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Research article

Differential induction of antioxidant stilbenoids in hairy roots of *Vitis rotundifolia* treated with methyl jasmonate and hydrogen peroxide

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ABSTRACT

Stilbenoids are polyphenolic phytoalexins that exhibit potential health applications in humans. Hairy root cultures of muscadine grape (*Vitis rotundifolia* Michx.) were used to study the biochemical and molecular regulation of stilbenoid biosynthesis upon treatment with 100 μM methyl jasmonate (MeJA) or 10 mM hydrogen peroxide (H_2O_2) over a 96-h period. Resveratrol, piceid, and ε-viniferin were identified in higher concentrations in the tissue whereas resveratrol was the most abundant stilbenoid in the medium under either treatment. An earlier increase in resveratrol accumulation was observed for the MeJA-treated group showing a maximum at 12 h in the tissue and 18 h in the medium. Furthermore, the antioxidant capacity of extracts from the tissue and medium was determined by the 2,2′-azinobis[3-ethylbenzthiazoline sulfonic acid] (ABTS) and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays showing correlation with the stilbenoid content. Fourteen candidate reference genes for qPCR were tested under the described experimental conditions and resulted in the selection of 5 reference genes. Quantitative analyses of transcripts for phenylalanine ammonia-lyase (*PAL*), resveratrol synthase (*RS*), and two stilbene synthases (*STS* and *STS2*) showed the highest RNA level induction at 3 h for both treatments with a higher induction for the MeJA treatment. In contrast, the flavonoid-related chalcone synthase (*CHS*) transcripts showed induction and a decrease in expression for MeJA and H_2O_2 treatments, respectively. The observed responses could be related to an oxidative burst triggered by the exposure to abiotic stressor compounds with signaling function such as MeJA and H_2O_2 which have been previously related to the synthesis of secondary metabolites.

1. Introduction

Plants are characterized for synthesizing a wide variety of metabolites with specific functions. An important group of these compounds are phenylpropanoids derivatives such as the stilbenoids which are produced via the phenylalanine/polymalonate route, the last step of which is catalyzed by the enzyme stilbene synthase (malonyl-CoA:4-coumaroyl-CoA malonyltransferase; EC 2.3.1.95; STS), a type III polyketide synthase. Stilbene synthases are closely related to chalcone synthase (naringenin-chalcone synthase; 2.3.1.74; CHS), the key enzymes of the flavonoid pathway and they share the same substrates. Stilbenoids are phytoalexins or defense compounds that accumulate as a protective response of the plant against biotic and abiotic stress such as pathogen attack, wounding, UV radiation exposure and treatment with chemicals (Kuc, 1995; Purkayashta, 1995).

Around 400 naturally occurring stilbenoids have been identified in at least 72 plant species from 33 unrelated families (Yu et al., 2005; Counet et al., 2006; Shen et al., 2009). Most of them share the basic structure of resveratrol (3,4′,5-trihydroxystilbene), which is the most studied stilbenoid (Fig. 1A). Resveratrol and its analogues are present in several economically important crops like grapevine (*Vitis vinifera*) and peanut (*Arachis hypogaea*). The presence of resveratrol in red wine has been associated with the

Abbreviations: ABTS, 2,2′-azinobis[3-ethylbenzthiazoline sulfonic acid]; BDS, Dunstan and Short medium; CHS, chalcone synthase; CTAB, cetyltrimethylammonium bromide; DIMEB, 2,6 dimethyl-β-cyclodextrin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FW, fresh weight; H_2O_2, hydrogen peroxide; DW, dry weight; EtOAc, ethyl acetate; HPLC, high performance liquid chromatography; JA, jasmonic acid; MeJA, methyl jasmonate; MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; MS, Murashige and Skoog medium; MSV, modified Murashige and Skoog medium; NF, normalization factor; PAL, phenylalanine ammonia-lyase; qPCR, quantitative polymerase chain reaction; ROS, reactive oxygen species; RQI, RNA quality indicator; RS, resveratrol synthase; Rt, retention time; SD, standard deviation; TAC, total antioxidant capacity; TEAC, Trolox equivalent antioxidant capacity; *V*, pairwise variation; *μ*, specific growth rate.

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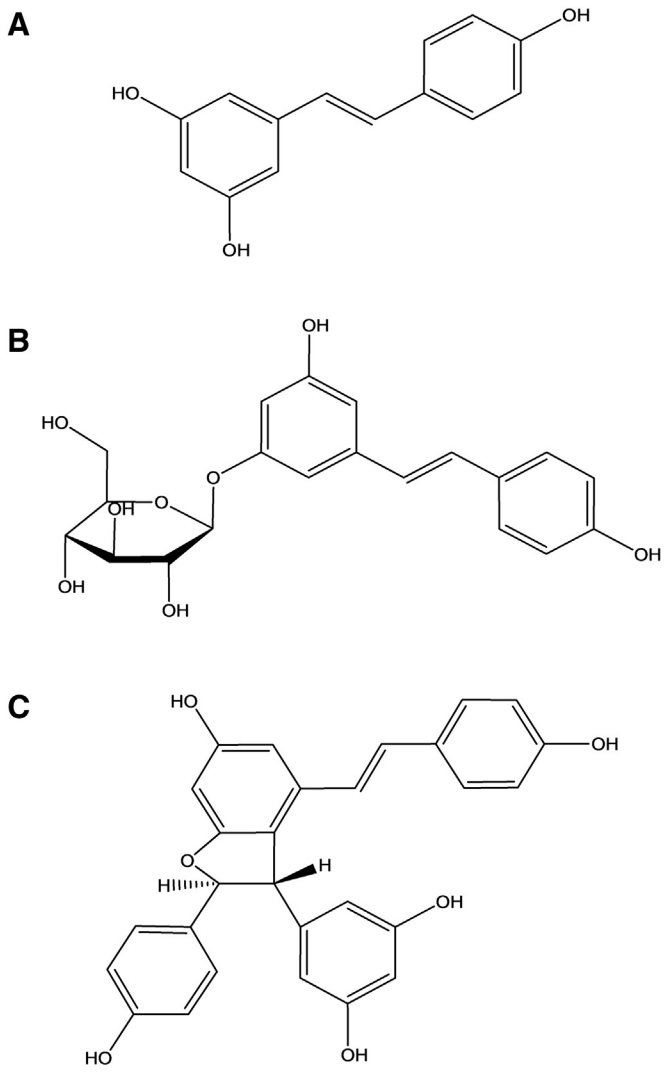

Fig. 1. Structures of selected stilbenoids synthesized by hairy roots of muscadine grape (*V. rotundifolia*). (A) Resveratrol; (B) piceid; (C) ε-viniferin.

reported health benefits found in populations where moderately drinking of wine is part of their diet (Renaud and de Lorgeril, 1992). Anticancer, anti-inflammatory, neuroprotective and antioxidant activities are among the biological properties in mammals that are attributed to resveratrol and its analogues such as piceid (Fig. 1B), viniferins (Fig. 1C), arachidins, and piceatannol (Romero-Pérez et al., 1999; Yáñez et al., 2006; Huang et al., 2010; Ko et al., 2013).

Muscadine grape (*Vitis rotundifolia*) is a member of the Vitaceae family, a crop with economic importance in its native region, the southeastern United States. The fruits are used for fresh consumption and in the elaboration of wines among several other food products. This species grows in a region where *V. vinifera* hardly grows making it an important substitute. Furthermore, *V. rotundifolia* shows greater resistance to diseases such as downy mildew (Staudt and Kassemeyer, 1995) which in turn has created interest in crossing it with *V. vinifera* varieties to introgress resistance into the grapevine background (Peressotti et al., 2010). Moreover, studies on the composition of extracts from the fruits have shown important levels of stilbenoids present in muscadine grape.

Previously, we have shown that hairy roots of peanut (Condori et al., 2010) and muscadine grape (Nopo-Olazabal et al., 2013) can be treated with abiotic elicitors to induce production of stilbenoids, including resveratrol and several of its analogues that have been described in fungal-challenged plant tissues. Therefore, hairy roots provide a reliable system to study the regulatory mechanisms that affect the biosynthesis of these specialized compounds in plants.

In this study two abiotic stressors methyl jasmonate (MeJA), a key compound in the defense-related jasmonate signal transduction pathway (Gundlach et al., 1992), and hydrogen peroxide (H_2O_2), one of the major reactive oxygen species in plant tissues (Asada, 1999; Moller, 2001) which have been suggested to play a key signaling role in plant responses to biotic and abiotic stresses such as pathogen attack, extreme temperatures, drought, radiation, ozone, and wounding (Prasad et al., 1994; Foyer et al., 1997; Orozco-Cárdenas et al., 2001; Neill et al., 2002) were compared on their ability to induce the synthesis of stilbenoids in muscadine grape hairy roots. Since stilbenoids may counteract the oxidative stress produced upon treatment with these distinct elicitors, we were interested in elucidating the antioxidant potential of stilbenoid-containing extracts from elicited-hairy roots. Given the fact that antioxidant methods can differ in their measurement of the total antioxidant capacity (TAC) (Yu et al., 2002; Chu et al., 2010), we assayed the culture medium and hairy root tissue extracts with two methods, the 2,2′-azinobis[3-ethylbenzthiazoline sulfonate] (ABTS), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay.

To study the expression of genes related to stilbenoid biosynthesis in *V. rotundifolia*, reference genes for this species had to be validated under the experimental conditions of this study. Taking advantage of the reference genes described for *V. vinifera*, we tested 14 candidate reference genes for qPCR in *V. rotundifolia*. These genes were evaluated in the time course experiments of hairy roots treated with MeJA and H_2O_2. In the subsequent study, the best reference genes were used to normalize the expression of selected genes associated with stilbenoid and flavonoid biosynthesis. Herein, we compare the levels of expression of these genes, the production of stilbenoids and their antioxidant capacity upon MeJA and H_2O_2 treatment and propose a mechanism by which these two elicitors may regulate the biosynthesis of these bioactive compounds.

2. Results

2.1. Growth kinetics of muscadine grape hairy roots in MSV medium

In order to study the growth performance and to identify the most adequate developmental stage for stilbenoid biosynthesis, muscadine grape hairy roots of line Fry-3A were grown in MSV medium during 45 days. Data obtained on the specific growth rate describes the increase in mass per unit time, and conductivity change correlates the root growth with the nutrient uptake from the culture medium. The inoculum was obtained from cultures of 21 days of age and consisted of ten root tips with a length of 4–5 cm containing two root primordia. The weight of the inoculum was approximately 0.1 g DW (1.5 g FW). A steady growth was observed up to day 39 when this stage ended and the stationary growth phase started (Fig. 2A). At the end of the growth kinetics (45 d) the root weight was approximately 0.6 g DW (9 g FW) and the average DW/FW ratio was 0.07 during the 45 d of the culture period with the highest variability at 21 d and 33 d (Fig. 3).

The roots showed a ropy phenotype and a mucilage that turned brown as the roots aged. The culture medium presented no change in color after root inoculation and it continued to show a clear appearance until the stationary growth phase was reached, at this point a slightly yellowish color was observed. The culture medium pH was set to 6 prior autoclaving it and dropped to an average of