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^{13}C NMR analysis of polyphenol biosynthesis in grape cells: Impact of various inducing factors

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Abstract

^{13}C NMR spectroscopy was used as a complement to high performance liquid chromatography (HPLC) or spectrophotometry to analyse stilbene and anthocyanin metabolism in grape cell cultures. Stilbene and anthocyanin biosynthesis are closely related. Anthocyanins are major components of grape quality whereas stilbenes play a crucial role in defence mechanisms and are strongly correlated to natural grapevine resistance. Stimulation of stilbene production by natural inducers offers an interesting alternative to the use of pesticides in plant protection strategies. NMR allowed us to analyse both stilbene and anthocyanin pathways by following step by step the incorporation of the labelled precursor [1-^{13}C] L-phenylalanine along the entire biosynthetic routes. However, several intermediates of the anthocyanin biosynthetic pathways remained unobservable, providing evidence for metabolic channelling. The effect of various elicitors on stilbene and anthocyanin biosynthesis was also investigated. In complement to quantification of the end-products by HPLC and spectrophotometry, ^{13}C NMR studies provided information at the biosynthetic level. Sucrose addition stimulated the biosynthesis of anthocyanins without influencing stilbenes. Methyljasmonate and fungal elicitor strongly increased stilbene production through the activation of enzymes from phenylalanine ammonia-lyase to stilbene synthase. However, methyljasmonate showed an inhibitory effect on anthocyanin biosynthesis, suggesting the existence of a competition between the two pathways. ^{13}C NMR spectroscopy, combined with analytical techniques such as HPLC and spectrophotometry, provides a very interesting tool to better understand the mechanisms underlying the production of secondary metabolites and the metabolic processes of plant resistance induction by elicitation.

Keywords: ^{13}C NMR spectroscopy; *Vitis vinifera* L.; Cell culture; Polyphenol biosynthesis; Plant resistance; Inducing factors

1. Introduction

Among the inducible plant defences developed following microbial attack [1,2], the synthesis of the antimicrobial compounds known as "phytoalexins" represents a major mechanism of disease resistance in many plant/pathogen interactions [3,4].

Although these molecules display an enormous chemical diversity [5–7], phytoalexins from the Vitaceae, such as grapevine (*Vitis vinifera* L.), seem to constitute a rather restricted group of polyphenolic secondary metabolites belonging to the stilbene family [8], whose skeleton is based on the *trans*-resveratrol structure (3,5,4′-trihydroxystilbene). *Trans*-resveratrol, as primary phytoalexin, is then transformed into several more potent antifungal stilbenes, like ε-viniferin, a resveratrol dimer [8]. A close relationship between stilbene phytoalexin accumulation and grapevine resistance to diseases has been demonstrated in many instances [9,10].

As grapevine (*V. vinifera* L.) represents one of the most important crop cultures in the world and is susceptible to many diseases, phytochemicals are intensively used in vineyards to prevent and limit pathogenic infections. Because such agricultural practice favors the development of pesticide-resistant strains [11] and has a negative impact on the environment and human health, considerable interest has been focused on the replacement of such pesticides by alternative crop protection strategies. In particular, attention has focused on the use of natural resistance inducers, also called elicitors, for preventing infection through the activation of the plant's own defence mechanisms [12–15].

Moreover, the ability of grape berries to synthesize stilbenes dramatically decreases during ripening, a phenomenon that is correlated with a higher vulnerability to pathogens. At the same time, as the berry begins to accumulate sugars, the biosynthe-

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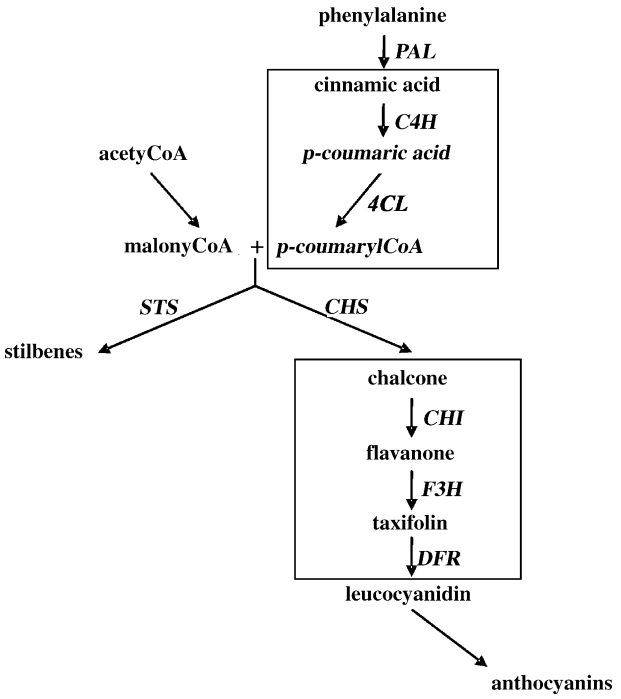

Fig. 1. Simplified stilbene and anthocyanin biosynthetic pathways. The boxes indicate reactions where metabolic channelling has been hypothesized. The enzymes are: PAL, L-phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol reductase; STS, stilbene synthase.

sis of anthocyanin pigments strongly increases, suggesting a competition between the two metabolic pathways [16]. Stilbene synthase (STS), the key enzyme leading to stilbene synthesis, is indeed closely related to chalcone synthase (CHS), the key enzyme of the biosynthesis of flavonoid-type compounds like anthocyanins. STS and CHS use the same substrates and catalyze the same condensing-type of enzyme reaction, forming two different products, respectively, resveratrol and chalcone [17,18] (Fig. 1).

In view of this, the development of protection strategies based on the induction of stilbene production by the plant has to include the study of their impact on both stilbene and anthocyanin biosynthesis, the latter molecules playing a crucial role in wine and table grape organoleptic qualities, such as taste and color.

In this context, we used an in vitro model of *V. vinifera* L. cell suspension cultures to provide an environment in which some aspects of defence responses could be examined more readily than in the whole plant. We studied both stilbene and anthocyanin metabolism from a qualitative and quantitative biosynthetic point of view by combining various analytical techniques: high performance liquid chromatography (HPLC) and spectrophotometry were used to quantify the end-products of interest, stilbenes and anthocyanins, whereas ^{13}C nuclear magnetic resonance (NMR) spectroscopy provided more detailed information about the overall pathway and the biosynthetic steps.

^{13}C NMR spectroscopy has previously been shown to be a powerful technique to investigate metabolic pathways [19,20] when coupled with the use of specifically ^{13}C-labelled substrates that allow the evolution of a specific carbon atom to be followed through many enzymatic transformations of the original compound [21].

For our experiments, *V. vinifera* L. cells were fed with L-[1-^{13}C] phenylalanine, a labelled precursor of both stilbene and anthocyanin pathways. Phenolic compounds such as stilbene and anthocyanins are aromatic secondary metabolites formed essentially via the shikimate and/or malonate pathways [22]. The first reaction in the phenylpropanoid pathway is catalyzed by phenylalanine ammonia-lyase (PAL) converting phenylalanine to *trans*-cinnamic acid, followed by cinnamate 4-hydroxylase (C4H) and coumarate CoA ligase (4CL) resulting in the synthesis of *p*-coumarylCoA, the direct precursor of both stilbene and anthocyanin biosynthesis. Therefore, phenylalanine was chosen as labelled precursor for our NMR experiments.

The final aim of this work was to study the influence of various elicitors on anthocyanin and stilbene biosynthesis in grape cells. Attention was focused on three elicitors: sucrose, for its capacity to stimulate anthocyanin biosynthesis in cultured grape cells [24–26]; methyljasmonate (MeJA) and fungal cell wall extracts, for which previous studies have shown the inductive effect on stilbene biosynthesis in cell suspension cultures of grape [27–29]. Elicitors have previously been employed to induce the biosynthetic pathways of secondary metabolites in order to elucidate the mechanisms that underlie the steps of their biosynthesis, distribution and accumulation [23]. Indeed, a better understanding and manipulation of plant secondary metabolite biosynthesis represents a major challenge for future applications in plant physiopathology.

2. Experimental

2.1. Plant material and culture conditions

Cell suspension cultures of *V. vinifera* L. cv. Gamay Fréaux var. Teinturier were established as previously described [30] and maintained under continuous fluorescent light (5000 lux) at $25 \pm 1\,^{\circ}C$ in 250 ml Erlenmeyer flasks containing 50 ml of cell suspension on an orbital shaker (100 rpm). The maintenance medium contained B5 macroelements, microelements and vitamins and was supplemented with $20\,g\,L^{-1}$ sucrose, $250\,mg\,L^{-1}$ casein hydrolysate, $0.1\,mg\,L^{-1}$ 1-naphtalene-acetic acid and $0.2\,mg\,L^{-1}$ kinetin. Cells were subcultured every week by inoculating the cells at a 1:5 (v/v) ratio into fresh medium. For experimental purposes, 7-day-old cells were inoculated (1:8, v/v ratio) into an induction medium containing the same sucrose concentration than the maintenance medium but 2-fold more $(NH_4)_2SO_4$, NaH_2PO_4 and $MgSO_4$ [30].

2.2. Feeding experiments

Unenriched L-phenylalanine (L-[1-^{12}C] phenylalanine) and L-[1-^{13}C] phenylalanine were purchased from Euriso-Top (Saint-Aubin, France), dissolved in DMSO-H_2O (15:85, v/v),