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The potential relationship of stilbene (resveratrol) synthesis to anthocyanin content in grape berry skins

by

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S u m m a r y : The relationship between the production of resveratrol, a phytoalexin related to grape disease resistance, and the anthocyanin content of grape berries in diverse *Vitis* species has been investigated. Previous studies have reported that the phytoalexin production potential of grapes suddenly declines at veraison. The results obtained here from assaying resveratrol and anthocyanins from grape berries in different developmental stages suggest that chalcone synthase (EC 2.3.1.74), the key enzyme involved in anthocyanin biosynthesis, may compete with stilbene (resveratrol) synthase (EC 2.3.1.-), such that the decrease of the ability of grapes to synthesize resveratrol in response to UV-irradiation observed after the onset of fruit ripening may be a consequence of the concomitant rise of anthocyanin accumulation in fruits.

K e y   w o r d s : resveratrol, anthocyanins, grapevine, chalcone synthase, stilbene, synthase.

A b b r e v i a t i o n s : CS = chalcone synthase; RS = resveratrol synthase.

Introduction

The resistance of grapevines (*Vitis vinifera* L.) to fungal penetration by *Botrytis cinerea* Pers., the causal organism for grey mould, appears to be due in part to an induced resistance mechanism involving the production of several phytoalexins, namely resveratrol (trans-3,5,4'-trihydroxystilbene) (Langcake and Pryce 1976; Hoos and Blaich 1988; Dercks and Creasy 1989; Jeandet *et al.* 1991; Liswidowati *et al.* 1991; Jeandet *et al.* 1992; Calderon *et al.* 1993; Jeandet *et al.* 1995 b) and a number of biosynthetically related compounds derived from resveratrol (pterostilbene and viniferins) (Langcake and Pryce 1977; Pezet and Pont 1988).

Stilbene phytoalexins, which are formed on the phenylalanine / polymalonate pathway, accumulate very rapidly in the seemingly healthy grape tissues adjacent to *B. cinerea* infected zones (Langcake and Pryce 1976). Short wavelength (254 nm) UV-light may also cause resveratrol to form in grape leaves or grape berries (Langcake and Pryce 1976; Fritzmeier and Kindl 1981). However, some results (Creasy and Coffee 1988; Jeandet *et al.* 1991) indicate that, in grapevines, the changes in phytoalexin production potential depend on the physiological stage of the plant; e.g. the ability of skin cells of *Vitis vinifera* grapes (var. Pinot noir) to synthesize resveratrol (Jeandet *et al.* 1991) suddenly decreases at veraison (i.e. the brief period corresponding to the beginning of anthocyanin accumulation in skins of red grapes). This observation together with the fact that anthocyanins also derive from the phenylalanine/polymalonate pathway and share common precursors with resveratrol led us to investigate the potential relationship of resveratrol production to anthocyanin content in the skin of different grape varieties. The present study suggests that chalcone synthase (EC 2.3.1.74), a key enzyme in flavonoid biosynthesis may compete with stilbene (resveratrol) synthase (EC 2.3.1.-), the key enzyme of stilbene phytoalexin synthesis, such that the decrease of the ability of grapes to synthesize resveratrol in response to UV-irradiation may be a consequence of the concomitant rise of anthocyanin accumulation in fruits. Maturing grape fruits provide a good model to study the functions of these two enzymes, one of them being constitutive (CS) while the other is only inducible (RS).

Red grapes contain flavonoid-type compounds of which anthocyanins and tannins are the most prominent. Because tannin content of grape skins is already high at veraison when anthocyanins appear (Ribéreau-Gayon and Glories 1982), only the changes of anthocyanins during ripening were considered in this study.

Using this model, we have also reexamined the hypothesis that a stress such as a short UV-exposure or infection by various pathogens might divert normal metabolism to stilbene formation by blocking flavonoid biosynthesis (Hillis and Ishikura 1969).

Materials and methods

P l a n t   m a t e r i a l : To investigate the potential relationship between the resveratrol formation and the anthocyanin content of grape skins in diverse species, fruits of the following vines growing in the fields of the botanical garden of the University were taken: *V. labrusca, V. aestivalis,* Concord (variety of *V. labrusca*), Vialla (hybrid of *V. labrusca*), 5 BB (*V. berlandieri* x *V. riparia* hy-

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brid) and Aramon (*V. vinifera*). Grape berries ranging from green to ripe were randomly sampled at weekly intervals beginning one month before veraison and until maturity, and then assayed for resveratrol.

Induction of resveratrol biosynthesis: For this study, UV-irradiation was used as an eliciting agent (LANGCAKE and PRYCE 1976; JEANDET *et al.* 1991). Whole berries were irradiated as previously described (i.e. at 254 nm for 15 min with a fluence rate of 0.36 J/cm^2) (JEANDET *et al.* 1991).

Grape skin analysis: A modification of the method of LANGCAKE and PRYCE (1976) was used to determine resveratrol and anthocyanins in grape berries. After separation of the skin and the flesh of the fruit, 1 g of induced fresh skin material from pooled sources was ground in a mortar and pestle with sand in 30 ml methanolic 0.1% HCl. Extracts were clarified by centrifugation at 10,000 *g* for 10 min. The supernatant was decanted and acidified to pH 1.0 by using 1 M HCl . Absorbancies of total anthocyanins were determined at 520 nm from an aliquot of the preceding solution using 1 cm quartz cells. After proper dilution, anthocyanins in the grape skins were expressed as mg of a mixture of acylated and non-acylated anthocyanin ($E_{1\%}^{1cm}$ = 500) (SOMERS and EVANS 1977). Methanol was then removed at 40 °C *in vacuo* and resveratrol extracted by phase partition between ethyl acetate and $NaHCO_3$ 3 % (v/v). The resulting organic phase was twice washed with water, concentrated and redissolved in ethyl acetate (1 ml per g fr. skin). Further characterization and quantification of resveratrol in grape skin extracts was carried out by GLC in the form of its trimethysilyl ether using a capillary column CP Sil 5CB (25 m x 0.32 mm). The temperature program was from 200 to 290 °C at 5 °C/min. The flow rate for the carrier gas was 1 ml/min of N_2. De Ros injector was kept at 300 °C. Resveratrol was detected by FID (300 °C) (Retention Time= 14.85 min) (JEANDET *et al.* 1991 and 1995 a). During sample preparation, extracts were constantly protected from light to avoid photochemical isomerization of *trans*-resveratrol to the *cis* form.

Results and discussion

The relationship between anthocyanin concentration in the grape skin and the capacity of grape berries to produce resveratrol after a short UV-exposure was studied during fruit ripening. Fig. 1 shows a decline in the phytoalexin production potential of fruits as soon as anthocyanins begin to accumulate in grape skin; i.e. at veraison. While grossly different levels in resveratrol concentrations are found among immature berries of the same vine variety, its appears that a negative relationship exists between resveratrol synthesis and anthocyanin concentration. Unripe clusters exhibited a high capacity for resveratrol production (100 to 500 µg per g fr. skin), far exceeding that of fruits collected from veraison until maturity (20 to 60 µg per g fr. skin). Thus, unlike the situation in immature fruits, resveratrol may not reach concentrations fungicidal to infection by *Botrytis* hyphae in ripe clusters (ED_{75} = 60 µg/ml) (DERCKS and CREASY 1989). This would explain why grapes change with respect to their resistance to grey mould during the latter phases of ripening (JEANDET *et al.* 1991).

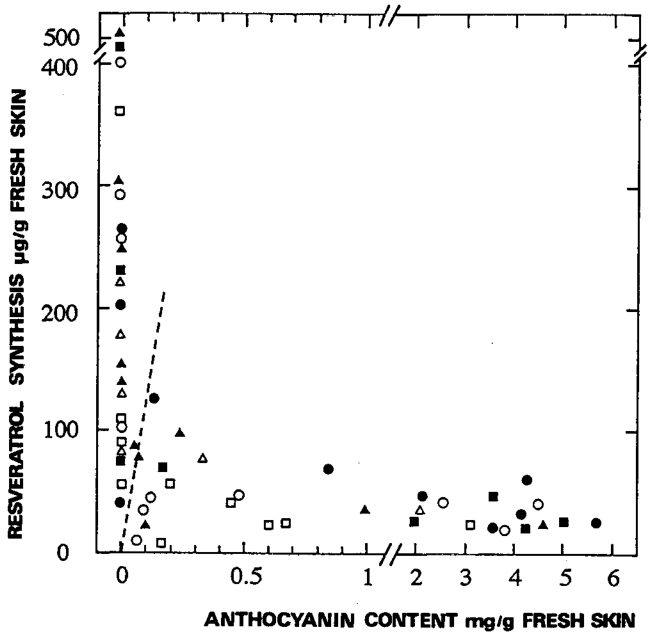

Fig. 1: Relationship between resveratrol production and anthocyanin content in grape berry skins of the following vines: *V. labrusca* (○); Concord (●); *V. aestivalis* (▲); 5 BB (*V. berlandieri* x *V. riparia*) (■); Vialla (□); Aramon (Δ). Berries coming from pre-veraison are on the left of the demarcation line (- - - -). Each point corresponds to the mean for a sample obtained by pooling several berries, which were in the same developmental stages.

The fact that resveratrol production is inversely related to anthocyanin biosynthesis in grape berries raises questions concerning the mechanism of stilbene accumulation versus that of anthocyanin in grape skin cells at veraison. Indeed, CS and RS are two closely related proteins whose activities may concurrently be enhanced in UV-irradiated grapevine cells (SCHRÖDER *et al.*1979; FRITZEMEIER and KINDL 1981) . Both enzymes use the same substrates (i.e. *p*-coumaroyl-CoA and malonyl-CoA) (LANGCAKE and PRYCE 1977; RUPPRICH and KINDL 1978; SCHRÖDER *et al.* 1979; ROLFS and KINDL 1984; SCHRÖDER *et al.* 1988; SCHRÖDER and SCHRÖDER 1990) and catalyze the same condensing-type of enzyme reaction, but nevertheless form two different products, respectively, naringenin chalcone **2** (see Fig. 2), the first C15 intermediate in the flavonoid route, and resveratrol **1** (see Fig. 2). These proteins possess a common scaffold for substrate recognition and for the condensing reaction (SCHRÖDER and SCHRÖDER 1990). Accordingly, analysis of the relationship between resveratrol production in grape skin cells and anthocyanin content suggests that CS may compete with RS for substrate binding, thus limiting phytoalexin production by grape skin cells in response to elicitation.

According to HRAZDINA *et al.* (1985), chalcone synthase activity enhances dramatically in grape berries at veraison.

Indeed, the intensive anthocyanin biosynthesis in the subepidermal layers of the berries at veraison results from an increased availability of precursors, especially of phenylalanine (HRAZDINA *et al.* 1985), derived from sugars via the shikimate pathway. The negative relationship existing between stilbene biosynthesis and flavonoid accumulation thus implies that changes in the activity of anthocyanin enzymes are directly related to the simultaneous accumulation of sugars in the grape berries, at veraison, though DARNÉ (1993) has argued that anthocyanins are synthesized before veraison from proanthocyanidins, which are mainly located in the seeds.

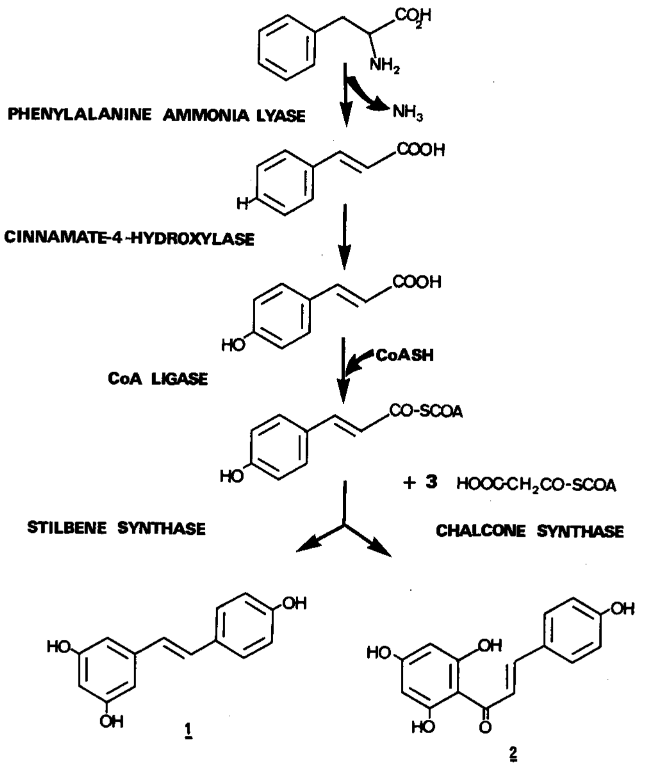

Fig. 2: Biosynthetic pathway from phenylalanine to resveratrol, **1**, and naringenin chalcone, **2**.

Finally, the fact that anthocyanin formation appears to limit resveratrol production in grape berries after the onset of fruit ripening cannot be reconciled with the possibility that infection and presumably other stresses divert normal metabolism to stilbene phytoalexin formation by blocking the flavonoid pathway, as has been suggested by HILLIS and ISHIKURA (1969). On the contrary, it seems likely from the results presented here that the two pathways function, but that the increased metabolic flow is channelled into anthocyanins rather than into stilbenes.

Conclusion

A direct negative relationship appeared between stilbene phytoalexin formation and anthocyanin content in grape skin cells. This in turn is supportive evidence that flavonoids and stilbenes, which derive from the same precursors, are intimately connected biogenetically. Our results obtained from biochemical studies, indirectly confirm molecular studies on the relationship existing between stilbene and chalcone synthases in grapes. Finally, this work has confirmed that grape berries lose their capacity to synthesize high amounts of resveratrol at veraison.

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