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Research Article

Leucoanthocyanidin reductase and anthocyanidin reductase gene expression and activity in flowers, young berries and skins of *Vitis vinifera* L. cv. Cabernet-Sauvignon during development

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ABSTRACT

Proanthocyanidins, or condensed tannins, are crucial polyphenolic compounds for grape and wine quality. Recently, significant advances were achieved in understanding the biosynthesis of their main subunits: (+)-catechin and (−)-epicatechin, produced by catalysis of leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), respectively. Expression studies had been published but no data were available on enzyme activity. In our work, we devised assays to measure LAR and ANR activity and determine their development throughout the growth of flowers, young berries, and skins of *Vitis vinifera* L. cv. Cabernet-Sauvignon. We also investigated the accumulation of compounds in these tissues and focused on the expression of both the structural genes and the transcription factors involved in regulating them: *VvMYB5a* and *VvMYBPA1*. Biosynthetic genes were expressed early and LAR and ANR were already active during flowering and at the beginning of berry growth, as well as during colour-change in skins. The profiles we determined correlated with total tannin, catechin, and epicatechin concentrations. The involvement of *VvMYB5a* and *VvMYBPA1* was confirmed and specific expression patterns were also established for *VvLAR* transcripts.

1. Introduction

Proanthocyanidins, also known as condensed tannins, are polyphenolic secondary metabolites synthesized *via* the flavonoid pathway. In plants, they act as protective factors for radiation [37], disease [12], and predators [3,18]. Their beneficial dietary effects on animal [28] and human [5] health are widely documented. The organoleptic properties they confer on fruits, i.e. bitterness and astringency [10], and their ability to interact with proteins and other polyphenols [4,6] make them crucial components in grape and wine quality. In grapes, they are mainly located in seeds and in stems but also in skins, where they are reactive and easily extractible. They accumulate early then decrease continuously in grape skins [13,16,31,35]. They contribute to the intensity and stability of wine colour and take part in wine structure.

The biosynthesis mechanism of proanthocyanidins, especially in the later stages, has not yet been completely elucidated, but the varied, positive effects of these compounds have recently attracted growing interest. Even if flavanol polymerization mechanisms are not yet known, significant progress has been made concerning monomer production. Xie et al. [40] and Tanner et al. [38] clearly established the roles of anthocyanidin reductase (ANR) and leucocyanidin reductase (LAR) in (−)-epicatechin and (+)-catechin synthesis, respectively. ANR (E.C. 1.3.1.77) converts anthocyanidins to 2,3-*cis*-flavanols *via* NAPDH-mediated reduction, thus inversing the stereochemistry of the pyran ring at C_3 and producing mainly (−)-epicatechin. LAR (E.C. 1.17.1.3) catalyzes the NADPH-dependent reduction of leucoanthocyanidins to 2,3-*trans*-flavanols, such as (+)-catechin.

Several recent studies have focused on the expression and regulation of *VvANR* and *VvLAR* in different grape-berry organs. They were highly expressed in leaves and flowers, as well as in skins and seeds up to the colour-change period, when the metabolic pathway shifted to anthocyanin production. These patterns were correlated to flavanol accumulation [19]. Important data were reported concerning the control of their expression. Until now, only two transcription factors related to tannin metabolism were identified in grape. *VvMYB5a* expression [15] early in berry development was apparently closely correlated to proanthocyanidin accumulation. According to Bogs et al. [8], *VvMYBPA1* specifically

Abbreviations: ANR, anthocyanidin reductase; DAA, days after anthesis; FW, fresh weight; LAR, leucoanthocyanidin reductase; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

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controlled flavanol synthesis by inducing *VvANR* and *VvLAR* expression.

Further research is still required to connect the elements involved in proanthocyanidin biosynthesis. No data are currently available on enzyme activities, which would complement the genomic approach.

In this work, we developed enzyme assays for LAR and ANR activity throughout grape skin development. Since we hypothesized that the continuous decrease in tannin content we measured in skins was linked to tannin metabolism in flowers (i.e. flower organs differentiate into the different grape-berry tissues), we also analyzed flowers and immature berries. At the same time, we correlated enzyme data with the expression of *LAR*, *ANR*, their transcription factors, VvMYB5a and VvMYBPA1, and catechin and epicatechin concentrations both as monomers and polymerized subunits.

2. Materials and methods

2.1. Plant material and sample collection

Vitis vinifera L. cv. Cabernet-Sauvignon flowers and berries were sampled from a commercial vineyard in the Pessac-Léognan appellation near Bordeaux (France) and collected at different development stages in 2006, according to the phenological stages defined by Eichhorn and Lorenz [17]. The north/south-oriented vineyard had been planted in 1990 and grafted onto 101-14 rootstock. Planting density was 6500 vines per ha, and the pruning method was Guyot double.

Flowers were collected at three stages: floral bud (stage 17), flowering onset corresponding to 50% of flowers at inflorescence (stage 21), and full flowering (stage 23).

Random samples of five grape clusters on ten vines were selected at ten phenological stages. Four green stages were collected: berry set (stage 27), pea-sized berries (stage 31), ten days after stage 31, and berry touch (stage 33), corresponding to 14, 24, 34, and 45 days after anthesis (DAA), respectively. Four samples were collected during colour-change: 10% red ripe (RR) berries (stage 35), 50% RR (stage 36), 80% RR, and 100% RR (stage 37), corresponding to 59, 61, 63, and 67 DAA, respectively. Two samples were taken during fruit ripening: 2 weeks after the end of the colour-change (83 DAA) and at maturity (harvest, 110 DAA, stage 38).

Table 1 summarizes the samplings made for this study.

Floral and berry samples were immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. Grape skins were carefully removed for analysis using razor blades, starting with pea-sized berries. Before this stage, the tissue is not sufficiently differentiated for the skins to be removed.

Table 1
Plant material collected for this study.

Stage of development	Number of days after anthesis	Phenological stage according to Ref. [17]
Floral bud	–	17
Flowering onset (50% flowers per inflorescence)	–	21
Full flowering	–	23
Berry set	14	27
Pea-sized berry	24	31
Pea-sized berry + 10 days	34	–
Berry touch	45	33
10% red ripe berries per bunch	59	35
50% RR berries per bunch	61	36
80% RR berries per bunch	63	–
100% RR berries per bunch	67	37
100% RR + 2 weeks	83	–
Maturity or harvest	110	38

2.2. Enzyme extraction

All procedures were carried out at 4 °C. LAR and ANR were simultaneously extracted using a method adapted from Ref. [14] to isolate dihydroflavonol 4-reductase (DFR). Previous reports [33,32] presented this method for simultaneous crude extraction of ANR and LAR and validated it for both enzymes. Moreover, this procedure, similar to the crude enzyme extract preparation method used in our laboratory [21], was also rapid, easy and removed anthocyanins from the enzyme extract. Plant material (2 g) was ground to powder in liquid nitrogen, homogenized in 3 mL lysis buffer (0.1 M HEPES pH 7.3, 1% sucrose (w/v), 1% PEG (w/v), 25 mM $CaCl_2$) and mixed with 200 mg PVPP. The homogenate was centrifuged at 20 000 g for 10 min and the supernatant incubated with Dowex 1 × 2 mesh 200 (Sigma, Saint Quentin Fallavier – France), equilibrated with the lysis buffer. After centrifugation at 20 000 g for 5 min, the supernatant was percolated through a Sephadex G-25 column (GE Healthcare – Amersham Biosciences, Orsay – France). The recovered suspension was used as crude extract to determine enzyme activity. Extractions were performed in triplicates.

2.3. Determining assay parameters

Protocols for LAR and ANR assays were adapted from Ref. [32] and from Ref. [40,33,32], respectively. The volume of reaction mixture was initially set (200 μL for LAR and 500 μL for ANR) and different concentrations of dihydroquercetin (Sigma, Saint Quentin Fallavier – France) or cyanidine (Extrasynthèse, Genay – France) were tested together with different volumes of crude extract, different pHs, and different temperatures with crude extracts from skins at two distinct phenological stages. Kinetic study was done to determine the optimum incubation time. For each assay, five independent assays were performed per phenological stage to confirm the efficiency of both methods. Negative controls, consisting of boiled crude extracts, showed neither production of catechin or epicatechin, nor substrate degradation.

The separation method described by Ref. [32] for HPLC analysis was adapted to quantify catechin and epicatechin.

2.4. LAR assay

LAR activity (expressed in pkatals g^{-1} FW) was determined by monitoring the conversion of dihydroquercetin to (+)-catechin. The assay mixture contained 10 μL dihydroquercetin (1 g L^{-1} in methanol), 10 μL NADPH 20 mM and 110 μL Tris–HCl buffer 0.1 M pH 7.5. The reaction was initiated by adding 70 μL crude extract, incubated at 25 °C for 30 min, and stopped by adding 200 μL ethyl acetate with vigorous vortexing. Extraction was repeated and the ethyl acetate phases were pooled and dried under nitrogen gas. Residues were dissolved in 100 μL HPLC-grade methanol for HPLC analysis. LAR products were separated on a Beckman Ultrasphere ODS (250 × 4.6 mm, 5 μm) reversed-phase column and eluted with acetic acid (5% v/v) in water (solvent A) and methanol (solvent B), according to the following program: 5% B from 0 min to 5 min, 5%–10% B from 5 min to 10 min, 10% from 10 min to 16 min, 10%–90% B from 16 min to 21 min, 90% B from 21 min to 31 min, 90%–5% B from 31 min to 36 min, and 5% B up to 45 min. The flow rate was set at 1 mL/min, the detection wavelength was 280 nm, and the injection volume was 50 μL. Identification and quantification were performed using an external (+)-catechin standard (Sigma, Saint Quentin Fallavier – France). Data represent the mean of three assays per extract ± standard deviation (SD).