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Rosa hybrida orcinol *O*-methyl transferase-mediated production of pterostilbene in metabolically engineered grapevine cell cultures

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ABSTRACT

Stilbenes are naturally scarce high-added-value plant compounds with chemopreventive, pharmacological and cosmetic properties. Bioproduction strategies include engineering the metabolisms of bacterial, fungal and plant cell systems. Strikingly, one of the most effective strategies consists in the elicitation of wild grapevine cell cultures, which leads to vast stilbene resveratrol accumulation in the extracellular medium. The combination of both cell culture elicitation and metabolic engineering strategies to produce resveratrol analogs proved more efficient for the hydroxylated derivative piceatannol than for the dimethylated derivative pterostilbene, for which human hydroxylase HsCYP1B1- and grapevine O-methyltransferase VvROMT-transformed cell cultures were respectively used. Rose orcinol O-methyltransferase (OOMT) displays enzymatic properties, which makes it an appealing candidate to substitute VvROMT in the combined strategy to enhance the pterostilbene production level by engineered grapevine cells upon elicitation. Here we cloned a *Rosa hybrida* OOMT gene, and created a genetic construction suitable for *Agrobacterium*-mediated plant transformation. OOMT's ability to catalyze the conversion of resveratrol into pterostilbene was first assessed *in vitro* using protein extracts of agroinfiltrated *N. benthamiana* leaves and transformed grapevine callus. The grapevine cell cultures transformed with *Rh*OOMT produced about 16 mg/L culture of pterostilbene and reached an extracellular distribution of up to 34% of total production at the best, which is by far the highest production reported to date in a plant system. A bonus large resveratrol production of ca. 1500–3000 mg/L was simultaneously obtained. Our results demonstrate a viable successful metabolic engineering strategy to produce pterostilbene, a resveratrol analog with enhanced pharmacological properties.

Introduction

Vitis vinifera is a species capable of producing stilbenes, all of which are derivatives of *trans*-resveratrol (*t*-R, 3,4′,5-trihydroxystilbene) [1]. These compounds are biosynthesized in grapevine tissues in specific developmental stages [2], and act as a defensive response against certain kinds of stress [3–5]. Biosynthetically, *t*-R derives from the phenylpropanoid pathway, formed by stilbene synthase through the condensation of *p*-coumaroyl-CoA with three units of malonyl-CoA [6]. Derivatives of *t*-R may subsequently form through photochemical and enzyme-catalyzed reactions [2,7], although several steps remain uncharacterized.

t-R has beneficial effects on human health, such as preventing or slowing down of a wide range of illnesses, including cancer, obesity and cardiovascular diseases, and extending the life span of various organisms [8–12]. However, transferring the beneficial properties of *t*-R *in*

Abbreviations: R, resveratrol; Pt, pterostilbene; Pn, piceatannol; *t*-, *trans*; *Hs*CYP1B1, human cytochrome P450 hydroxylase 1B1; *Vv*ROMT, *Vitis vinifera* resveratrol *O*-methyltransferase; *Vv*GSTU-2, *Vitis vinifera* glutathione S-transferase tau 2; *Rc*OOMT1, *Rosa chinensis* orcinol *O*-methyltransferase 1; *Rh*OOMT4, *Rosa hybrida* orcinol *O*-methyltransferase 4; OMT, *O*-methyltransferase; STS, stilbene synthase; HC-Pro, potyvirus helper component-proteinase; GFP, green fluorescent protein; HA, haemagglutinin; MRM, multiple reaction monitoring; MBCD, methylated β-cyclodextrins; MeJA, methyl jasmonate; SAM, S-(5′-Adenosyl)-L-methionine chloride dihydrochloride; TCA, trichloracetic acid; EDTA, Ethylenediaminetetraacetic acid; PVPP, Polyvinylpolypyrrolidone; PMSF, Phenylmethanesulfonyl fluoride; PVDF, Polyvinylidene fluoride; FW, fresh weight

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vitro to *in vivo* systems is restricted by its limited oral bioavailability [13]. Natural, but low abundance, *t*-R analogs, such as polyhydroxy- and polymethoxy-derivatives *t*-piceatannol (*t*-Pn) and *t*-pterostilbene (*t*-Pt), respectively, display significantly greater bioavailability [14,15], while maintaining or enhancing their anti-cancer and cancer chemo-preventive activities [16,17].

As the value of stilbenes is high, many efforts are being made to produce them on a large scale. With *t*-R, synthetic biology approaches in microbial systems, such as yeast [18] and *E. coli* [19], have been intensively explored. However, one of the most effective strategies is to use grapevine cell cultures elicited with methylated β-cyclodextrins (MBCD), either alone or combined with methyl jasmonate (MeJA) [4,20,21]. The feasibility of resveratrol analog production has also been demonstrated in a plant system by a direct metabolic engineering approach combined with elicitation to overexpress homologous (*Vv*ROMT) and heterologous (*Hs*CYP1B1) genes that encode enzymes to convert *t*-R into *t*-Pt and *t*-Pn, respectively, in grapevine cell cultures [22]. Although this bioproduction system is very promising, one major challenge is to increase the conversion rate of (elicitation-induced) *t*-R into analogs, especially methylated ones. The heterologous expression of alternative methyltransferase enzymes in grapevine cells could be a promising approach to improve *t*-Pt production yields.

Plant O-methyltransferases (OMTs) constitute a large family of enzymes that methylate the hydroxyl groups of a variety of secondary metabolites, including phenylpropanoids, flavonoids and alkaloids. However, very few plant OMT genes have been isolated and characterized to potentially exhibit resveratrol O-methyltransferase activity. Genes from sorghum [23,24] and rice [25] have been used in both metabolically engineered plants and microbial systems, and have shown limited reaction specificity on *t*-R. Schmidlin et al. [7] applied a candidate gene approach and discovered *Vv*ROMT by searching grapevine EST collections for *Rosa chinensis* orcinol O-methyltransferase 1 (*Rc*OOMT1), as they claimed that purified recombinant *Rc*OOMT1 could methylate *t*-R. *Rose* sp. OOMTs have been shown to catalyze the methylation of orcinol, the structural analog of t-R, to yield the volatile phenolic ether 3,5-dimethoxytoluene, the structural analog of t-Pt [7], which is also a major compound of rose scent [26,27] (Fig. 1). In this work, we provide experimental evidence for the ability of rose OOMT to catalyze the conversion of *t*-R into *t*-Pt, and show that it is a good alternative candidate to *Vv*ROMT [22] for a combined elicitation strategy with metabolically engineered grapevine cell cultures to improve the *t*-Pt production yields in this system.

Material and methods

Plant material

vinifera L. cv. Gamay calli were kindly supplied by Drs. J. C. Pech and A. Latché (ENSA, Toulouse, France) in 1989. *Vitis vinifera* L. cv. Monastrell albino calli were established in 1995 as previously described [28]. These cell lines were maintained in both solid and liquid cultures in Gamborg B5 medium as described elsewhere [4]. *Nicotiana benthamiana* plants were obtained from seeds that were germinated and grown in potting soil in a greenhouse at 25 °C with a 16 h light/8 h dark photoperiod until they were 3–5 weeks old. Petals of rose (*Rosa hybrida*) were obtained from the Torretes Biological Station botanical garden at the University of Alicante located in Ibi, Alicante (Spain) in September 2015. They were snap-frozen in LN_2 and transported to the laboratory at −20 °C.

RNA isolation and cloning of RhOOMT

Total RNA was isolated as previously described [29] from 0.5 g of rose petal and quantified in a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Only the RNA samples with a 260/280 ratio between 1.9 and 2.1 were used. Residual genomic DNA was removed by DNase I digestion with DNase I RNase-Free (Thermo Scientific). First-strand cDNA was synthesized from 1 µg of total RNA by a cDNA synthesis kit (RevertAid First Strand cDNA Synthesis Kit, ThermoScientific) according to the manufacturer's instructions. The OOMT coding region (Acc. AJ439744.1) was PCR-amplified from rose petal cDNA using specific primers according to Scalliet et al. [27], excluding restriction sites (Fw: 5′-ATGGAAAGGCTAAACAGCTTTAGACACCTTAAC-3′ and Rev: 5′-TCAAGGATAAACCTCAATGAGAGACCTTAAACC-3′). The amplification reactions consisted of 1 cycle at 94 °C for 5 min and 30 cycles at 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, followed by an extension cycle of 10 min at 72 °C. Amplified DNA fragments were cloned into pGEM®-T Easy (Promega, Madison, WI,

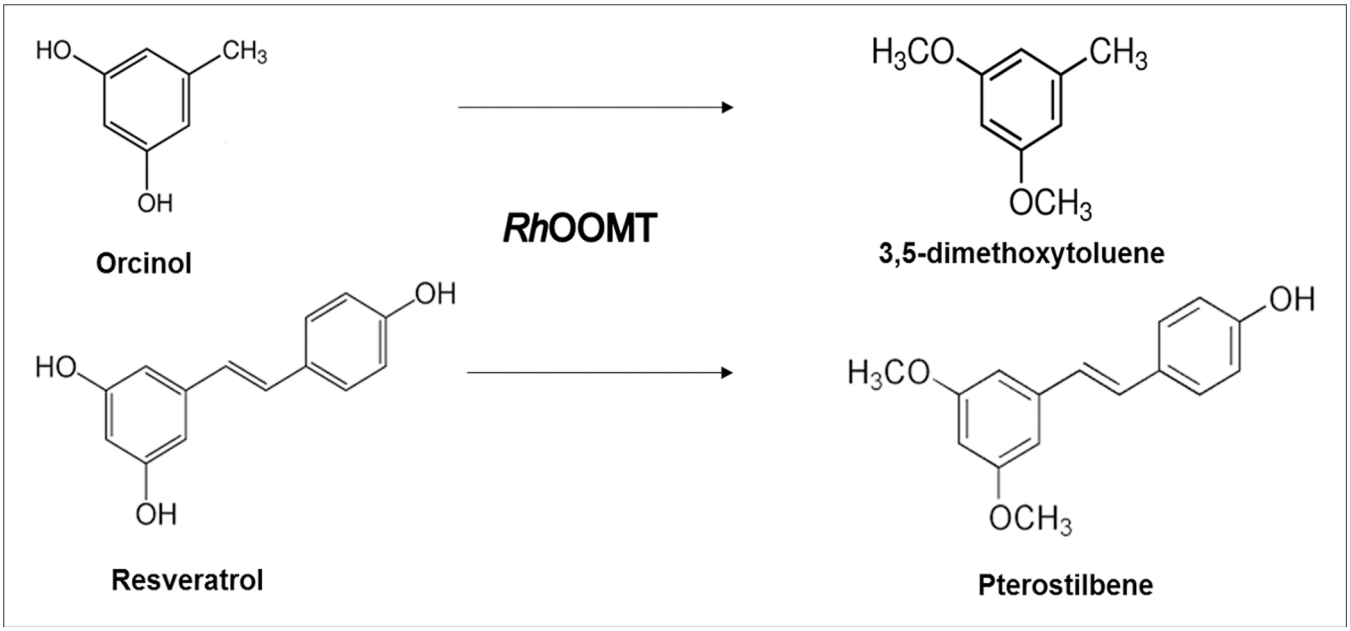

Fig. 1. Dimethylation reactions catalyzed by RhOOMT. The enzyme catalyzes the methylation of two hydroxyl groups in meta position of a phenyl group substituted in position 1, using SAM as methyl group donor. Resveratrol is a structural analog of the natural substrate orcinol, thus it might potentially be used by RhOOMT to generate the structural analog of 3,5-dimethoxytoluene, pterostilbene.