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Induction of *trans*-resveratrol and extracellular pathogenesis-related proteins in elicited suspension cultured cells of *Vitis vinifera* cv Monastrell☆

Sarai Belchí-Navarro[a], Lorena Almagro[a], Ana Belén Sabater-Jara[a], Francisco Fernández-Pérez[a], Roque Bru[b], Maria Angeles Pedreño[a,*]

[a] Department of Plant Biology, Faculty of Biology, University of Murcia, Campus Universitario de Espinardo, E-30100 Murcia, Spain
[b] Department of Agrochemistry and Biochemistry, University of Alicante, Campus Universitario de San Vicente de Raspeig, Alicante, Spain

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ABSTRACT

Suspension-cultured cells of *Vitis vinifera* cv Monastrell were used to investigate the effects of methyljasmonate, ethylene and salicylic acid separately or in combination with cyclodextrins on both *trans*-resveratrol production and the induction of defense responses.

The results showed that the addition of methyljasmonate or ethylene to suspension-cultured cells jointly treated with cyclodextrins and salicylic acid provoked a decrease of *trans*-resveratrol levels suggesting that salicylic acid has a negative and antagonistic effect with methyljasmonate or ethylene on *trans*-resveratrol production. Likewise, the exogenous application of these compounds induced the accumulation of pathogenesis-related proteins. Analysis of the extracellular proteome showed the presence of amino acid sequences homologous to an specific β-1,3-glucanase, class III peroxidases and a β-1,4-mannanase, which suggests that these signal molecules could play a role in mediating defense-related gene product expression in *V. vinifera* cv Monastrell. Apart from these inducible proteins, other proteins were found in both the control and elicited cell cultures of *V. vinifera*. These included class IV chitinase, polygalacturonase inhibitor protein and reticuline oxidase-like protein, suggesting that their expression is constitutive being involved in the modification of the cell wall architecture during cell culture growth and in the prevention of pathogen attack.

Introduction

Plants respond to pathogen attack and/or external stresses through constitutive and inducible mechanisms. Structural barriers, or strategically positioned reservoirs of antimicrobial compounds, represent preformed constitutive defenses against tissue colonization. Induced defense responses include the activation of defense-related genes, leading to the reinforcement of plant cell walls, and the accumulation of phytoalexins and pathogenesis-related protein (PR-proteins) as part of a mechanism activated by pathogen infection or treatment with external chemical elicitors (Sabater-Jara et al., 2010; Almagro et al., 2012).

A sequential event in elicitor-induced defense responses involves the production of jasmonates, ethylene (ET), and salicylic acid (SA). These compounds coordinate the activation of a large set of defense responses, and when applied exogenously, they are able to induce resistance in both plants and suspension-cultured cells (SCC). In this sense, jasmonic acid and its more active derivative methyl jasmonate (MeJa) are considered signal molecules that act as key compounds of the signal transduction pathway involved in the induction of the biosynthesis of secondary metabolites which takes part in plant defense reactions (Almagro et al., 2009). Thus, the production of secondary metabolites increases in SCC elicited with jasmonates (Zhao et al., 2005). In this way, the production of stilbenes in *V. vinifera* SCC increases by exogenous MeJa application (Tassoni et al., 2005). Likewise, ET is involved in many physiological processes such as fruit ripening and senescence in plants, also it is one of the major regulators of plant defense responses, and its biosynthesis is stimulated in response to environmental stresses as well as in infection by pathogens and wounding, inducing some genes and defense-related compounds (van Loon et al., 2006; Bari and Jones, 2009). The exogenous ET application to SCC of *Coffea arabica* and *Thalictrum rugosum* induced the biosynthesis of polyphenols and alkaloids as well as enzymes involved in their biosynthetic reactions (Cho et al., 1988). Similarly, Touno et al. (2005) observed that ET induced phenylalanine ammonia lyase activity and triggered the formation of shikonine in shoot cultures of *Lithospermum erythrorhizon*.

Abbreviations: CDs, cyclodextrins; ET, ethylene; MeJa, methyl jasmonate; PR-proteins, pathogenesis-related proteins; SA, salicylic acid; SCC, suspension cultured cells.

☆ This work is dedicated *in memoriam* of Prof. Alfonso Ros-Barceló, our mentor and friend.

* Corresponding author. Tel.: +34 868 887000; fax: +34 868 883963.
E-mail address: mpedreno@um.es (M.A. Pedreño).

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On the other hand, SA is required for the rapid and local activation of defense responses that are mediated by several resistance genes against some pathogens, pests and abiotic stress, and for the establishment of systemic acquired resistance (Grant and Lamb, 2006; Anand et al., 2008; Bari and Jones, 2009). In addition, the exogenous application of SA induces resistance in plants against different pathogens (Almagro et al., 2009; Bari and Jones, 2009; Mandal et al., 2009). Indeed, several pathogenesis related-proteins (PR-proteins) whose expression is SA-dependent are commonly used as reporters of SA-dependent defense. In this context, a great number of class III plant peroxidases are generally induced by SA (Martínez et al., 2000), but this behavior is not universal since some peroxidases are not responsive to SA (Hiraga et al., 2000). Also, Repka (2001) observed the accumulation of PR-proteins and key enzymes of the phenylpropanoid pathway in *V. vinifera* cv Limberger SCC stimulated with SA. Moreover, SA is involved in some signal transduction pathways in which enzymes catalyzing biosynthetic reactions are induced to form defense compounds of phenolic nature (Ding et al., 2002). In fact, the addition of 50 μM SA to *Vitis amurensis* callus cultures increased both phenylalanine ammonia lyase and stilbene synthase gene expression, and *trans*-R levels. Similarly, Dong et al. (2010) showed that SA increased both phenolic compounds and the activities of phenylalanine ammonia lyase, tyrosine amino transferase and antioxidative enzymes in SCC of *Salvia miltiorrhiza*.

On the other hand, special attention has been paid to the use of cyclodextrins (CDs) which are cyclic oligosaccharides consisting of seven α-D-glucopyranose residues linked by α (1 → 4) glucosidic bonds, as potential elicitors inducing defense responses (Bru et al., 2006; Lijavetzky et al., 2008). The ability of CDs to induce defense responses in grapevine SCC was suggested by our previous results that pointed out the stilbene accumulation, gene expression and changes in peroxidase activity (Bru et al., 2006; Lijavetzky et al., 2008). The way in which CDs act, could be due to their chemical similarity with the alkyl-derived oligosaccharides that are released from the cell walls during fungal attack (Bru et al., 2006). They have a hydrophilic external surface and hydrophobic central cavity that can trap apolar compounds, including *trans*-resveratrol (*trans*-R, *trans*-3,5,4′-trihydroxystilbene), which is a inducible phytoalexin in *Vitis* species belonging to the stilbene family. This compound is constitutively synthesized in woody tissues, whereas it is induced in non-woody organ/tissues (Bavaresco and Fregoni, 2001) and grapevine SCC as the result of both abiotic and biotic stress (Belchí-Navarro et al., 2012). While the defense responses triggered by elicitors in grapevine is fairly well characterized at the chemical level, the response at the protein level is largely unknown. Proteomics of the extracellular matrix (secretome) of elicited grapevine SCC provides a powerful tool to approach this problem since the identification of differentially expressed proteins may bridge the gap in our understanding of the role played by this plant compartment in defense against pathogen attack.

Although MeJa, ET and SA have been considered as inducers of defense responses, nothing is known about the effect of ET and SA on *V. vinifera* SCC elicited with CDs. For this reason, the objective of this work is to investigate the effects of these compounds separately or in combination with CDs on *trans*-R production in SCC of *V. vinifera* cv Monastrell, and explore the secretome in the presence of SA and CDs.

Materials and methods

Plant materials

Vitis vinifera cv Monastrell calli were established in our laboratory in 1990 as described by Calderón et al. (1993). Since then, calli have been maintained and grapevine SCC derived from them have been routinely maintained by periodical subcultures as described by Belchí-Navarro et al. (2012).

Elicitation experiments on V. vinifera cv Monastrell SCC

Elicitation experiments were performed in triplicate using 12–14 days old Monastrell SCC. At this stage of cell development, 20 g of cell fresh weight (that is, 1 g cell dry weight (DW)) were washed and transferred into 250 mL flasks which contained 100 mL of fresh medium supplemented with either 50 mM CDs (CAVASOL®, Wacker Quimica Ibérica S.A.) or 100 μM MeJa (Duchefa, Spain) or 100 μM SA or 1 mM ET (using ethephon (2-chloroethylphosphonic acid) which is often used to generated ET) separately, or in combination. The SCC was incubated for up to 96 h in a rotary shaker (110 rpm) at 25 °C in darkness. After elicitation, cells were separated from the culture medium under a gentle vacuum, and the extracellular medium was used for measuring the *trans*-R content and identified the extracellular proteins.

Extraction, isolation and identification of trans-resveratrol

Aliquots of the extracellular medium were diluted with water and methanol to a final concentration of 80% methanol (v/v). For this, 20 μl of diluted and filtered (Anopore 0.2 μm) samples were analyzed in a HPLC-DAD (Waters 600E, Waters 996) as described by Belchí-Navarro et al. (2012).

Protein extraction and electrophoretic analysis

Aliquots of the spent media were extensively dialyzed against 5 mM sodium acetate buffer (pH 5.0) to remove salts remaining in the culture medium. Then, in order to purify the extracellular proteins, 100 mL of spent medium was treated with $(NH_4)_2SO_4$ up to 95% saturation. The precipitated protein was suspended in 50 mM Tris–HCl, pH 7.5, and dialyzed in this buffer overnight at 4 °C. The dialyzed sample was concentrated in Amicon Ultra (Millipore) and used for the electrophoretic assays. SDS-PAGE was performed on 10% (w/v) polyacrylamide gels using a MiniProtean® 3 Cell electrophoresis kit (Bio-Rad) as described by Sabater-Jara et al. (2010). Proteins were visualized by silver staining.

Protein digestion and sample preparation

Bands of interest were excised manually from gels, deposited in 96-well plates and processed automatically in a Proteineer DP (Bruker Daltonics, Bremen, Germany) as described by Sabater-Jara et al. (2010).

LC–ESI-MS/MS analysis

Samples were analyzed by liquid chromatography coupled to electrospray ion-trap mass spectrometry tandem MS (nanoLC–ESI-MS/MS) as described by Sabater-Jara et al. (2010).

For protein identification, LC–ESI-MS/MS spectra were transferred to BioTools 2.0 interface (Bruker Daltonics) to search in the NCBInr database using a licensed version of the Mascot v.2.2.04 search engine (www.matrixscience.com; Matrix Science, UK). Search parameters were set as described by Sabater-Jara et al. (2010). In all protein identifications, the probability scores were greater than the minimum score fixed as significant, with a *p*-value minor than 0.05.