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Enhancement of phytosterols, taraxasterol and induction of extracellular pathogenesis-related proteins in cell cultures of *Solanum lycopersicum* cv Micro-Tom elicited with cyclodextrins and methyl jasmonate

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ABSTRACT

Suspension-cultured cells of *Solanum lycopersicum* cv Micro-Tom were used to evaluate the effect of methyl jasmonate and cyclodextrins, separately or in combination, on the induction of defense responses. An extracellular accumulation of two sterols (isofucosterol and β-sitosterol) and taraxasterol, a common tomato fruit cuticular triterpene, were observed. Their levels were higher in Micro-Tom tomato suspension cultured cells elicited with cyclodextrins than in control and methyl jasmonate-treated cells. Also, their accumulation profiles during the cell growth phase were markedly different. The most striking feature in response to cyclodextrin treatments was the observed enhancement of taraxasterol accumulation. Likewise, the exogenous application of methyl jasmonate and cyclodextrins induced the accumulation of pathogenesis-related proteins. Analysis of the extracellular proteome showed the presence of amino acid sequences homologous to pathogenesis-related 1 and 5 proteins, a cationic peroxidase and a biotic cell death-associated protein, which suggests that methyl jasmonate and cyclodextrins could play a role in mediating defense-related gene product expression in *S. lycopersicum* cv Micro-Tom.

Introduction

The analysis of plant responses to biotic stress in terms of biochemical and molecular changes provides unique information to interpret the mechanisms, both passive and active, that plants have developed to resist diseases and pests. The active and induced defense responses include the production of phytoalexins and pathogenesis-related proteins (PR-proteins), the reinforcement of the cell wall through the cross-linking of cell wall structural proteins, lignification and callose deposition. Therefore, the cell wall is a dynamic compartment that plays a critical role not only in determining cell shape and growth but also in interacting with environmental factors including those required for nutrition and defense responses to abiotic stress and pathogen attacks (Pignocchi et al., 2006; Seifert and Blaukopf, 2010). In suspension cultured cells (SCC), elicitors induce defense responses that resemble those triggered by pathogen attack, such as the synthesis of phytoalexins and PR-proteins that may accumulate in the extracellular space. Therefore, SCC under elicitation constitute a useful model system of reduced complexity to investigate these defense responses since the spent medium of a SCC can be regarded as a continuous cell wall that provides a convenient, continuous and unique source of cell wall proteins, and where all the occur particular aspects of cell wall metabolism occur, including those involved in defense. Likewise, the advantage of using elicited SCC is that the culture medium can be easily separated from cells without cell disruption to recover the free extracellular proteins and metabolites (Martínez-Esteso et al., 2009).

Another sequential event in elicitor-induced defense responses is the production of jasmonates that coordinate the activation of a large set of defense responses, and when applied exogenously, they are able to induce resistance in plants. Jasmonate-dependent responses are associated with both transcriptional reprogramming and post-transcriptional modifications and, under stress, some jasmonate-regulated genes have been identified, including those encoding for PR-proteins. In fact, the exogenous application of methyl jasmonate (MJ) to tobacco and pepper cell cultures induces the transcript accumulation of genes that encode PR-proteins such as chitinases and glucan endo-1,3-beta-glucosidases (β-1,3-glucanases) and peroxidases (Sabater-Jara et al., 2010; Almagro et al., 2009). MJ also triggers the accumulation of secondary metabolites in *Solanaceae* cell cultures, *e.g.* capsidiol and nicotine in

Abbreviations: CDs, cyclodextrins; MJ, methyl jasmonate; MT, Micro-Tom; PR-proteins, pathogenesis-related proteins; SCC, suspension cultured cells.

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Nicotiana tabacum (Mandujano-Chavez et al., 2000; Goossens et al., 2003) and solavetivone in *Capsicum annuum* (Sabater-Jara et al., 2010) and *Hyoscyamus muticus* (Singh et al., 1998).

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 (Morales et al., 1998). The way in which CDs act is related to their chemical similarity with the alkyl-derived oligosaccharides that are released from the cell walls during fungal attack (Bru et al., 2006). The first evidence for the potential role of CDs to elicit defense responses was reported by Morales et al. (1998). More recently, Zamboni et al. (2009) showed, using transcriptomic approaches, that CDs modulate gene expression of specific biosynthetic pathways involved in the reinforcement of the cell wall and the accumulation of both phytoalexins and PR-proteins. Furthermore, CDs in combination with MJ synergistically enhance sesquiterpene and phytosterol biosynthesis in *C. annuum* SCC (Sabater-Jara et al., 2010). Within the Solanaceae, the accumulation of extracellular sesquiterpenoids upon addition of fungal or yeast elicitors to the cell cultures is well documented (Vögeli and Chappell, 1988; D'Harlingue et al., 1995). However, no elicitation studies with CDs and MJ have been conducted with tomato, an important crop widely used as a model system for studying the role of jasmonates in plant defense responses (Howe, 2004; Peña-Cortés et al., 2005).

The dwarf rapid-cycling of "Micro-Tom" (MT) tomato (*Solanum lycopersicum*) has been shown to be a representative plant model of the Solanaceae and other crops with fleshy, berry-type fruits (Meissner et al., 1997; Aoki et al., 2010). MT tomato is currently being used for plant genetic (Meissner et al., 1997; Martí et al., 2006), genomic (Dan et al., 2006; Aoki et al., 2010), and physiological and developmental studies (Martí et al., 2006; Gómez et al., 2009).

Based on this evidence, the goal of this work was to analyze the inductor effect of MJ and CDs, alone or in combination, on MT tomato SCC, exploring the changes in both secondary metabolite and PR-protein content that are produced in these elicited cell cultures as defense responses.

Materials and methods

Cell line initiation, culture conditions and cell growth and viability measurements

Germinated tomato (*Solanum lycopersicum* L. cv Micro-Tom) seeds, originally from Dr. A. Levy, at the Weizmann Institute of Science (Israel), were planted individually in 5 cm-diameter plastic pots containing a moist mixture of peat:vermiculite (1:1) and cultured in a growth chamber as previously described (Gómez et al., 2009). Calli were induced from immature mesocarp tissue of MT fruits using MS basal medium (Murashige and Skoog, 1962), supplemented with Morel vitamins (Morel and Wetmore, 1951), casein hydrolysate (250 mg L^{-1}), sucrose (30 g L^{-1}), α-naphtalen acetic acid (4.6 μM), kinetin (0.5 μM) and agar (7.5 g L^{-1}). Friable calluses were maintained at 25 ± 3 °C, under a 16 h light/8 h dark photoperiod with a light intensity of 6 Wm^{-2}, and subcultured every 21 days. After 6 months, SCC were induced by transferring 20 g of friable callus into 250-mL Erlenmeyer flasks containing 100 mL of the same medium without agar, and they were maintained in an orbital shaker (110 rpm). Subculturing was performed on a 16-day cycle by diluting the cells with an equal volume of culture medium. SCC were grown under the same conditions as calli. The growth of SCC was measured by weighing cell fresh weight. Cell viability was assessed by incubating the cells for 1–2 min in fresh culture medium containing 100 μg mL^{-1} fluorescein diacetate (Huang et al., 1986) and observing the fluorescence emission by living cells with a DMRB Leica optical microscope (λ_{exc} = 490 nm, λ_{emi} = 520 nm) using a Leica filter I3.

Additionally, the biomass growth index (ratio of maximum to initial cell density, X_{max}/X_0) and the biomass productivity [$P_r = (X_{max} - X_0)/t$ (X_{max} = maximum cell density, X_0 = initial cell density, and t = the time for maximum cell density] were determined (Wang et al., 2001).

Elicitation experiments

Elicitation experiments were carried out on 10-day-old MT tomato SCC. The elicitors assayed were MJ (0.1 mM, Sigma–Adrich) and CDs (50 mM, CAVASOL®, Wacker Quimica Ibérica S.A.). Elicitors were sterilized by filtering through 0.22-μm sterile filters (Millipore). The effect of elicitors was assessed separately or in combination. Briefly, washed cells were weighed (20 g fresh weight) and suspended in 100 mL of sterile fresh medium containing 50 mM CDs, 0.1 mM MJ, or 50 mM CDs + 0.1 mM MJ. Based on our experience and on previous results (Bru et al., 2006; Almagro et al., 2010; Sabater-Jara et al., 2010), the timing of elicitor treatment was 96 h. After elicitation, cells were separated from the culture medium under gentle vacuum and the spent medium was used for extracting and analyzing both terpenoid metabolites and extracellular proteins.

Extraction, isolation and identification of terpenoid metabolites

Terpenoid metabolites were extracted from 20 mL of the extracellular medium by phase partitioning with ethylacetate, as previously described (Sabater-Jara et al., 2010). GC/MS analysis was carried out using a 6890N gas chromatograph (Agilent) equipped with a mass spectrometric detector (Agilent 5973N) and an Agilent 19091S-433 HP-5MS capillary column, 30 m × 0.25 mm i.d., 0.25 μm film thickness. One μL of each sample was injected on column into a flow of helium gas held constant at 1.0 mL min^{-1}. The oven temperature was programmed from 60 to 325 °C. Terpenoid metabolites were identified by comparing the retention times and mass spectra with spectral data of commercial standards (isofucosterol and β-sitosterol, Sigma-Aldrich) and from the NBS 75 K spectral library.

Extraction and identification of extracellular proteins

Extracellular proteins were precipitated with ammonium sulphate (95%, w/v saturation), resuspended in 50 mM Tris–HCl, pH 7.5, and extensively dialyzed against the same buffer overnight at 4 °C. Five μg of proteins (140 ng μL^{-1}) from each treatment were loaded into a 10% (w/v) polyacrylamide gel using a MiniProtean® 3 Cell electrophoresis unit (Bio-Rad) for SDS-PAGE (Laemmli, 1970). Proteins were stained with ammoniacal silver nitrate solution (Silver Staining Kit, GE Healthcare). Band intensities were quantified using ImageJ software (version 1.34s, NIH, Bethesda, MD; http://rbs.info.nih.gov/ij). Selected protein bands were excised, alkylated and digested with sequencing grade trypsin (Promega, Madison, WI) as previously described (Sabater-Jara et al., 2010). The resulting peptides were analyzed by liquid chromatography coupled to electrospray ion-trap mass spectrometry tandem MS (nano LC-ESI-MS/MS) using an Ultimate 3000 nano LC (Dionex, Amsterdam, The Netherlands) and a 75 μm I.D., 100 mm reversed-phase column, at a flow rate of 300 nl min^{-1}, coupled to a Bruker HCT Ultra ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany) working in dynamic exclusion mode. For protein identification, LC-ESI-MS/MS spectra were transferred to BioTools 2.0 interface (Bruker Daltonics) to search in the non-redundant NCBI database using a licensed version of the Mascot v.2.2.04 search engine (www.matrixscience.com MatrixScience, London,