mentioned above, and washed with MS liquid medium everyday.

Transformed calli were selected on the Gelrite-solidified medium consisted with MS basal components supplemented with 0.2 M sucrose, 50 μM kinetin, 50 mg l^{-1} geneticin and 250 mg l^{-1} Claforan (pH 5.6). Gene incorporation was confirmed by standard PCR amplification technique with a primer set of 35S promoter (5'-ATCTCCACTGACG-TAAGGGATGACG-3') and *LGT*-GR (5'-TCAA-TACTGTACACGTGTCCGTCG-3') sequences. Thermal condition was 30 cycles of 1 min at 94 ℃, 1 min at 58 ℃ and 1 min at 72 ℃.

Accumulation of *CitLGT* transcript was analyzed by RT-PCR with the 1st strand cDNA as a template. Total RNA isolation and 1st strand cDNA synthesis from transformed calli were performed by the methods described previously (Kita *et al.*, 2000). A set of primers used for RT-PCR amplification was *LGT-GF* (5'-ATGGGAACTGAATCTCTTG-TTCAT-3') and *LGT*-GR. Thermal condition was same with that of PCR.

Results and Discussion

Limonid metabolisms in suspension cell culture

In order to investigate the metabolism of limonoids in embryogenic callus cells, nomilin or limonin was added into liquid medium, and limonoid contents of cultured cells were analyzed. As shown in **Table 1** and the HPLC-chromatogram (**Fig. 2**), a

Table 1. Limonoids detected in the callus culture added with nomilin.

Cell clone	Limonoid				
	I[1]	IV	II	III	calamin group[2]
C. sinensis					
cv. Washington navel	+	-	+	+	-
cv. Trovita orange	+	-	+	+	-
cv. Valencia orange	+	-	+	+	-
C. tangerina	+	-	+	+	-
C. reticulata					
cv. Ohta ponkan	+	-	+	+	-
C. junos	+	+	+	+	-
C. ichangensis	+	+	+	+	-
C. hystrix	+	+	+	+	-
C. madurensis	+	+	+	+	+
F. crassifolia	+	+	+	-	+
F. hindsii	+	+	+	-	+

[1]See **Fig.1.**

[2]See text.

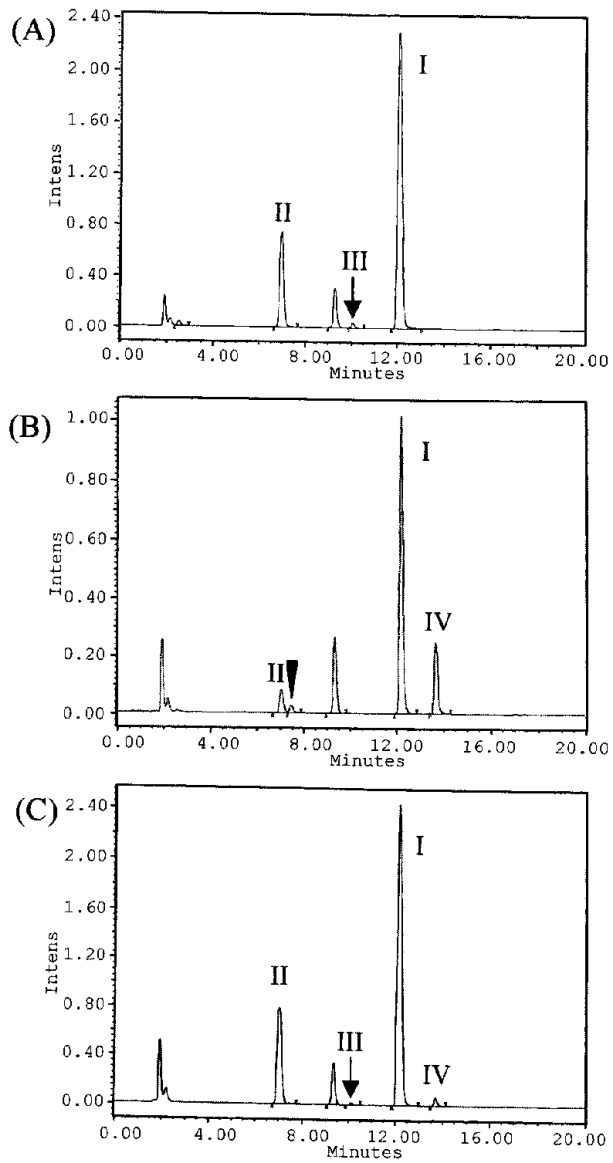

Fig. 2 HPLC profiles of limonoids in suspension cells cultured with nomilin.
I, II, III and IV: See **Fig. 1**. An arrowhead in (B) points a peak of a mixture composed of methyl deacetylnomilinate and unidentified compound similar to calamin, which is only observed in *Fortunella*. (A) *C. sinensis* cv. Trovita orange; (B) *F. crassifolia*; (C) *C. junos*.

variety of limonoids were detected over the clearly detectable levels in the cells cultured with nomilin. The cultured cells of sweet oranges (*Citrus sinensis* cvs. Washington navel, Trovita orange and Valencia orange) and mandarins (*C. tangerina* and *C. reticulata*) contained obacunone (II) and limonin (III) along with nomilin (I), corresponding that fruits and seeds of those species contained such major limonoids (Ozaki *et al.*, 1991). The cells of Trovita orange contained 1410 ppm nomilin per 1 g dried cells. The same cells contained 487 ppm obacunone and 44 ppm limonin, which correspond to 34.5% and 3.1% of nomilin, respectively. From the cells of two *Fortunella* species (*F. crassifolia* and *F. hindsii*) and *Fortunella*-related hybrid Calamondin (*C. madurensis*), calamin group limonoids, such as methyl deacetylnomilinate (VI), calamin (VII) and cyclocalamin (VIII), were detected in addition to obacunone. Although limonin was detected in Calamondin cells, two *Fortunella* species did not produce it at the detectable levels. The suspension cells of *C. ichangensis*, *C. junos* and *C. hystrix* produced obacunone, limonin and deacetylnomilin (IV). However, they did not produce their specific major limonoid, ichangensin (IX, keto and X, ketal). It appears that the conversion from nomilin to deacetylnomilin would be regulated basically in a genotype-specific manner in cultured cells same as intact fruits and seeds, suggesting that nomilin-acetyl esterase, which is unique to this species, is active in the cultured cells as well as in various tissues (Herman, *et al.*, 1989). However, enzyme activities involved in dehydroxylation of deacetylnomilinate followed by decarboxylation would be repressed in cultured cells.

All of the suspension cells did not metabolize limonin added to the media (data not shown). Together with the results of nomilin feeding experiment, this indicates that the major pathway from nomilin to limonin via obacunone is unidirectional in cultured cells, which is consistent with previous reports (Herman and Hasegawa, 1985; Hasegawa and Herman, 1986).

Limonoid metabolisms during embryogenesis from callus of C. aurantium

It is generally known that secondary metabolism is repressed in nondifferentiated cells and this repression can be reversed during the course of differentiation as in organogenesis and embryogenesis. Gavish *et al.* (1989) used embryo cultures of *C. paradisi* in the experiments of biotransformation of flavanones in the cell culture, and showed that the endogenous production of naringin in *Citrus* cell culture is developmentally regulated. However, there has been a little information for the limonoid metabolism in the cell culture. Limonin contents were rapidly decreased in calli from fruit flavedo, albedo, juice vesicle, leaf, stem and cotyledon of *Citrus*, but they were recovered in the regenerated shoots from callus (Barthe *et al.*, 1987).

In this study, limonoid metabolisms were investigated in both embryoid-inductive and cell-proliferating conditions (**Table 2**). Embryogenesis of *C. aurantium* has been induced on the embryogenesis medium. Embryoids with green color were observed after 4 weeks on the same medium, while original

Table 2. Limonoids of *C. aurantium* cells after 4 weeks culture.

Added Limonid	Culture condition	Limonoid I[1)]	IV	II	III
Nomilin	Embryogenesis	+	+	+	+
	Cell proliferation	+	+	+	+
Limonin	Embryogenesis	-	-	-	+
	Cell proliferation	-	-	-	+
Control (DMSO)	Embryogenesis	-	-	-	-
	Cell proliferation	-	-	-	-
-	Before culture	-	-	-	-

[1)] See **Fig. 1.**

callus continued to proliferate on the subculture medium. Before the embryo formation had occurred, no limonoids were detectable in such cultures without feeding of limonoids. When limonin was added to the culture media, only limonin (III) was recovered from the cells on both embryoid-inductive and cell-proliferating conditions. In contrast, deacetylnomilin (IV), obacunone (II) and limonin (III) as well as nomilin (I) were detected when nomilin was added into both media, independent from the cell differentiation state. These results showed that there were no differences between cell-proliferating and embryoid-inductive conditions in nomilin or limonin biotransformation.

Since neither proliferating callus cells nor embryoids produced limonoids without nomilin addition to the medium, the suppression of limonoid biosynthesis would occur prior to the biosynthesis of nomilin independent from embryogenesis. This corresponds to the report by Hasegawa *et al.* (1980) in which *de novo* synthesis of limonoids from acetate in the seeds could not be demonstrated. Since nomilin biosynthesis occurs only in phloem of stem of intact plant (Hasegawa *et al.*, 1986; Ou *et al.*, 1988), further development of young embryoids to plantlets with well-organized vascular system would be required for biosynthesis of limonoids from acetate *in vitro*.

Limonoid metabolism in CitLGT-introduced cells

Because the glucosylation of limonoids is a natural debittering process, regulation of biosynthesis of limonoid glucosides is one of major targets for genetic modification to produce *Citrus* trees that have fruits free of limonoid bitterness (Kita *et al.*, 2000; Omura *et al.*, 2000).

Transgenic cells of *C. sinensis* cv. Washington navel, in which *CitLGT* encoding UDP-D-glucoside: limonoid glucosyltransferase (Kita *et al.*, 2000) was ectopically expressed, were established via *Agrobacterium* infection. RT-PCR analysis

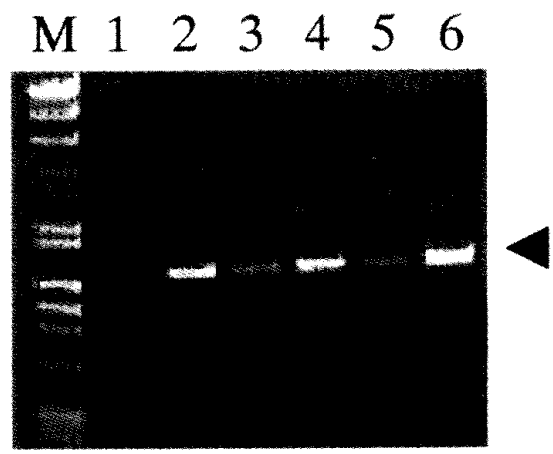

Fig. 3 RT-PCR pattern of Washington navel callus transformed with *CitLGT* gene.
RT-PCR products were electrophoresed on agarose gel and stained with ethidium bromide. An arrowhead indicates 1536bp target position of *CitLGT* gene. M: λ *Hin*dIII + ϕX174 *Hae*III, Lane 1: untransformed callus, Lane 2-6: transgenic callus (Lane 2: 4-1, Lane 3: 5-1, Lane 4: 6-1, Lane 5: 7-1 and Lane 6: 5-10).

indicated that transgenic callus clones were transcriptionally active for *CitLGT*, whereas control callus was not (**Fig. 3**). Production of limonoid glucosides was tested in independent transgenic cell lines. In the first experiment, transformed calli of 3 independent clones were cultured and limonin 17-β-D-glucopyranoside (LG) was detected in 2 clones when limonin was added into medium (**Table 3**). Following experiment using other 5 lines showed that only one line produced LG (data not shown). Limonin was produced from nomilin in each cell line (**Table 3**), showing metabolism of limonoid aglycones in consistent with the results of non-transformed cells. However, LG was not produced when nomilin was added into medium, even in the cell lines in which LG was produced from limonin. This might be due to the relatively low level contents of limonin produced from nomilin in

Table 3. Limonids detected in the *CitLGT*-introduced callus of Washington navel.

Cell line	Added limonoid	Limonoid I[1]	III	LG[2]
4-1	Control (DMSO)	-	-	-
	Nomilin	+	+	-
	Limonin	-	+	+
5-1	Control (DMSO)	-	-	-
	Nomilin	+	+	-
	Limonin	-	+	+
5-10	Control (DMSO)	-	-	-
	Nomilin	+	+	-
	Limonin	-	+	-

[1] See **Fig. 1.**

[2] limonin 17-β-D-glucopyranoside

cultured cells, as mentioned above, and/or to relatively low activity of limonin GTase derived from the transgene. We also observed that the native limonoid GTase showed very low activity, when it was isolated from fruit tissues (Hasegawa *et al.*, 1997) and when its cDNA was expressed as a fusion protein with GST in *E. coli* (Kita *et al.*, 2000). In addition, the fact that only a few lines could produce LG from limonin added into medium suggests several factors by which limonoid glucoside accumulation is regulated besides transcript accumulation of *CitLGT*. However, this is the first finding that the callus cells of *Citrus* produced limonoid glucosides if transformed with a cDNA encoding the limonoid GTase. Prior to this report, callus cells of *Citrus* have not been used for the assay system of the bio-functional substances, but our results support that citrus cell culture is a useful tool for the evaluation of limonoid metabolism.

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