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Peroxidase isoenzymes as markers of cell de-differentiation in grapevines (*Vitis vinifera*)

by

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S u m m a r y : The expression, and the tissue localization and the subcellular localization of peroxidase isoenzymes were studied during the development of mesocarp and hypodermal tissues of *Vitis vinifera* fruits cvs Airen and Monastrell until fruit softening. In addition the de-differentiation process of mesocarp tissues to form callus cultures was investigated. Both grapevine cultivars contain, as the only component of peroxidase polymorphism in the whole fruit, the peroxidase isoenzyme B_5 (the sole component of the peroxidase isoenzyme group HpI BPrx in grapevines), which is both developmentally-regulated and tissue-specific. The establishment of cell cultures from the mesocarp of grapevine fruits is accompanied by the *de novo* expression of one acidic (APrx) and one basic (LpI BPrx) peroxidase isoenzyme group, whose particular isoenzyme composition is cultivar-specific and dependent on subcellular location (soluble or bound to cell wall). These results suggest that, during the establishment of grapevine cell cultures from mesocarp tissues of grapevine fruits, there is a differential expression of the peroxidase isoenzyme groups APrx and LpI BPrx located in both soluble and cell wall-bound fractions. This *de novo* gene expression probably expresses at molecular level the totipotency of grapevine somatic cells, which is linked to the de-differentiation process associated with the *in vitro* culture.

K e y w o r d s : grapevine, cell cultures, cell de-differentiation, peroxidase, isoenzyme expression, subcellular localization.

Introduction

One of the unique characteristics of plant cells is totipotency, in which properly cultured non-dividing, differentiated somatic cells start dividing, producing callus which eventually regenerates plants via direct organogenesis or somatic embryogenesis (Siminis *et al.* 1993). Thus, plant cells are characterized by high plasticity in their developmental pattern, being capable of regenerating the whole plant from a single somatic cell (Rasol 1991). This makes of both organogenesis and embryogenesis an unique tool for regenerating plants with induced systemic resistance to microbial diseases once this will be induced in (or gained by) cell cultures. This is of special importance since induced resistance is not seed transmissible, but it is transmitted to regenerants via tissue culture (Madamanchi and Kuc 1991).

We have managed to induce in grapevine cell cultures obtained from the susceptible but agronomically important cultivar *Vitis vinifera* cv. Monastrell, the particular reactions of disease resistance to oomycetes which cannot be expressed by the wild-type plant (Calderón *et al.* 1993 a and 1994 b). Organogenesis and/or embryogenesis of the induced and de-differentiated cells appears to be the next step in obtaining disease resistant plants (Calderón *et al.* 1993 a).

Somatic embryogenesis has been described in grapevine suspension cell cultures for several cultivars (Thevenot *et al.* 1992). Recent results have shown that, as in other plant cell cultures (Joersbo *et al.* 1989; Cordewener *et al.* 1991; Bapat *et al.* 1992) specific extracellular peroxidase isoenzymes (EC 1.11.1.7) can modify grapevine somatic embryo differentiation, promoting embryogenic development (Thevenot *et al.* 1992), and thus acting as markers of the embryogenic success.

However, the de-differentiation process, which is linked to the expression of totipotency by grapevine somatic cells, remains largely unknown in molecular and biochemical terms. For this reason, and due to the particular properties of some peroxidase isoenzymes in promoting embryogenic development, the aim of the present work was to study the expression and subcellular localization of peroxidase isoenzymes during the de-differentiation of grapevine cell cultures in relation to their expression in the plant material source, the grape berry. This organ, which is susceptible to diseases caused by oomycetes, has previously been used successfully for the establishment of grapevine cell cultures capable of being brought to a disease resistant state (Calderón *et al.* 1993 a).

Material and methods

P l a n t m a t e r i a l : The *Vitis vinifera* L. cultivars Airen and Monastrell were grown in the field at the Hacienda Nueva Viticultural Experimental Station of the C.R.I.A. (Murcia, Spain) and in a vineyard in Campillo del Negro-Chinchilla (Albacete, Spain). Samples of fruit clusters were taken at 7-day intervals, for a total of 8 sampling dates within the growing season from bloom until veraison. Fruit clusters were transferred to the laboratory and samples, except those used to establish *in vitro* cultures, were frozen immediately at -30 °C until use.

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Peroxidase fractions in the whole fruit: Berries (10 g) were fractioned into hypodermis and mesocarp after removal of the skin with a scalpel. The hypodermal and mesocarp tissues were homogenized with a mortar and pestle at 4 °C as described by CALDERÓN *et al.* (1993 b). The homogenate was centrifuged at 20,000 *g* for 15 min, and the supernatant dialyzed overnight against 50 mM Tris-HCl buffer (pH 7.5). The dialyzed extracts constituted the soluble protein fraction used in further studies.

In vitro culture: Berries were used as starting material. At several developmental stages, berries containing soft seeds, were washed in distilled water, and then shaken in a 7 % (w/v) aqueous solution of $Ca(ClO)_2$ for 15 min (HAWKER *et al.* 1973). After 3 washes in sterile water, the berries were cut into quarters, the seeds removed and the quarters (five per 10 x 2 cm culture dish) were placed on 5 ml of sterile autoclaved Murashige and Skoog's medium (MURASHIGE and SKOOG 1962) supplemented with 250 mg l^{-1} casein hydrolysate, 20 g l^{-1} sucrose, 0.8 % (w/v) agar (Plant cell culture tested, Sigma Chemical Co.), 1.0 µM kinetin and 0.5 µM α-naphthaleneacetic acid (pH 5.8). Cultures were maintained in the dark at 25 °C. For both grape cultivars, calli developed from mesocarp tissues within 15 d and continued to grow for 1 month. After subculture in 250 ml flasks, calli obtained from 5-8-week-old grapes were grown as white friable non-pigmented callus for 3 years or more.

Peroxidase activity in callus cultures: Twenty-day-old grapevine calli were used for these studies. Homogenization of grapevine calli and subcellular fractionation of peroxidase activity was carried out as described by GARCÍA-FLORENCIANO *et al.* (1991). Three subcellular fractions were obtained: 1) a soluble (non-sedimentable) fraction, 2) a membrane fraction and 3) a cell wall fraction. Membrane and cell wall-bound peroxidases were solubilized as reported by GARCÍA-FLORENCIANO *et al.* (1991).

Determination of peroxidase activity: Peroxidase activity was determined with 4-methoxy-α-naphthol, as described by FERRER *et al.* (1990).

Isoelectric focusing and peroxidase staining: Separation of peroxidase isoenzymes by isoelectric focusing was carried out as described by CALDERÓN *et al.* (1990). Staining of peroxidase isoenzymes was performed using 4-methoxy-α-naphthol (FERRER *et al.* 1990).

Results

Developmental and tissue-specific expression of peroxidase isoenzymes in grapevine fruits: Peroxidase activity is not detectable in unripe (4-6 weeks post-anthesis) Airen and Monastrell berries until fruit softening (ZAPATA *et al.* 1995), suggesting that peroxidase is a developmentally regulated enzyme in grapevine fruits. This developmental regulation of peroxidase is in accordance with the described role of this enzyme in phenolic turnover and degradation (CALDERÓN *et al.* 1992 a; ROS BARCELÓ *et al.* 1994), since maximal expression of the enzyme takes place at veraison (8 weeks post-anthesis).

In a similar way to the whole enzymatic activity, the expression of peroxidase isoenzymes is also developmentally regulated in the berry (Fig. 1). Only at softening, fruits of both Airen (Fig. 1 A) and Monastrell (Fig. 1 B) express the peroxidase isoenzyme B_5, the sole component of the peroxidase isoenzyme group HpI BPrx in grapevines (PEDREÑO *et al.* 1993).

The peroxidase isoenzyme B_5 and, therefore, the peroxidase isoenzyme group HpI BPrx, is not only developmentally regulated, but is also tissue specific. Thus, in both Airen (Fig. 1 A) and Monastrell (Fig. 1 B) at softening, the basic peroxidase isoenzyme B_5 is mainly associated with hypodermal tissues.

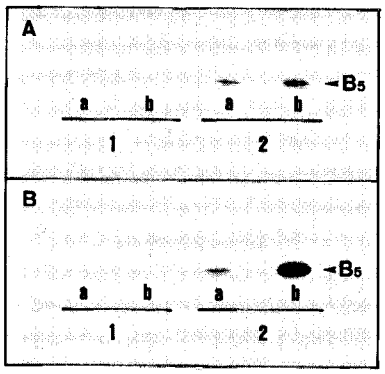

Fig. 1: Isoenzyme patterns of peroxidase activity present in the mesocarp (lane a) and hypodermis (lane b) of the *Vitis vinifera* cultivars Airen (A) and Monastrell (B), for 6-week post-anthesis (1) and 8-week post-anthesis (2) fruits.

Grapevine cell cultures: Both in Airen and Monastrell berries growth is described by a double sigmoid curve, of which two periods of rapid growth are separated by a period of reduced growth (Fig. 2). COOMBE (1960) and HALE (1968) stated that during the first period of rapid growth the berry increases in size by cell division and expansion while, in the second period, the increase in size is due only to cell expansion. The second growth period is

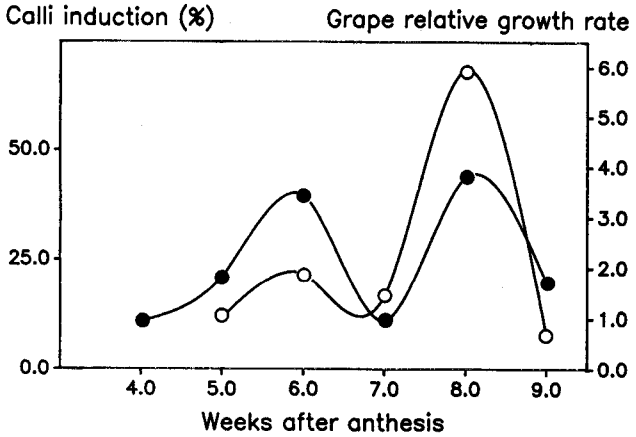

Fig. 2: Relationship between percentage of calli induction (●), and relative growth rate (○), and the development of grapevine fruits expressed as weeks post-anthesis.

also characterized by tissue softening and a change in pigmentation (loss of green color and the development of anthocyanin pigments in the case of the red cv. Monastrell).

Coinciding with these two periods of rapid growth of the fruit, is the ability of fruit explants to develop callus tissues (Fig. 2), the de-differentiation process being mainly located in mesocarp tissues, the hypodermal tissues being recalcitrant to de-differentiation. Callus obtained from mesocarp tissues grown in the dark shows that a concentration of 1.0 μM kinetin and 0.5 μM α-naphthaleneacetic acid are optimal for growth.

Peroxidase isoenzyme patterns in grapevine cell cultures: Callus induction from mesocarp tissues of Airen and Monastrell fruits and growth of the cell culture in a Murashige and Skoog solid medium leads to the *de novo* expression of one acidic (APrx) and one basic (LpI BPrx) peroxidase isoenzyme group in both the cell wall-bound and soluble fractions (Figs. 3 and 4), whose particular isoenzyme compositions are somewhat different (see Discussion). Therefore, with the exception of peroxidase isoenzyme B_5, which, although in lower proportions, is also present in mesocarp tissues (Figs. 1 A and 1 B), all remaining peroxidase isoenzymes were *de novo* expressed in cell cultures (Figs. 3 and 4).

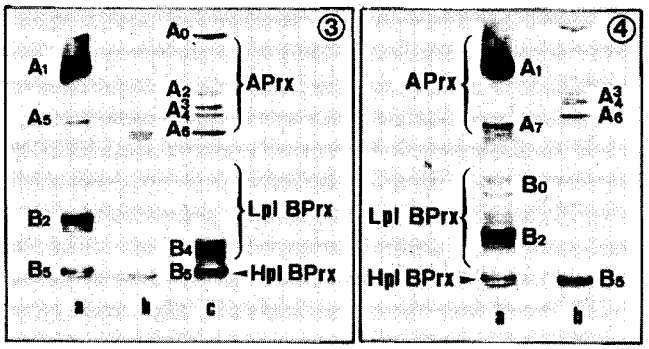

Fig. 3 (left) and Fig. 4 (right): Isoenzyme patterns of peroxidase activity present in soluble (lane a), membrane (lane b) and cell wall (lane c) fractions from calli developed from *V. vinifera* cv. Airen berries (**3**) and cv. Monastrell berries (**4**).

Discussion

Peroxidase isoenzyme patterns in grapevines, as in other plant species (Pedreño *et al.* 1993), may be classified, according to the pI, into 3 principal groups: APrx, LpI BPrx and HpI BPrx. These peroxidase isoenzyme groups differ in their subcellular localization. Thus, while APrx and LpI BPrx are exclusively located in the cell wall and in cell wall free-spaces (Calderón *et al.* 1992 b and 1994 a; Pedreño *et al.* 1993), HpI BPrx are located in both, cell walls and vacuoles (Calderón *et al.* 1992 b; Pedreño *et al.* 1993).

Peroxidase isoenzyme groups in grapevines not only differ in their subcellular localization but also in their responsiveness to abiotic and biotic elicitors. Thus, the peroxidase isoenzyme group APrx, more precisely the peroxidase isoenzyme A_1, is responsive to the abiotic elicitor UV-C in *Plasmopara viticola*-resistant *Vitis* genotypes (Zapata *et al.* 1994), whereas the peroxidase isoenzyme group LpI BPrx, represented by the peroxidase isoenzyme B_3, is responsive to biotic elicitors, such as those obtained from the soil fungus *Trichoderma viride* (Calderón *et al.* 1994 b). This fungus is used in the biological control of grapevine diseases (Calderón *et al.* 1993 a), and its secreted products may be effectively used to induce disease resistance in grapevines. Nevertheless, and unlike the elicitor-inducing properties of peroxidase isoenzyme groups APrx and LpI BPrx, the peroxidase isoenzyme group HpI BPrx is a constitutive (non-inducible) marker of disease resistance in the vegetative organs (leaves and stems) of grapevines (Calderón *et al.* 1992 c).

Unlike in leaves and stems, in which the HpI BPrx isoenzyme group is constitutive (Calderón *et al.* 1992 c), in berries of Airen and Monastrell the HpI BPrx isoenzyme group is both tissue-specific and developmentally regulated (Figs. 1 A and 1 B). In this sense, HpI BPrx is the only component of peroxidase polymorphism in these grapes, and is differentially expressed at fruit softening principally in hypodermal tissues (Figs. 1 A and 1 B).

HpI BPrx is also present, although to a lesser extent, in mesocarp tissues of both cultivars where, similarly to that which occurs in hypodermal tissues, it is also developmentally regulated (Figs. 1 A and 1 B). The establishment of cell cultures from the mesocarp tissues of Airen and Monastrell leads to the *de novo* expression of the peroxidase isoenzyme groups, APrx and LpI BPrx (Figs. 3 and 4). These peroxidase isoenzyme groups are not present in the explant source (Figs. 1 A and 1 B), and therefore may be considered as markers of this de-differentiation process. In this sense, the isoenzyme groups APrx and LpI BPrx may be considered as markers of the process of de-differentiation that is linked to the expression of totipotency by grapevine cell cultures.

The isoenzyme molecular composition of the APrx and LpI BPrx groups in Airen and Monastrell callus cultures is a function not only of the subcellular localization but also of the grapevine cultivar. Thus, the APrx isoenzyme group, in soluble fractions of the Airen cultivar, is composed mainly of the peroxidase isoenzymes, A_1 and A_5, whereas, in the cell wall-bound fraction, the APrx isoenzyme group is composed of the set of A_0, A_2, A_3, A_4 and A_6 peroxidase isoenzymes, the peroxidase isoenzymes A_1 and A_5 being absent (Fig. 3, lanes a and c). Likewise, in soluble fractions of the Airen cultivar, the LpI BPrx isoenzyme group is composed mainly of the peroxidase isoenzyme B_2 whereas, in the cell wall-bound fraction, the LpI BPrx isoenzyme group is only composed of the B_4 peroxidase isoenzyme (Fig. 3, lanes a and c).

In Monastrell callus cultures, the soluble peroxidase isoenzyme A_7 (Fig. 4, lane a) appears as the equivalent of the soluble peroxidase isoenzyme A_5 of Airen cell cultures (Fig. 3, lane a). In the case of the peroxidase isoenzyme group LpI BPrx, it is important to note that the cell wall-bound peroxidase isoenzyme B_4, previously detected in Airen cell cultures, was absent in Monastrell cell cultures (Fig. 4, lane b). This leads to the conclusion that peroxidase isoenzyme patterns of the soluble and the cell wall-bound

fraction obtained from both Airen and Monastrell callus cultures are not identical for both cultivars, although the explant source shows identical peroxidase isoenzyme expression (Figs. 1 A and 1 B). This indicates that the peroxidase isoenzyme groups found in the cell culture are, to some extent, cultivar-specific.

In conclusion, these results suggest that, during the establishment of grapevine cell cultures from mesocarp tissues of grapevine berries, a differential expression of the peroxidase isoenzymes located in soluble and cell wall-bound fractions takes place, and that this expression is cultivar-specific. As a result of this alteration in gene expression, the peroxidase isoenzyme groups APrx and LpI BPrx are *de novo* expressed in the culture, probably expressing at molecular level the totipotency of grapevine somatic cells, which is linked to the de-differentiation process associated with *in vitro* culture.

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