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Changes and subcellular localizations of the enzymes involved in phenylpropanoid metabolism during grape berry development

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Summary

The phenylpropanoid pathway yields a variety of phenolics that are closely associated with fruit qualities in addition to structural and defense-related functions. However, very little has been reported concerning its metabolism in fruit. This experiment was designed to assess changes of eleven phenolic acids in grape berry (*Vitis vinifera* L. cv. Cabernet Sauvignon) and explore both the activities and amounts of three key enzymes – phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate:coenzyme A ligase (4CL) – catalyzing the biosynthesis of these compounds during berry development. Finally, the subcellular localizations of the enzymes within berry tissues were also investigated using immuno-gold electron microscopic technique. The results indicated that the contents of gallic, protocatechuic, gentisic and caffeic acid all changed drastically during berry development, while other compounds containing p-hydroxybenzoic, vanillic, syringic, chlorogenic, p-coumaric, ferulic and sinapic acid varied only slightly. Activities of PAL, C4H and 4CL showed similar pattern changes with two accumulated peaks throughout berry development. In addition, their activities all showed a highly positive correlation with the total contents of phenolic acids, whereas the immunoblotting analysis showed that changes in enzyme activities were independent of the enzyme amounts. Results from the subcellular-localization study revealed that PAL was mainly present in the cell walls, secondarily thickened walls, and

Abbreviations: ATP, adenosine triphosphate; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate /nitro blue terazolium; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate:coenzyme A ligase; CoA-SH, coenzyme A; EDTA, ethylenediamine tetra-aceticacid; G-6-PNa_2, glucose-6-phosphate sodium salt; NADP-Na_2, nicotinamide adenine dinucluotide phosphate sodium salt; PAL, phenylalanine ammonia-lyase; PMSF, phenylmenthylsulfonyl fluride; PVP, polyvinyl pyrroldione; Tris, tris(hydroxymethyl)-amino methane

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the parenchyma cells of the berry mesocarp cells, C4H was found primarily in the chloroplast (plastid) and nucleus and 4CL predominantly in the secondarily thickened walls and the parenchyma cells of mesocarp vascular tissue.

Introduction

Phenolics are important secondary metabolites in grape berries that play a critical role in determining the quality of both the berry and any wine produced using the berries. Because they contribute to wine characteristics such as color, flavor, astringency and bitterness, their biosynthesis and modulation in fruits have attracted close attentions (Chamkha et al., 2003).

The phenylpropanoid pathway is an important pathway in secondary plant metabolism, catalyzing the conversion of phenylalanine to a myriad of phenolic secondary metabolites unique to plants (phenolic acids, flavonoids, lignins, and stilbenes). In addition, the enzymes such as phenylalanine ammonia-lyase (PAL, E.C. 4.3.1.5), cinnamate-4-hydroxylase (C4H, E.C. 1.14.13.11) and 4-coumarate: coenzyme A ligase (4CL, E.C. 6.2.1.12) are considered to be crucial to phenylpropanoid metabolism (Weisshaar and Jenkins, 1998; Brenda, 1999). Numerous reports have demonstrated that phenylpropanoid derivatives are capable of protecting plants against various biotic and abiotic stresses such as extremes in temperatures, UV-B light, wounding and ozone, along with several specific roles in pathogen defense response, anti-herbivory, ultraviolet screening, radical scavenging (Dixon and Paiva, 1995; Solecka and Kacperska, 2003). They are also important structural components in cell walls (Robbins, 2003). Although there has been a substantial amount of research on phenylpropanoid metabolism, little attention has been given to characterizing its role in fruit development, particularly in grape berries.

The aim of the present work was focused on analyzing the changes in eleven phenolic acids and three key enzymes (PAL, C4H and 4CL) during grape berry development, as well as exploring the subcellular localizations of the three enzymes in berry tissues.

Materials and methods

Samples and sample preparation

Grape berries (*Vitis vinifera* L. cv. Cabernet Sauvignon) were collected from a commercial vineyard in the western suburbs of Beijing, P.R.C. Sampling took place per 10 d from full bloom to harvest. Each sample consisted of 50 clusters picked randomly from 50 different plants located in 50 different rows, and clusters from different plants were picked during each sampling. The samples were transported to the laboratory where about 600 berries were separated from clusters and sorted based on size, weight and the absence of physical injuries or infections. Once separated, the berries were immediately pre-cooled and then washed with distilled water before air-drying. Fifty berries from this group were weighed and the rest of the berries frozen in liquid nitrogen and stored at −80 °C until analysis. All chemicals were purchased from the Sigma Corporation (St. Louis, MO 63178, USA) unless otherwise noted.

Determination of protein, total phenols, total flavonoids and UV-320 absorbance

Protein content was estimated according to the method described in Bradford (1976) using BSA as a standard. Total phenols was determined spectrophotometrically using Folin-Ciocalteu's reagent as described by Singleton and Rossi (1965), and the results were expressed as mg of gallic acid per berry. Total flavonoids was measured according to the method of Wolfe et al. (2003) and the results were expressed as mg of catechin per berry. Absorbance at 320 nm was assayed as described by Campos-Vargas et al. (2005) and expressed as OD value per berry.

Extraction and determination of phenolic acid contents

The 11 phenolic acids were extracted as described by Hakkinen et al. (1998) but with some modifications. Tissues containing epidermis and flesh (the seed discarded from the 50 berries) were first ground in liquid nitrogen and then 10 g of the sample was rinsed with 50 mL of extraction solution (70% methanol+1.2 M HCl+80 mg ascorbic acid) into a 100 mL round-bottom bottle. The solution was carefully blended and then sonicated for 2 min. The remaining air in the bottle was replaced by nitrogen gas. After shaking for 16 h in a water bath at 35 °C in dark, the extract was cooled to room