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Metabolic Engineering of Plant Cells for Biotransformation of Hesperedin into Neohesperidin, a Substrate for Production of the Low-Calorie Sweetener and Flavor Enhancer NHDC

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Neohesperidin dihydrochalcone (NHDC) is a seminatural, safe, low-calorie sweetener, bitterness blocker, and flavor enhancer with unique properties and applications for the food, beverage, pharmaceutical, and animal feed industries. Current production is limited by the availability of the substrate neohesperidin, a flavonoid that accumulates to significant levels only in the inedible bitter citrus species. We propose a process to convert hesperidin, a tasteless flavonoid extracted from orange peels that are abundant byproducts of the vast orange juice industry, into neohesperidin using metabolic engineering and biotransformation via three steps: (i) extraction of hesperidin from orange peels, (ii) hydrolysis of sugar moieties, and (iii) biotransformation of hesperidin hydrolysis products into neohesperidin. We overcame the current technological bottleneck in biotransformation of hesperidin hydrolysis products into neohesperidin using metabolically engineered plant cell cultures expressing a recombinant flavanone-7-*O*-glucoside-2-*O*-rhamnosyltransferase. A small-scale production experiment established the feasibility of the proposed process.

KEYWORDS: Citrus; flavonoid; neohesperidin; NHDC; biotransformation; sweetener; metabolic engineering; rhamnosyltransferase

INTRODUCTION

The genus *Citrus* is an important resource for natural products with direct benefits to humans ranging from health-benefiting compounds (*1*−*3*) to flavor and aroma compounds used by the food, beverage, and cosmetics industries (*1*, *4*). Compounds of applied interest include flavonoids belonging to the flavanone subgroup, which accumulate to considerable levels in the leaves and fruit of citrus species where their biological role in planta is not clear (*5*, *6*). The bitter flavanone neohesperidin, abundant mainly in inedible citrus fruit species, is the substrate for the commercial production of neohesperidin dihydrochalcone (NHDC), a seminatural and generally regarded as safe low-calorie sweetener with unique properties (*7*−*9*). NHDC is approximately 1500-fold sweeter than sucrose, is stable under heat and a wide pH range, and has also been documented to function as a bitterness blocker and flavor enhancer with diverse uses in the food, beverage, pharmaceutical, and animal feed industries (*8*−*10*). NHDC for human consumption is usually combined with other sweeteners resulting in (i) a synergistic sweetening effect allowing for lower overall concentrations of the sweetening compounds and (ii) masking of sweetener aftertastes to derive a flavor resembling sucrose (*9*, *11*, *12*).

NHDC has been a commercial product for many years; however, production is limited by the availability of the substrate neohesperidin, which accumulates to significant levels only in inedible citrus species such as *Citrus aurantium* (bitter orange) and other citrus hybrids that are especially grown for this purpose (*11*, *13*). Neohesperidin has also been detected in *Citrus aurantium* callus cultures (*14*); however, the growth rate of citrus callus/cell cultures is extremely low (*15*), and therefore, this source does not have commercial potential. NHDC may also be produced by chemical conversion of naringin (the dominant bitter flavonoid in grapefruit peels) into neohesperidin, but the amount of grapefruit peels is limiting (*11*). An isomeric flavanone, the rutinoside hesperidin, is the predominant fla-

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vanone in orange peels (over 90%) (*16*), as well as lemon and mandarin peels, and is readily available in large quantities as a byproduct of the vast orange juice industry. However, hesperidin (hesperetin-7-*O*-rutinoside) could not be converted into neohesperidin (hesperetin-7-*O*-neohesperidoside) by chemical or biological modification using the tools available up to now. In this work, we demonstrate a process for conversion of orange peel hesperidin into neohesperidin via biotransformation by metabolically engineered plant cell suspension cultures.

MATERIALS AND METHODS

Genetic Transformation of Plant Cell Cultures with a 1,2-Rhamnosyltransferase Encoding Gene. Construction of a plasmid containing the gene *Cm1,2RhaT* (GeneBank accession no. AY048882) under the control of the CAMV 35S promoter and transformation of tobacco cell suspension cultures were previously described (*17*). Transformation of a carrot cell suspension culture using the same plasmid was as follows: 3 mL of a 6 day old carrot cell suspension culture was added to 12 mL of MSD medium and inoculated with 300 μL of a culture of *Agrobacterium* harboring the binary plasmid (OD600 0.4−0.7). Cocultivation was done for 4 h without shaking in the dark and for 3 days with shaking (100 rpm) under fluorescent light at 28 °C. Following cocultivation, carrot cells were strained through a sterile 100 μm mesh and washed with 300 mL of medium to reduce levels of *Agrobacterium*. Strained carrot cells were resuspended in 50 mL of MSD medium [4.4 g/L MS medium (Duchefa, Haarlem, The Netherlands), 9.9 mg/L thimaine-HCl, 0.5 mg/L folic acid, 0.5 mg/L D-(+)-biotin, 800 mg/L casein hydrolisate, 30 g/L sucrose, and 0.1 mg/L 2,4-D, pH 5.7−5.8] containing antibiotics [10 μg/mL G-418, 250 μg/mL vancomicin, and 250 μg/mL claforan] and were grown with shaking under fluorescent light at 28 °C. After 2 weeks, 1 mL of the cell suspension culture was diluted into 20 mL of MSD medium containing antibiotics. The latter step was repeated three times in intervals of 1 week to obtain an *Agrobacterium*-free transformed cell suspension culture.

Biotransformation of Flavonoids Using Transgenic Tobacco and Carrot Cell Suspension Cultures. Transgenic cell lines (tobacco and carrot) expressing *Cm1,2RhaT* were used for biotransformation studies as follows. To initiate biotransformation, transgenic and wild-type cell cultures were diluted (400 μL of BY2 or 1 mL of carrot cell suspension culture into 20 mL of new media) and grown in Erlenmeyer flasks for 4 days. Flavonoid aglycones or glycosides were dissolved in DMSO to a concentration of 30 mM and added to the flasks to a final concentration of 0.15 mM. The cultures were grown for an additional 48 h before harvest. The cell cultures and media were boiled together for 15 min, cooled to room temperature, and then extracted with water-saturated *n*-butanol. The butanol phase was transferred to a new tube and dried. In these small-scale lab experiments, *n*-butanol was used for convenience reasons, while methanol/ethanol would most probably be the solvents of choice in large-scale experiments. Commercial flavanones were purchased from Sigma-Aldrich (Milwaukee, WI). Hesperetin-7-*O*-glucoside (HG) standard was generously supplied by Dr. Jose Antonio Del Rio.

Extraction of Hesperidin from Orange Peels and Hydrolysis. Hesperidin was extracted from ripe orange (*Citrus sinensis* var. Shamouti) peels by a modification of a published protocol (*18*) as follows: Orange peels (100 g) were pulverized in 500 mL of saturated $Ca(OH)_2$ using a homogenizer (the pH was measured to be ~12). The homogenate was transferred to a bottle and flushed with nitrogen before closing. The homogenate was heated to 60 °C for 1 h and left to cool slowly, and the pH was adjusted to ~12 by the addition of $Ca(OH)_2$. Peel particles were filtered out of the homogenate using gauze and filter paper. The homogenate was centrifuged at 20000*g* for 15 min, and the supernatant was collected and filtered through a nylon membrane (0.45 μm). The pH of the now clear solution was lowered to 3.5 and incubated at room temperature overnight to induce ring C closure and to precipitate the hesperidin. The precipitate was collected by centrifugation at 20000*g* for 15 min, and the supernatant was discarded. The precipitate was washed with 10 mL of acetone and dried into a powder.

For hydrolysis, 50 mg of hesperidin was dissolved in 500 μL of DMSO that was then added to 25 mL of preheated (65 °C) 0.25% aqueous H_2SO_4 and immediately autoclaved for two consecutive cycles at 121 °C for 25 min. Hydrolysis products were allowed to precipitate overnight at room temperature, the supernatant was discarded, and the precipitate was dried in a lyophilizer.

Analysis of Flavanone-Glycoside Products by Thin-Layer Chromatography (TLC). Biotransformation products were resuspended in DMSO and analyzed by TLC. The 1−3 μL samples were loaded on Silica gel 60 TLC plates (Merck, Darmstadt, Germany) and separated in solvent (*n*-butanol:methanol:ethyl acetate:dichloromethane, 1:1:1:1) in a standard TLC chamber. TLC plates were dried, and fluorescence was developed by spraying with 1% $AlCl_3$ in methanol (*19*). The plates were photographed with a Nikon Coolpix 5000 digital camera (Nikon, Japan) while irradiating the TLC plate with 302 nm UV. The putative neohesperidin spot, which appeared only in the transgenic cell line biotransformation extract, was scraped from the TLC plate, eluted with methanol, dried, and verified by liquid chromatography/mass spectrometry (LC/MS) (see below).

Analysis of Flavanone-Glycoside Products by LC-MS. LC-MS analyses were conducted using an Agilent 1100 high-performance liquid chromatography (HPLC) coupled to a Bruker Esquire (Billerica, MA) ion-trap mass spectrometer. HPLC separations were achieved using a reversed-phase, C18, 5 μm, 4.6 mm × 250 mm column (J. T. Baker, Phillipsburg, NJ). The mobile phases consisted of (A) 0.1% aqueous acetic acid and (B) acetonitrile. Samples were eluted with a linear gradient of 95% A:5% B to 5% A:95% B over 90 min at a flow rate of 0.8 mL/min. Negative-ion electrospray ionization (ESI) mass spectra were acquired using a source potential of 3000 V and a capillary offset potential of −70.7 V. Nebulization was achieved using nitrogen gas delivered at a pressure of 70 psi. Desolvation was aided by a counter current gas of nitrogen at a pressure of 12 psi and a capillary temperature of 360 °C. Mass spectra were recorded over a 50−2200 *m*/*z* range. The Bruker ion-trap mass spectrometer was operated using an ion current control preset at 20000, a maximum acquire time of 100 ms, and a trap drive setting of 60. Tandem mass spectra were obtained using automated LC/MS/MS following the selection of the two most abundant ions above *m*/*z* 200 as precursor ions for MS/MS. Tandem spectra were then acquired using an isolation width of 2.0, a fragmentation amplitude of 0.83, and a threshold setting of 5000. The ion charge control was set at 2000 with a maximum acquire time set at 100 ms.

RESULTS AND DISCUSSION

Designing a Process for Conversion of Orange Peel Hesperidin into Neohesperidin. We propose a process to convert hesperidin extracted from orange peels into neohesperidin using metabolic engineering and biotransformation via three steps (**Figure 1**): (i) extraction of hesperidin, (ii) full or partial hydrolysis of hesperidin sugar moieties, and (iii) biotransformation of hesperidin hydrolysis products into neohesperidin. (i) Various methods for the extraction of hesperidin from orange peels have been described (*18*, *20*) and are appropriate for industrial applications. (ii) Hesperidin can be enzymatically hydrolyzed to hesperetin-7-*O*-glucoside (HG) and hesperetin using hesperidinase (a mixture of α-L-rhamnosidase EC 3.2.1.40 and β-D-glucosidase EC3.2.1.21); however, the solubility of hesperidin in aqueous solutions at temperatures compatible with enzymatic activity is very low, making this approach somewhat of a challenge for industrial applications. Alternatively, efficient hydrolysis of hesperidin into HG and hesperetin can be achieved using dilute acid catalysis under high temperatures (*21*), which allows for efficient solubilization of hesperidin in the aqueous solution. This process is simple and is straightforward for application in the industry. (iii) A tobacco cell suspension culture (*22*) containing endogenous 7-*O*-glucosyltransferase activity (*23*) was transformed with the gene *Cm1,2RhaT* encoding a flavanone-7-*O*-glucoside-2-*O*-rhamnosyltransferase and was shown to convert naringenin and

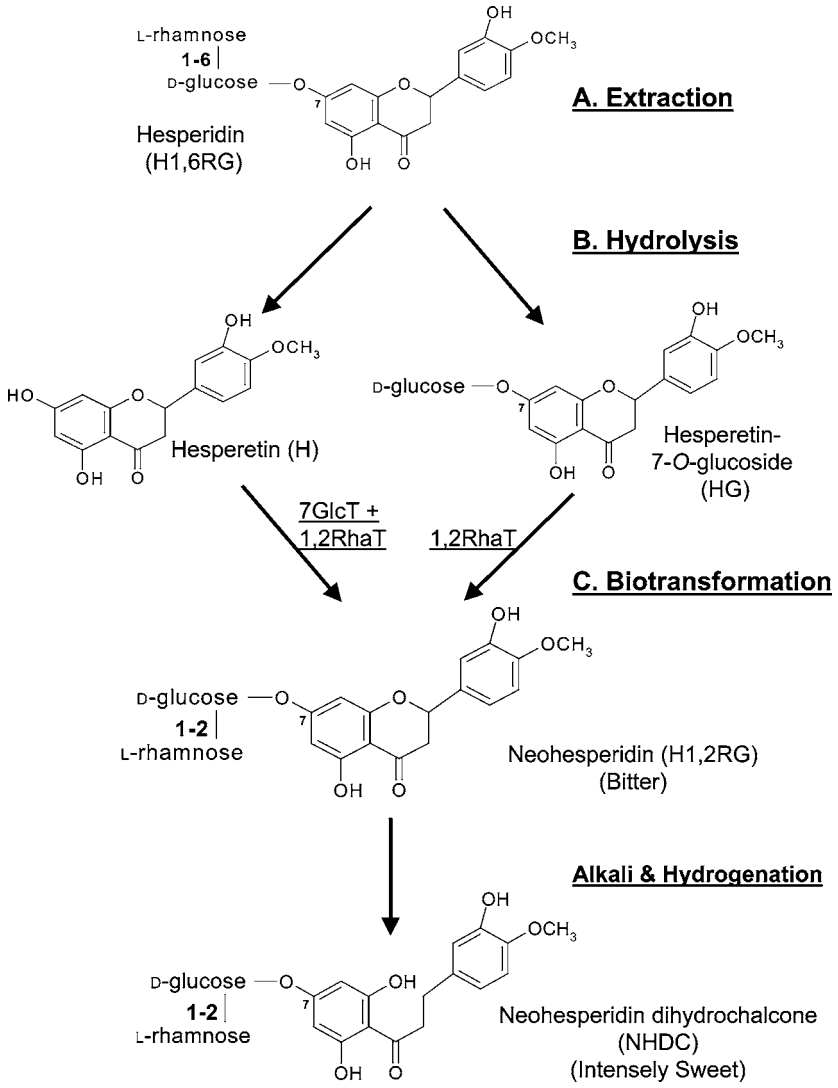

Figure 1. Scheme for conversion of heseridin from orange peels into neohesperidin and ultimately the sweetener NHDC. (**A**) Extraction of hesperidin from orange fruit peel. (**B**) Hydrolysis of hesperidin into HG and hesperetin. (**C**) Biotransformation of hesperidin hydrolysis products by transgenic cell suspension culture expressing 1,2RhaT. HG is biotransformed in one enzymatic step into neohesperidin by the recombinant 1,2RhaT (*17*). Hesperetin is biotransformed in two enzymatic steps into neohesperidin by an endogenous 7-*O*-glucosyltransferase (*23*) and the recombinant 1,2RhaT (*17*). Neohesperidin is converted into the dihydrochalcone NHDC by alkaline catalysis conditions followed by reductive hydrogenation (*7*).

naringenin-7-*O*-glucoside into naringin (*17*). Because modifications on flavonoid ring B appear not to substantially affect the affinity of the enzyme to the flavonoid substrate (*17*), it is expected that HG is a substrate for the enzyme encoded by *Cm 1,2RhaT*. This expectation is further supported by the fact that the 1,2RhaT enzyme purified from pummelo leaves was active on both naringenin-7-*O*-glucoside and HG substrates (*24*). Therefore, we propose to use cell suspension cultures transformed with *Cm 1,2RhaT* for biotransformation of HG and hesperetin into neohesperidin in one or two enzymatic steps, respectively (**Figure 1**).

Conversion of neohesperidin into NHDC was first demonstrated by Horowitz and Gentili (*7*). This simple and established procedure (*11*) isomerizes neohesperidin into a chalcone under alkaline conditions, and the reductive hydrogenation in the presence of a catalyst completes the conversion to NHDC (**Figure 1**).

Transgenic Plant Cell Culture Lines Expressing a Flavonoid 1,2-Rhamnosyltransferase Convert Commercial Hesperetin into Neohesperidin. Tobacco (BY2) and carrot cell suspension cultures were transformed with the gene *Cm1,2RhaT* (*17* and Materials and Methods respectively), encoding a citrus flavanone-7-*O*-glucoside 1,2-rhamnosyltransferase, under the control of a CAMV 35S promoter. We tested the potential of the transgenic cell suspension culture lines expressing *Cm1,-2RhaT* to biotransform a commercial sample of the flavanone aglycone hesperetin into neohesperidin. Wild-type (BY2) and transgenic (BY2-1,2RhaT) cells were fed with the substrate hesperetin for 48 h. Flavonoids were extracted and analyzed by TLC (not shown) and LC/MS (**Figure 2**). Two flavanone peaks were detected in extracts from both BY2 and BY2-1,-2RhaT cell lines fed with hesperetin. These peaks were identified, based on retention time and mass spectral comparison to authentic standards to be HG and hesperetin-diglucoside (HGG). An additional peak was detected only in extracts of the transgenic cell line BY2-1,2RhaT (**Figure 2**, compare panels **b** and **d**) and was identified as neohesperidin (H1,2RG). Similar results were obtained using the transgenic carrot cell suspension culture (data not shown). The results demonstrate that metabolically engineered plant cell suspension cultures expressing recombinant *Cm1,2RhaT* can biotransform hesperetin, the hesperidin aglycone, into neohesperidin.

Conversion of Orange Peel Hesperidin into Neohesperidin. A small-scale production experiment was conducted to establish

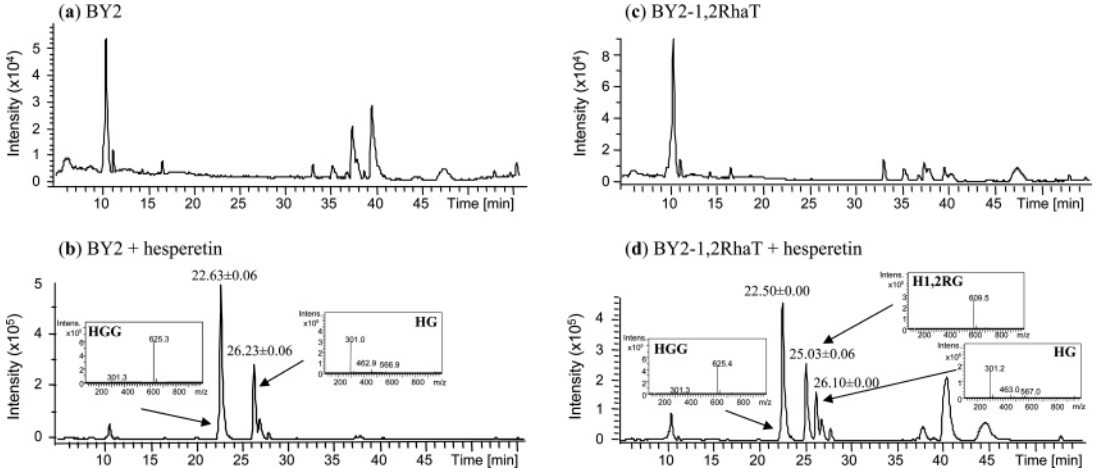

Figure 2. Biotransformation of commercial hesperetin into neohesperidin. Tobacco BY2 wild-type and transgenic cell cultures were fed with hesperetin for 48 h followed by flavonoid extraction and analysis by HPLC/MS as follows: (**a**) extract of BY2 cells, (**b**) extract of BY2 cells fed with hesperetin, (**c**) extract of BY2-1,2RhaT cells, and (**d**) extract of BY2-1,2RhaT cells fed with hesperetin. Peaks were identified based on retention times and mass spectral comparison to authentic standards: HG, hesperetin-7-*O*-glucoside; H1,2RG, hesperetin-7-*O*-neohesperidoside (neohesperidin); HGG, hesperetin diglucoside.

the feasibility of the process presented above for conversion of orange peel hesperidin into neohesperidin. Each stage was monitored by analysis of the products by TLC (**Figure 3a**). Hesperidin was extracted from orange peels by a simplified protocol (see Materials and Methods) and was found to comigrate with a commercial standard of hesperidin (**Figure 3a**, lanes 1 and 2). Because we do not have the facilities to conduct acid-catalyzed hydrolysis experiments at 140 °C necessary for obtaining high solubility and hydrolysis of hesperidin (*21*), the crude orange peel hesperidin preparation was solubilized in DMSO and subjected to dilute acid catalysis at 121 °C in an autoclave. Hydrolysis products were collected and were found to consist of a mixture of hesperetin and hesperetin-7-*O*-glucoside (**Figure 3a**, lane 4). The hydrolysis products were added to the media of wild-type (BY2) and transgenic (BY2-1,2RhaT) cells. Biotransformation results were similar to those described above for commercial hesperetin. Extracts of both wild-type and transgenic cells contained a spot comigrating with HG as well as a slower migrating spot (**Figure 3a**, lanes 5 and 6) corresponding to HGG shown in **Figure 2**. However, only the transgenic cell line BY2-1,2RhaT contained a third spot comigrating with neohesperidin (**Figure 3**, compare lanes 5 and 6 and see the neohesperidin standard in lane 7). The putative neohesperdin spot was extracted from the silica gel plate and confirmed to be neohesperidin by comparison to a commercial standard using LC/MS (**Figure 3b**).

The data represent a "proof-of-concept" for production of neohesperidin (and consequently NHDC) from orange peel hesperidin by biotransformation using metabolically engineered plant cell suspension cultures. While bacteria and yeast are often the systems of choice as biological "factories", they lack the biosynthetic pathway to produce UDP-rhamnose, which is the rhamnose source for neohesperidin biosynthesis. In contrast, plants cells produce UDP-rhamnose and are therefore the only currently available systems to produce neohesperidin by this approach. Tobacco BY2 cells and carrot cells demonstrate relatively high growth rates as cell suspension cultures (*22*) and are therefore appropriate for commercial applications.

While the data show our approach to neohesperidin production is feasible at the laboratory level, much research is needed to advance toward application. Two apparently correlated drawbacks that need to be worked out are (i) inconsistent levels of neohesperidin production and (ii) a significant proportion of

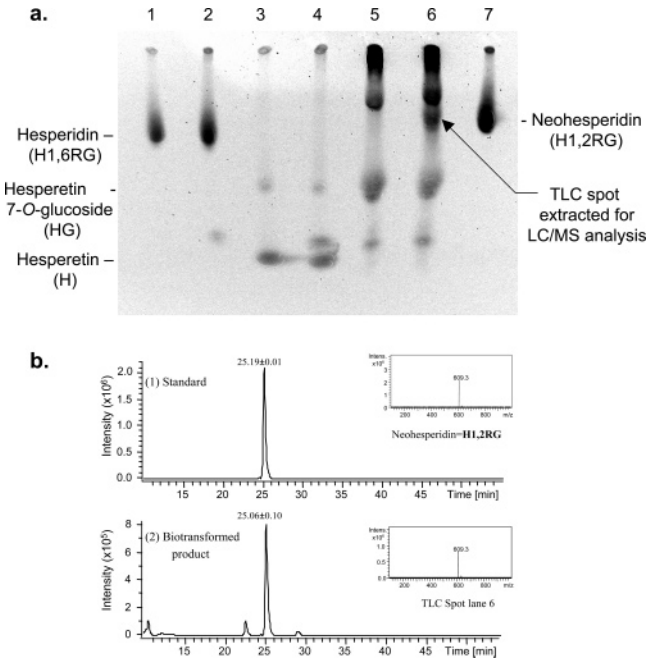

Figure 3. Small-scale production of neohesperidin from orange peel hesperidin. (**a**) Hesperidin was extracted from orange peels and hydrolyzed as described in the Materials and Methods, and hydrolysis products were biotransformed by BY2 wild-type and transgenic cell culture lines. Flavonoids were extracted and separated by TLC as follows: 1, hesperidin standard; 2, hesperidin extracted from orange peels; 3, hesperetin standard (contains contamination with HG); 4, orange peel hesperidin hydrolysis products; 5, extract of BY2 cells fed with hydrolysis products; 6, extract of BY2-1,2RhaT cells fed with hydrolysis products; and 7, neohesperidin standard. Plates were developed and photographed under UV light as described in the Materials and Methods. (**b**) The spot from lane 6 comigrating with neohesperidin (see arrow) was extracted from the silica gel plate as described in the Materials and Methods and analyzed by LC/MS relative to a neohesperidin standard as follows: 1, neohesperidin standard; and 2, TLC spot extracted from lane 6.

the hydrolyzed hesperidin substrate is converted into a diglucoside at the expense of the desired neohesperidin product (see **Figures 2** and **3**). We note that based on our observations, use of a freshly transformed cell culture for biotransformation

yielded mostly neohesperidin, while stably transformed cultures maintained for several weeks or months yielded increasing amounts of diglucoside at the expense of neohesperidin. Because the transgenic cell suspension cultures that we use are likely composed of several independent transformation events and are therefore not clonal, we speculate that cells highly expressing *Cm1,2RhaT* may be growing slower than low expressing cells, leading eventually to cultures expressing predominantly lower levels of the enzyme. The "selection" for cells expressing lower levels of *Cm1,2RhaT* could explain the decrease in neohesperidin production efficiency correlated with an increase in the competing diglucoside. One potential approach to maintain a favorable ratio of neohesperidin/diglucoside is to develop plant cell suspension culture clonal lines based on single transformation events and selecting for the high *Cm1,2RhaT* expressers. A stable clonal cell line highly expressing *Cm1,2RhaT* would also be expected to ensure consistent neohesperidin production efficiency levels. An additional potential approach to improve the neohesperidin/diglucoside ratio may be to block production of the undesired diglucoside by silencing the endogenous genes that encode the relevant glycosyltransferases, some of which have recently been described in tobacco (*25*). This approach may be feasible, provided that these enzymes are not essential for cell viability.

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