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Research article

Enhanced extracellular production of *trans*-resveratrol in *Vitis vinifera* suspension cultured cells by using cyclodextrins and coronatine

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ABSTRACT

In the present work the effect of cyclodextrin and coronatine on both *trans*-resveratrol production and the expression of stilbene biosynthetic genes in *Vitis vinifera* L. cv Monastrell suspension cultured cells were evaluated. The results showed the maximum level of *trans*-resveratrol produced by cells and secreted to the culture medium with 50 mM cyclodextrins and 1 μM coronatine. Since the levels of *trans*-resveratrol produced in the combined treatment were higher than the sum of the individual treatments, a synergistic effect between both elicitors was assumed. In addition, all the analysed genes were induced by cyclodextrins and/or coronatine. The expression of the *phenylalanine ammonia lyase* and *stilbene synthase* genes was greatly enhanced by coronatine although an increase in the amount of *trans*-resveratrol in the spent medium was not detected. Therefore, despite the fact that *trans*-resveratrol production is related with the expression of genes involved in the biosynthetic process, other factors may be involved, such as post-transcriptional and post-traductional regulation. The expression maximal levels of *cinnamate 4-hydroxylase* and *4-coumarate-CoA ligase* genes were found with cyclodextrins alone or in combination with coronatine suggesting that the activity of these enzymes could be not only important for the formation of intermediates of *trans*-R biosynthesis but also for those intermediates involved in the biosynthesis of lignins and/or flavonoids.

1. Introduction

Vitis vinifera produces stilbenes, which are a small group of compounds characterized by a 1,2-diphenylethylene backbone. Most plant stilbenes are derivatives of the monomeric unit *trans*-resveratrol (*trans*-R, 3,5,4′-trihydroxystilbene, Jeandet et al., 2014). The formation of stilbenes is considered to be a part of the general defense mechanism since they display strong antifungal and antimicrobial activities (Pezet., 2004; Bru et al., 2006; Adrian and Jeandet, 2012). In fact, *trans*-R is produced in both grapevine vegetative tissues and berries as well as in suspension cultured cells (SCC) in response to abiotic and biotic stress (Cantos et al., 2003; Wang et al., 2010). Since *trans*-R was also postulated to be involved in the health benefits associated with a moderate consumption of red wine (Siemann and Creasy 1992), it is one of the most extensively natural products studied. In fact, hundreds of studies have reported the beneficial effects of *trans*-R on neurological system (Okawara et al., 2007), cardiovascular diseases (Bradamante et al., 2004), preventing carcinogenesis (Vang et al., 2011; Fernández-Pérez et al., 2012) and as an antiaging agent in the treatment of age-related human diseases (De la Lastra and Villegas 2005).

On the other hand, *trans*-R is biosynthesized from phenylalanine which is a key intermediate linking the primary metabolism and the secondary metabolism. Thus, the first step in the stilbene biosynthesis pathway consists in the transformation of phenylalanine into cinnamic acid in a reaction catalysed by the enzyme phenylalanine ammonia lyase (PAL). The consecutive action of cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL) transforms cinnamic acid into 4-coumaroyl-CoA, which is the

Abbreviations: 4CL, 4-coumarate-CoA ligase; C4H, cinnamate 4-hydroxylase; Cor, coronatine; DW, dry weight; FW, fresh weight; JA–Ile, isoleucine-conjugated form of jasmonic acid; PAL, phenylalanine ammonia lyase; STS, stilbene synthase; SCC, suspension cultured cells; *trans*-R, *trans*-resveratrol; CD, β-cyclodextrins.

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common precursor of most of the phenolic compounds found in plants: lignins, flavonoids and stilbenoids. Then, one molecule of 4-coumaroyl-CoA is condensed consecutively with three malonyl-CoA units to produce either *trans*-R, through the action of stilbene synthase (STS), or naringenin chalcone by the action of chalcone synthase (Almagro et al., 2013 and references therein).

Stilbene biosynthesis is induced in response to a wide range of biotic and abiotic elicitors, which in turn, activate the expression of genes encoding stilbene biosynthesis pathway (Ferri et al., 2009; Tassoni et al., 2005). Stilbene biosynthesis is triggered by signalling molecules such as methyl jasmonate (Kiselev 2011). In this way, upon perception of jasmonate signals, the plant cell activates several defense mechanisms, reflected in a massive reprogramming of gene expression which leads to both the activation of stilbene biosynthesis and the expression of pathogenesis related-proteins (Tassoni et al., 2005; Donnez et al., 2011; Kiselev 2011; Belchí-Navarro et al., 2012). Moreover, coronatine (Cor) is a phytotoxin produced by *Pseudomonas syringae* (Bender et al., 1999), that acts as a mimic molecule of the isoleucine-conjugated form of jasmonic acid (JA–Ile) (Katsir et al., 2008). This compound is important for its potential to act as both plant growth regulator and elicitor of plant secondary metabolism (Onrubia et al., 2013).

As Cor resembles the JA–Ile their mode of action could be similar. The actions of Cor include the induction of jasmonic acid biosynthesis, inhibition of root elongation, hypertrophy, chlorosis, ethylene emission, secondary metabolite production, among others (Tamogami and Kodama 2000; Yao et al., 2002; reviewed in Uppalapati et al., 2005). However, although the action of jasmonic acid or methyl jasmonate on secondary metabolite biosynthesis has been widely studied (Tassoni et al., 2005; Lee-Parson et al., 2004), there are relatively few reports on the effect of Cor on secondary metabolite production. In this sense, Onrubia et al. (2013) observed that Cor enhanced both taxane production and expression of genes encoding enzymes involved in the biosynthesis of taxanes in *Taxus media* SCC. In addition, Cor also increased the accumulation of benzo[c]phenanthridine alkaloid and glyceollins in *Eschscholzia californica* and *Glycine max* L SCC, respectively (Haider et al., 2000; Fliegmann et al., 2003).

On the other hand, β-cyclodextrins (CD) are cyclic oligosaccharides that chemically resemble to the alkyl-derived pectic oligosaccharides naturally released from the cell walls during fungal attack (Bru et al., 2006). They act as true elicitors since they provoke stilbene accumulation and induce the accumulation of new gene products like peroxidases, β-1,3-glucanases and chitinases (Morales et al., 1998; Martínez-Esteso et al., 2009). They have a hydrophilic external surface and hydrophobic central cavity that trap *trans*-R, forming inclusion complexes (Morales et al., 1998). In addition, the high levels of *trans*-R whih is secreted and accumulated in the culture medium have no toxic effect on the cell lines, allowing successful subcultures. In fact, CD act not only as inducers of *trans*-R biosynthesis but also as promoters of adducts that remove *trans*-R from the medium, reducing feedback inhibition and *trans*-R degradation and allowing its accumulation in high concentrations (Almagro et al., 2011). Interestingly, Lijavetzky et al. (2008) demonstrated that the combined use of CD and methyl jasmonate enhanced the production of *trans*-R, which was strongly associated to an increased expression of *STS*, *PAL*, *C4H*, *4CL* genes in grapevine SCC. However, the effect of Cor in combination with others elicitors has never been studied on both *trans*-R production and phenylpropanoid pathway gene expression.

Taking into account all of the above, in the present study we have analysed the influence of CD and/or Cor on the expression profiles of several biosynthetic pathway genes associated with the production of *trans*-R in *V. vinifera* Monastrell SCC.

2. Material and methods

2.1. Plant material

V. vinifera L. cv Monastrell calli were established in our laboratory in 1990 as described by Calderón et al. (1993). Since then, calli have been maintained at 25 °C in the dark in 250 mL flasks containing 100 mL of fresh growth medium (Gamborg B_5) supplemented with Morel vitamins (Morel, 1970), 0.25 g L^{-1} casein hydrolysate, 20 g L^{-1} sucrose, 0.2 mg L^{-1} kinetin, 0.1 mg L^{-1} 1-naphthaleneacetic acid and pH adjusted at 6.0. Grapevine calli were subcultured on solid growth medium every month. *V. vinifera* SCC were initiated by inoculating friable callus pieces (20 g fresh weight (FW)) in 250 mL Erlenmeyer flasks containing 100 mL of liquid growth medium adjusted to pH 6.0 and, maintained in a rotary shaker (110 rpm) at 25 °C in the dark. *V. vinifera* SCC were routinely maintained by periodical subcultures every 15 days.

2.2. Elicitor treatments

Elicitation experiments were performed in triplicate using 15 day old *V. vinifera* SCC. At this stage of cell development, 4 g of FW of washed cells were transferred into 100 mL flasks, suspended in 20 mL of fresh growth medium supplemented with either 50 mM CD (Wacker Chemie, Spain) or 0.5, 1 or 2 μM Cor (Sigma, Spain) alone or in combination, and maintained at 25 °C in the dark in a rotary shaker (110 rpm). Control treatments without elicitors were always run in parallel. After elicitation, cells were separated from the culture medium by filtration, rapidly washed with cold distilled water, weighted and frozen at −80 °C until use. The elicited culture medium was used for *trans*-R quantification.

2.3. Quantification of trans-resveratrol in both culture medium and cells

For this, 20 μL of the spent medium were analysed in a HPLC-DAD (Waters 600E, Waters 996) as described by Belchí-Navarro et al. (2012). In addition, 50 mg of freeze-dried cells were extracted overnight in 4 mL methanol at 4 °C. The cell extract was diluted with water to a final concentration of 80% (v/v) methanol. Then, 20 μL of the diluted extract was filtered (Anopore 0.2 μm) and analysed in a HPLC-DAD (Waters 600E, Waters 996) as described by Bru et al. (2006) using a Spherisorb ODS2 C-18 column (250 × 4.6 mm, 5 μm). *trans*-R was identified at 306 nm and quantified by comparison with authentic standard of >99% purity (Sigma–Aldrich, Spain).

2.4. Gene expression analyses

RNA isolation, cDNA synthesis and real-time quantitative PCR.

Total RNA was extracted from frozen cells (0.5 g FW) by means of the TRIZOL reagent (Invitrogen) following the manufacture's recommendations. RNA quality was verified with a Bioanalyzer instrument (Bioanalyzer 2100, Agilent Technologies). RNA concentration was determined optically with a Helios Gamma spectrophotometer (Thermo Scientific). Only RNA preparations with A260/A280 ratios of 1.8–2.0 and A260/A230 ratios of 2.0 were used for subsequent analysis. RNA integrity was verified by 2% agarose gel electrophoresis RNAase free followed by GelRed staining. First strand cDNA was synthesized from 0.2 μg of total RNA using the iScriptTM Select cDNA Synthesis Kit (Bio-Rad) with the Oligo (Dt) primers mix.

The qRT-PCR procedures were performed using the primers designed by Lijavetzky et al. (2008). The cDNA samples were analysed with an iCycler (Bio-Rad) apparatus using SYBR Green PCR