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Constitutive expression of extracellular peroxidase isoenzymes capable of oxidizing 4-hydroxystilbenes during the growth cycle of grapevine suspension cell cultures

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Abstract

The constitutive expression of peroxidase isoenzymes which are capable of oxidizing 4-hydroxystilbenes was studied during the growth cycle of suspension cell cultures from grapevine (*Vitis vinifera* L. cv. Monastrell) berries. The results showed that the growth of suspension cell cultures is accompanied by the constitutive expression of the peroxidase isoenzymes HSPrx 1 and HSPrx 2, previously characterised by their properties for oxidizing 4-hydroxystilbene, the expression of these isoenzymes being characteristic of Monastrell vines brought to a resistant state. However, although the differential catalytic properties of these grapevine isoenzymes are also shown by the horseradish peroxidase, no immunological relationships have been found between the peroxidases from either source.

Introduction

Studies on the expression of resistance mechanisms have involved whole plants, tissue slices, callus cultures and protoplasts (Bell 1981, Daub 1986, Gotthardt and Grambow 1992). Resistance is combined result of multiple biochemical and structural components (Bell 1981), and consequently any single putative component of resistance should be evaluated individually with many experimental approaches when determining its role in the total defense system.

Peroxidase is the enzyme responsible for ε-viniferin synthesis from resveratrol (3,5,4′-trihydroxytransstilbene) (Langcake and Pryce 1977), a reaction which leads to the synthesis of the most potent phytoalexin in *Vitaceae* (Langcake and Pryce 1977). In this context, it has been found that grapevine peroxidase isoenzyme B_5, previously named HSPrx 1, is constitutively expressed in *Vitis* spp. in direct relation

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with disease resistance to *Plasmopara viticola* (Calderón *et al.* 1992). Similarly, peroxidase isoenzyme A_1, previously named HSPrx 2, is differentially expressed in direct relation to induced disease resistance in leaves of axillary bud cultures of *Vitis* spp., after an exposure to UV-C (Zapata *et al.* 1993a).

The capacity of peroxidase to oxidize 4-hydroxystilbenes is not restricted to peroxidases from *Vitis* spp., because the horseradish peroxidase also oxidizes 4-hydroxystilbenes to viniferin-type compounds (see Langcake and Pryce 1977, Calderón *et al.* 1990a). The aim of the present work was to study the expression of peroxidase isoenzymes capable of oxidizing 4-hydroxystilbenes (HSPrx) in the grapevine cell cultures during their growth cycles, and their immunological cross-reactivity against antibodies to horseradish peroxidase.

Materials and methods

***In vitro* cultures:** Unripe berries (*Vitis vinifera* L. cv. Monastrell) were washed in distilled water and then shaken in a 7 % (m/v) aqueous solution of $CaClO_2$ for the period of 15 min. They were cut into quarters, the seeds removed and the quarters placed in a sterile Murashige and Skoog's medium (Murashige and Skoog 1962) supplemented with 250 mg l^{-1} casein hydrolysate, 20 g l^{-1} sucrose, 0.2 mg l^{-1} kinetin and 0.1 mg l^{-1} α-napthylacetic acid. Cultures were kept in the dark at 25 °C.

Suspension cell cultures and peroxidase fractions: Using friable calli subcultured for 40 months, the grapevine cells were cultured in suspension in the above described culture medium in a 100 ml flask with orbital shaking (125 min^{-1}). The suspension cultured cells were grown at 25 °C in the dark for 20 d.

After removal of the cell material by centrifugation, the spent medium fraction was used for measuring conductivity and to characterize peroxidase. Conductivity was determined at 25 °C using a *Crison 525 Conductivity Meter* and a *109803000 Standard Conductivity Cell*. To characterize peroxidase activity, the spent medium fraction was dialyzed overnight against 50 mM Tris-HCl buffer, pH 7.5, and the dialyzed fraction constituted the enzyme fraction used in further studies.

Cross-reactivity and immunoprecipitation assays of grapevine peroxidase: Antisera specific for horseradish peroxidase isoenzymes were obtained from *Sigma Chemical Co.* (Madrid, Spain). They were developed in goat (P 5774) or rabbit (P 7899) using purified horseradish peroxidase as immunogen.

Immunoprecipitation of grapevine peroxidase and horseradish peroxidase (type II, *Sigma Chemical Co.*) by antibodies against horseradish peroxidase was determined by the method of Marucci (1973). For this purpose, various amounts of antibody were incubated with peroxidase for 16 h at 4 °C. The residual peroxidase activity of the peroxidase-antibody mixture was then determined as described further. Inhibition was calculated by comparing the catalytic activity of enzyme in the presence of antibody to a reference standard containing enzyme alone at time zero.

Determination of peroxidase activity: Determination of the peroxidase activity with 4-methoxy-α-naphthol was carried out as described by Ferrer *et al.* (1990). Enzyme activity was expressed in nkat using an $\varepsilon_{593} = 2.1 \times 10^4$ nkat M^{-1} cm^{-1} for the dye product.

Isoelectric focusing and peroxidase staining: Separation of peroxidase isoenzymes by isoelectric focusing was carried out as described by Calderón *et al.* (1990b). Peroxidase isoenzymes were stained with 4-methoxy-α-naphthol as described by Ferrer *et al.* (1990).

Results and discussion

The cultured cells continue to grow during the whole culture period, this growth being accompanied by a continuous decrease in the conductivity of the spent medium, which reveals changes in nutrient uptake (Fig. 1).

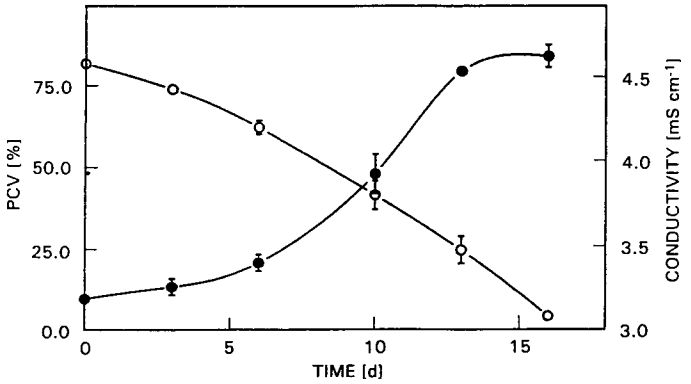

Fig. 1. Changes in the packed cell volume (*closed circles*) and conductivity of the spent medium (*open circles*) during the growth cycle of suspension cultured cells of Monastrell vines. Values are means ± SE (n = 3).

During both the pre-exponential and exponential growth phase of these cultures, the total peroxidase activity released into the culture media increased gradually and reached maximum values at the end of the post-exponential growth phase (Fig. 2). A similar variation in the level of extracellular peroxidase activity has been found for suspension cell cultures of *Capsicum annuum* (Cuenca *et al.* 1989) and Gamay grapevine cultures (García-Florenciano *et al.* 1991).

In contrast to pepper (Cuenca *et al.* 1989) and Gamay grapevine (García-Florenciano *et al.* 1991) suspension cell cultures, an analysis of the isoenzyme patterns of peroxidase secreted to the culture medium reveals no drastic changes during the growth cycle (Fig. 3). In fact, both HSPrx 1 and HSPrx 2 were constitutively expressed throughout the culture period. Only minor increases in

intensity of both isoperoxidase bands were observed during the culture ageing (Fig. 3).

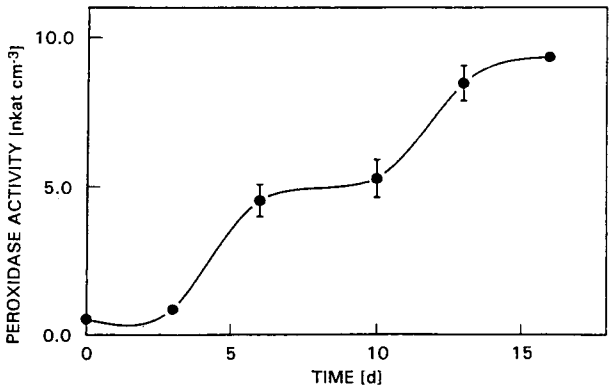

Fig. 2. Changes in the total peroxidase activity secreted to the culture medium during the growth cycle of suspension cultured cells of Monastrell vines. Values are means ± SE (n = 3).

These results suggest that HSPrx 1, a constitutive isoenzyme of Monastrell berries (Zapata *et al.* 1993b) is also constitutively expressed during the growth of suspension cell cultures, while HSPrx 2, an isoenzyme not expressed in the grape berry (Zapata *et al.* 1993b), is *de novo* constitutively expressed in the cell culture. This, in turn, suggests that Monastrell grapevine cell cultures express constitutively some of the biochemical characteristics of Monastrell vines that are brought to a resistant state (Zapata *et al.* 1993a).

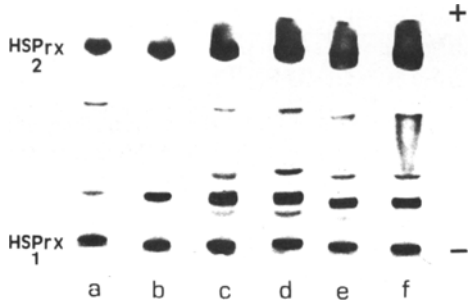

Fig. 3. Isoenzyme patterns of peroxidase secreted to the culture medium at 0 (*a*), 3 (*b*), 6 (*c*), 10 (*d*), 13 (*e*) and 16 (*f*) d of culture during the growth cycle of suspension cultured cells of Monastrell vines.

Unlike other grapevine peroxidase isoenzymes, both HSPrx 1 (Calderón *et al.* 1992) and HSPrx 2 (Zapata *et al.* 1993a) are able to oxidize 4-hydroxystilbenes to viniferin-type compounds. This property is also shared by peroxidase isoenzymes from horseradish (Calderón *et al.* 1992). For this reason, the immunological cross-reactivity of grapevine peroxidase isoenzymes with antibodies raised against

horseradish peroxidase was studied. Antisera specific for horseradish peroxidase were thus used to determine the immunological relationship between grapevine and horseradish peroxidase. This was carried out by immunotitrations of grapevine peroxidase (Fig. 4). This illustrates that although complete inhibition curves were found for horseradish peroxidase using a goat antiserum prepared against horseradish peroxidase, in no instance did goat antiserum prepared against horseradish peroxidase decrease the catalytic activity of grapevine peroxidase (Fig. 4). This was not due to the antiserum source, since similar results were obtained with rabbit immunoglobulins (data not shown).

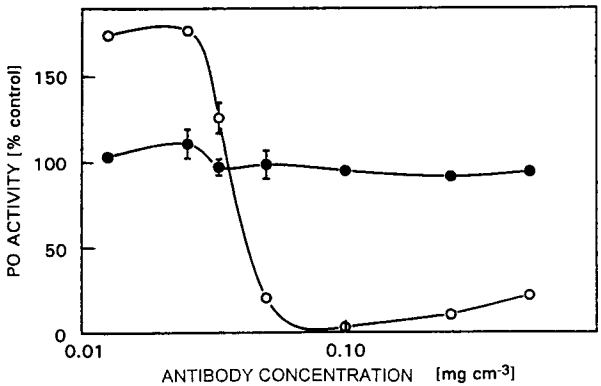

Fig. 4. Immunotitration of horseradish (*open circles*) and grapevine (*closed circles*) peroxidase with goat antiserum raised against purified horseradish peroxidase. Values are means ± SE (n = 3).

In conclusion, these results suggest that during the growth cycle of grapevine cell cultures, a constitutive expression of the peroxidase isoenzymes capable of oxidizing 4-hydroxystilbenes HSPrx 1 and HSPrx 2 takes place, and in consequence that Monastrell grapevine cell cultures express constitutively some of the biochemical characteristics of Monastrell vines that are brought to a resistant state. However, although the differential catalytic properties displayed by these grapevine isoenzymes are also shown by horseradish peroxidase, no immunological relationships were found between peroxidases from either source.

References

Bell, A.A.: Biochemical mechanisms of disease resistance. - Annu. Rev. Plant Physiol, **32**: 21-81, 1981.

Calderón, A.A., Pedreño, M.A., Ros Barceló, A., Muñoz, R.: Zymographic screening of plant peroxidase isoenzymes oxidizing 4-hydroxystilbenes. - Electrophoresis **11**: 507-508, 1990b.

Calderón, A.A., Zapata, J.M., Pedreño, M.A., Muñoz, R., Ros Barceló, A.: Levels of 4-hydroxystilbene-oxidizing isoperoxidases related to constitutive disease resistance in *in vitro*-cultured grapevine. - Plant Cell Tissue Organ Cult. **29**: 63-70, 1992.

Cuenca, J., García-Florenciano, E., Ros Barceló, A., Muñoz, R.: Sequential release of both basic and acidic isoperoxidases to the media of suspension cultured cells of *Capsicum annuum*. - Plant Cell Rep. **8**: 471-474, 1989.

Daub, M.E.: Tissue culture and the selection of resistance to pathogens. - Annu. Rev. Phytopathol. **24**: 159-186, 1986.

Ferrer, M.A., Calderón, A.A., Muñoz, R., Ros Barceló, A.: 4-Methoxy-α-naphthol as a specific substrate for kinetic, zymographic and cytochemical studies on plant peroxidase activities. - Phytochem. Anal. **1**: 63-69, 1990.

García-Florenciano, E., Calderón, A.A., Muñoz, R., Ros Barceló, A.: Epigenetic control of extracellular auxin catabolism in grapevine cells cultured in suspension. - Vitis **30**: 57-69, 1991.

Gotthardt, U., Grambow, H.J.: Near-isogenic wheat suspension cultures: establishment, elicitor induced peroxidase activity and potential use in the study of host/pathogen-interactions. - J. Plant Physiol. **139**: 659-665, 1992.

Langcake, P., Pryce, R.J.: A new class of phytolaexins from grapevines. - Experientia **33**: 151-152, 1977.

Marucci, A.A.: Inhibition of horseradish peroxidase by specific antisera. - Immunochemistry **10**: 278-280, 1973.

Murashige, T., Skoog, F.: A revised medium for rapid growth bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Zapata, J.M., Calderón, A.A., Pedreño, M.A., Muñoz, R., Ros Barceló, A.: Control by UV-C of peroxidase isoenzymes in axillary bud cultures of *Vitis* species differing in fungal resistance to *Plasmopara viticola*. - Biol. Plant. **36**: 133-138, 1994a.

Zapata, J.M., Calderón, A.A., Pedreño, M.A., Muñoz, R., Ros Barceló, A.: Restricted genetic expression of the enzymatic polymorphism of peroxidase in grapevines (*Vitis vinifera*). - Agrochimica, in press, 1994b.

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