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Proline Accumulation in Developing Grapevine Fruit Occurs Independently of Changes in the Levels of Δ^1-Pyrroline-5-Carboxylate Synthetase mRNA or Protein[1]

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Mature fruit of grapevine (*Vitis vinifera*) contains unusually high levels of free proline (Pro; up to 24 μmol or 2.8 mg/g fresh weight). Pro accumulation does not occur uniformly throughout berry development but only during the last 4 to 6 weeks of ripening when both berry growth and net protein accumulation have ceased. In contrast, the steady-state levels of both the mRNA encoding *V. vinifera* Δ^1-pyrroline-5-carboxylate synthetase (VVP5CS), a key regulatory enzyme in Pro biosynthesis, and its protein product remain relatively uniform throughout fruit development. In addition, the steady-state protein levels of Pro dehydrogenase, the first enzyme in Pro degradation, increased throughout early fruit development but thereafter remained relatively constant. The developmental accumulation of free Pro late in grape berry ripening is thus clearly distinct from the osmotic stress-induced accumulation of Pro in plants. It is not associated with either sustained increases in steady-state levels of P5CS mRNA or protein or a decrease in steady-state levels of Pro dehydrogenase protein, suggesting that other physiological factors are important for its regulation.

The high levels of free Pro observed in some plant tissues and organs suggest that this amino acid may have an important function in normal plant growth and development. Very high levels of free Pro have been reported in the flowers and seeds of Arabidopsis (Chiang and Dandekar, 1995; Savouré et al., 1995), in inflorescences and siliques of *Brassica napus* (Flasinski and Rogozinska, 1985), in ovules of broad bean (Venekamp and Koot, 1984), in pollen grains of petunia and tomato (Zhang et al., 1982; Fujita et al., 1998), and in the mature fruits of citrus species (Clements and Leland, 1962), pear species (Ulrich and Thaler, 1955), and grapevine (*Vitis vinifera*; Lafon-Lafourcade and Guimberteau, 1962; Kliewer, 1968; Ough and Stashak, 1974). The role of free Pro in the development or function of these organs remains unknown. Similarly, the regulation and temporal patterns of Pro biosynthesis and accumulation during normal plant development, in the absence of abiotic stress, remain essentially uncharacterized.

Most of the research concerning Pro metabolism in plants has been focused on its accumulation in vegetative tissues in response to abiotic stresses such as drought and salinity. Stress-induced accumulation occurs predominantly through the enhanced biosynthesis of Pro from Glu via the pathway catalyzed by P5CS and P5CR rather than from Orn via the pathway catalyzed by OAT and P5CR (Boggess et al., 1976; Rhodes and Bressan, 1986; Delauney and Verma, 1993). The onset of stress-induced Pro accumulation is correlated with transcriptional activation of the gene encoding P5CS, which is the key regulatory and rate-limiting enzyme in this biosynthetic pathway (Hu et al., 1992; Delauney and Verma, 1993; Kishor et al., 1995; Savouré et al., 1995; Yoshiba et al., 1995; Zhang et al., 1995; Peng et al., 1996; Strizhov et al., 1997). Transcriptional regulation of the gene encoding PDH, the first enzyme in the pathway of Pro degradation, has also been implicated in the control of free Pro levels during the abiotic stress response (Kiyosue et al., 1996; Peng et al., 1996; Verbruggen et al., 1996). Transcription of PDH is repressed during osmotic stress but is activated during poststress recovery, when the enzyme plays a key role in the rapid reduction of Pro levels.

The high levels of free Pro found in some plant tissues and organs in the absence of abiotic stress raises the question of whether Pro accumulation during normal plant development occurs by activation of pathways or processes, that are independent of those that operate in response to abiotic stress. In this study we examined developing grape berries of *V. vinifera* to address this question. We measured the changes in berry amino acids throughout fruit development and demonstrated that free Pro accumulation occurs only in the final stages of fruit ripening. We also cloned a grapevine cDNA encoding P5CS (VVP5CS) and monitored the steady-state levels of P5CS mRNA during this period. Previous studies of P5CS gene expression focused almost exclusively on assessment of mRNA levels, but because mRNA levels do not necessarily reflect final

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Abbreviations: degrees Brix, refractive index measure of total soluble solids; OAT, ornithine δ-aminotransferase; P5CR, Δ^1-pyrroline-5-carboxylate reductase; P5CS, Δ^1-pyrroline-5-carboxylate synthetase; PDH, Pro dehydrogenase.

levels of the gene product, we also expressed the cDNA in *Escherichia coli* and prepared specific antibodies to enable measurement of steady-state levels of the P5CS protein in grape berries. In contrast to previous studies of stress-induced Pro accumulation in plants, our results indicate that Pro accumulation in developing grape berries is not regulated by changes in P5CS mRNA or protein levels, or by changes in PDH protein levels, and that other physiological factors must be involved.

MATERIALS AND METHODS

Fruit Sampling

Mature fruit of grapevine (*Vitis vinifera* L. cvs Muscat Gordo Blanco and Gewurtztraminer) was obtained from the Alverstoke vineyard (Adelaide, South Australia). Fruit for developmental studies was harvested from 30 cv Chardonnay vines (C.A. Henschke and Co. vineyard, Lenswood, South Australia) and 16 cv Cabernet Sauvignon vines (Waite variety block, University of Adelaide, South Australia). To obtain homogeneous samples, bunches at apparently similar stages of development were identified 2 weeks postflowering and tagged. Ten of these tagged bunches were taken at each sampling point, although no more than 10% of the total number of bunches on any one vine was harvested to ensure no significant changes in crop load. A berry subsample of 50 was used to determine the average berry weight and the degrees Brix, as described previously by Tattersall et al. (1997). The remaining berries were stored at −70°C prior to further analyses.

Amino Acid Extraction and Analysis

Berries were frozen in liquid N_2 before being ground to a fine powder with a mortar and pestle. Free amino acids were extracted from 0.4 to 0.6 g of tissue by a modification of the method of Bieleski and Turner (1966). Extraction was performed in 1 mL of 12:5:3 (v/v) methanol:chloroform: water for 20 min. The extract was centrifuged at 12,000*g* for 5 min; the supernatant was diluted 1:5 (v/v) with 0.25 M borate buffer, pH 8.5, and then derivatized with 9-fluorenylmethylchloroformate before separation on a reverse-phase C_{18} column (150- × 4.6-mm i.d., Hypersil, Runcorn, Cheshire, UK) using an HPLC (GBC Scientific Equipment Co., Arlington Heights, IL) according to the manufacturer's instructions. Data were analyzed using a WinChrom 1.2 (Scientific Software, Pleasanton, CA) chromatography management system.

RNA Isolation and cDNA Synthesis

Total RNA was extracted as described previously by Tattersall et al. (1997). First-strand cDNAs were generated with RNase H reverse transcriptase (Superscript II, GIBCO-BRL) according to the manufacturer's instructions using 2 μg of RNA isolated from 10-week-postflowering berries as the template and a $(dT)_{15}$ primer. Based on amino acid homologies among P5CS sequences of *Vigna aconitifolia*, Arabidopsis, and *Escherichia coli* ProA/ProB (Fig. 2), degenerate oligonucleotides were designed to amplify by PCR a partial *V. vinifera* P5CS (*VVP5CS*) cDNA clone. The oligonucleotides were 3BP5CS (5′-AARCARAARCAYCARRAY GAYAT-3′) and 7P5CS (5′-GTYTCCATIGCRTTRCAIGC-3′). PCR reaction mixtures contained 1 unit of *Taq* polymerase (GIBCO-BRL), buffered according to the manufacturer's instructions, 50 pmol of each primer, and 2 μL of the first-strand cDNAs as the template in a final volume of 25 μL. A 1.1-kb PCR product was generated by incubating the reaction mixture at 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 30 s, 72°C for 1 min and 30 s, and a final extension step of 72°C for 7 min. The PCR fragment was purified from TAE-buffered (40 mM Tris-acetate, pH 8.0, and 1 mM Na_2EDTA) agarose gels using Bresaclean (Bresatec, Adelaide, Australia) ligated into the pGEM-T vector (Promega) and transformed into *E. coli* JM109 cells (Promega). DNA sequences were determined according to the method of Sanger et al. (1977).

cDNA Library Screening

The 1.1-kb partial cDNA clone of VVP5CS, labeled with [^{32}P]dCTP (Bresatec) using a Megaprime kit (Amersham) was used to screen a *V. vinifera* cv Shiraz cDNA library (constructed from 10-week-postflowering berry RNA in the Lambda-ZAPII vector (Stratagene) after transfer to a membrane (Hybond-N^+, Amersham) according to the manufacturer's instructions. The complete DNA sequence of a full-length *VVP5CS* clone was determined using the method of Sanger et al. (1977).

Southern Hybridization Analysis

The method of Steenkamp et al. (1994) was used to extract genomic DNA from grapevine leaves. DNA (10 μg) was digested with the specified restriction enzymes according to the manufacturer's instructions (Promega). The DNA was electrophoresed in a 0.8% (w/v) agarose gel (buffered in TAE), blotted onto a membrane (Hybond-N^+), and fixed in a UV cross-linker according to the manufacturer's recommendations. The blot was incubated at 60°C for 4 h in prehybridization solution (5× SSC, 0.5% [v/v] SDS, 5× Denhardt's reagent [Sambrook et al., 1989], and 100 mg mL^{-1} denatured and sheared salmon-sperm DNA). Denatured DNA probe, labeled with [^{32}P]dCTP as described above, was incubated with the blot for 17 h at 60°C. The membrane was washed at 60°C at medium stringency (1× SSC and 0.1% [w/v] SDS) and high stringency (0.1× SSC and 0.1% [w/v] SDS) according to the method of Meinkoth and Wahl (1984). Hybridizing DNA was detected with a phosphor imaging screen (Kodak) and analyzed using a phosphor imager (Storm 860, Molecular Dynamics, Sunnyvale, CA) and ImageQuant software (Molecular Dynamics).

Northern Analysis

Total RNA (15 μg) was denatured and resolved by electrophoresis through a 1.25% (w/v) agarose gel containing 6% (v/v) formaldehyde in Mops buffer, pH 7.0 (Sambrook

et al., 1989). The gel was blotted onto a membrane (Hybond-N) and fixed using a UV cross-linker. After hybridization at 65°C in prehybridization solution, the membrane was washed at 65°C in 0.1× SSC and 0.1% (w/v) SDS, and the labeled probe was detected as described for Southern analysis. Quantification of transcript level was performed using ImageQuant software.

Synthesis of VVP5CS in *E. coli*

The VVP5CS pET-14b expression construct was created by introducing 5′-*Nde*I and 3′-*Bam*HI restriction sites into the VVP5CS encoding cDNA using PCR with Vent^R DNA polymerase (New England Biolabs, Beverly, MA) and the primers VVP5CSN (5′-GTTAACATATGGACGCCATG GACCCAACTCGA-3′) and VVP5CSC (5′-AGCCGGATC CTTAGGGCTGCAAAGTAAGCTCCTT-3′). The PCR product was digested with *Nde*I and *Bam*HI, ligated into the pET-14b vector (Novagen, Madison, WI), and transformed into *E. coli* BL21 (DE3) cells (Novagen) containing the pLysS plasmid (Sambrook et al., 1989).

Recombinant VVP5CS protein (bearing an NH_2-terminal hexahistidine tag) was produced by inoculating 1.0 L of Luria broth (100 μg mL^{-1} ampicillin and 40 μg mL^{-1} chloramphenicol) with a transformed colony and incubating with shaking at 37°C until $A_{600\ nm}$ equaled 0.5. Expression of the recombinant protein was initiated by addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, followed by incubation at 37°C for 5 h (or 16°C for 16 h for enhanced levels of soluble VVP5CS) before processing of the cells by the lysozyme method of Sambrook et al. (1989). Recombinant VVP5CS was purified using Talon Metal Affinity Resin (CLONTECH, Palo Alto, CA) according to the manufacturer's instructions, except that protein was eluted in buffers containing 20% (v/v) glycerol.

P5CS Assay

The P5CS assay was carried out essentially as described by Garcia-Rios et al. (1997), except that the assay was conducted at 37°C in a reaction mixture containing 5 mM or 50 mM Glu, 75 mM Tris-Cl (pH 7.3), 18.75 mM $MgCl_2$, 5 mM ATP, 0.4 mM NADPH, and 20 μg of purified recombinant VVP5CS.

Immunological Techniques

Antigen for polyclonal antibody production was prepared by excising recombinant VVP5CS from 10% SDS-PAGE gels and developing it into a slurry, as described by Harlow and Lane (1988). For the initial immunization 350 μg of antigen was mixed with an equal volume of Freund's complete adjuvant (GIBCO-BRL) and injected subcutaneously into a New Zealand White rabbit. Booster injections were given three times, at 6 weekly intervals, using approximately 150 μg of the antigen mixed with an equal volume of Freund's incomplete adjuvant. Blood was taken from the rabbit's ear 10 d after the third injection, and the serum containing polyclonal antibodies to VVP5CS was collected. The serum was supplemented with 0.02% (w/v) sodium azide and stored at −70°C.

Protein Extraction

Frozen berries were homogenized and 1 to 2 g was added to 2 to 4 mL of protein extraction buffer (500 mM Tris-HCl, pH 8.0, 5% [w/v] SDS, 10 mM DTT, and 10 mM sodium diethyldithiocarbamate), incubated at 95°C for 5 min, and centrifuged at 12,000*g* for 5 min (Tattersall et al., 1997). The supernatant was stored at −20°C until analysis by SDS-PAGE and immunoblotting.

SDS-PAGE and Western Analysis

Proteins were resolved by SDS-PAGE in 12% Tris-Gly gels (Fling and Gregerson, 1986). Proteins were visualized with Coomassie Brilliant Blue R-250 staining or blotted onto nitrocellulose membranes (MSI Laboratories, Westboro, MA) using a semidry transfer unit (LKB, Broma, Sweden), as described by Harlow and Lane (1988). The blots were probed with rabbit anti-VVP5CS serum or anti-AtPDH (Arabidopsis PDH) serum (kindly supplied by N. Verbruggen, University of Gent, Belgium) and by horseradish peroxidase-labeled goat anti-rabbit IgG. The blot was incubated with ECL detection reagents (Amersham) and exposed to Hyperfilm-MP (Amersham). Quantification of relative protein levels was performed using ImageQuant software.

RESULTS

Pro Constitutes a Significant Proportion of the Free Amino Acid Pool of Ripe Grape Berries

Analysis of the free amino acids in the ripe fruit of four different cultivars of *V. vinifera* (Table I) demonstrated notable differences in the concentrations of Pro and other members of the Glu family of amino acids (Gln, Glu, and Arg) consistent with previous studies (Lafon-Lafourcade and Guimberteau, 1962; Kliewer, 1968; Ough and Stashak, 1974). Pro accumulated to particularly high levels in berries of both cv Chardonnay (16 μmol/g fresh weight, equivalent to 1.8 mg/g fresh weight) and cv Cabernet Sauvignon (24 μmol/g fresh weight, equivalent to 2.8 mg/g fresh weight).

Pro Accumulation Occurs Relatively Late in Grape Berry Development

To examine the dynamics of Pro accumulation during normal berry development, the free amino acid composition was assessed during the 16 weeks from flowering to fruit maturity (Fig. 1). Veraison, or the beginning of fruit ripening, occurred at 8 weeks postflowering when there was a rapid increase in the accumulation of soluble solids (predominantly Suc; Coombe, 1973). Significant changes in

Table I. *Free Pro is present at high concentrations in ripe fruit from several cultivars of V. vinifera*

Amino acids were extracted from mature berries of cvs Chardonnay (25 degrees Brix; CH), Cabernet Sauvignon (25 degrees Brix; CS), Gewurztraminer (24 degrees Brix; GW), and Muscat Gordo Blanco (22 degrees Brix; MG).

Amino Acid	CH	CS	GW	MG
μmol/g fresh wt				
Asp	0.47	0.44	0.45	0.23
Glu	1.48	0.44	0.76	1.12
Hyp	0.09	0.31	0.16	0.14
Asn	0.21	0.11	0.01	0.03
Gln	1.21	1.35	2.47	2.27
Ser	0.49	1.21	0.11	0.45
His	0.67	0.24	0.11	0.36
Gly	0.21	0.87	1.37	0.02
Thr	1.49	0.96	0.01	0.41
Ala	1.67	0.95	1.92	0.82
γ-Aminobutyrate	1.62	1.32	1.91	0.68
Pro	16.03	23.69	10.66	6.34
Tyr	0.56	0.57	0.46	0.15
Arg	2.11	2.14	8.97	8.01
Ile	0.71	0.64	0.28	0.14
Leu	0.26	0.34	0.11	0.18
Val	1.02	0.55	0.08	0.26
Met	0.71	0.44	0.35	0.04
Phe	0.83	0.29	0.08	0.01
Orn	0.07	0.04	0.04	0.02
Lys	0.27	0.05	0.12	0.14

the concentrations of all of the Glu family of amino acids occurred throughout fruit development. In particular, the concentration of free Pro increased dramatically during the later stages of fruit ripening (12–16 weeks postflowering) when its accumulation paralleled the increasing sugar concentration. The concentration of free Arg increased slightly at veraison and then remained somewhat stable, whereas the concentration of Glu, and Gln in particular, decreased throughout fruit development.

Cloning of a Grapevine P5CS cDNA Expressed in Developing Fruit

A full-length cDNA of 2376 bp encoding P5CS was isolated from a 10-week-postflowering *V. vinifera* cv Shiraz berry cDNA library (*VVP5CS*, accession no. AJ005686). *VVP5CS* encodes an 82.6-kD protein with homology to P5CS sequences from a number of other plant species, including *V. aconitifolia* P5CS (76% amino acid identity) and Arabidopsis AtP5CS-1 (79% amino acid identity), as well as the γ-glutamyl phosphate reductase and γ-glutamyl kinase domains of ProB and ProA in *E. coli* (Fig. 2; Hu et al., 1992; Savouré et al., 1995; Yoshiba et al., 1995; Maggio et al., 1996; Igarashi et al., 1997).

The product of the *VVP5CS* cDNA, when expressed in *E. coli* and purified by affinity chromatography, had P5CS activity as demonstrated by the ATP and Glu-dependent consumption of NADPH (Garcia-Rios et al., 1997). The production of active enzyme was not straightforward because of its initial insolubility and instability but could be enhanced by postinduction incubation of the *E. coli* cultures at 16°C and by the inclusion of 20% (v/v) glycerol in all buffers. Under these conditions the specific activity of the enzyme was 0.96 μmol min^{-1} mg^{-1} (at 37°C, 50 mM Glu), which is similar to that reported for the two component activities of mothbean P5CS (Zhang et al., 1995). In the presence of 5 mM Glu, VVP5CS was sensitive to feedback inhibition by Pro, with a 50% reduction in activity at 25 mM Pro (Fig. 3). In the presence of 50 mM Glu, less inhibition by Pro was seen, with only a 33% reduction in activity at 75 mM Pro.

VVP5CS Is Encoded by a Single Gene in the Grapevine Genome

Southern hybridization analyses were performed to determine whether VVP5CS is encoded by a single gene or whether other closely related genes exist in the grapevine genome. Washing at two different levels of stringency for detection of sequences with greater than 65% or 95% identity, respectively (Meinkoth and Wahl, 1984), produced identical patterns of hybridization (Fig. 4). The single bands detected after digestion with *Xba*I and *Sty*I and the two bands detected after digestion with *Hin*dIII are consistent with VVP5CS being encoded by a single gene because the partial cDNA clone used as the probe contained no *Xba*I or *Sty*I sites and only one *Hin*dIII site. The three bands detected after digestion with *Pst*I (in addition to the higher band of undigested DNA) are one more than might be expected considering that the partial cDNA clone contained only one *Pst*I site. However, the extra band could be

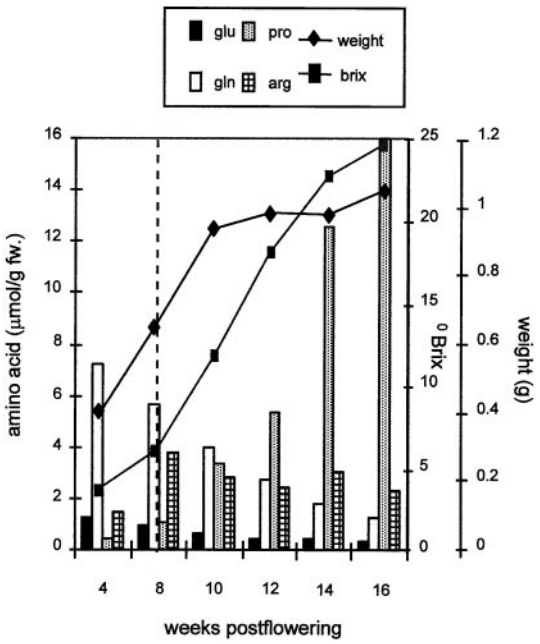

Figure 1. Physical and chemical changes in *V. vinifera* cv Chardonnay fruit from 4 to 16 weeks postflowering (full maturity). Veraison is indicated by a dashed line at 8 weeks postflowering. Concentrations of free Pro, Arg, Glu, and Gln are expressed as micromoles per gram fresh weight (fw.) of fruit.

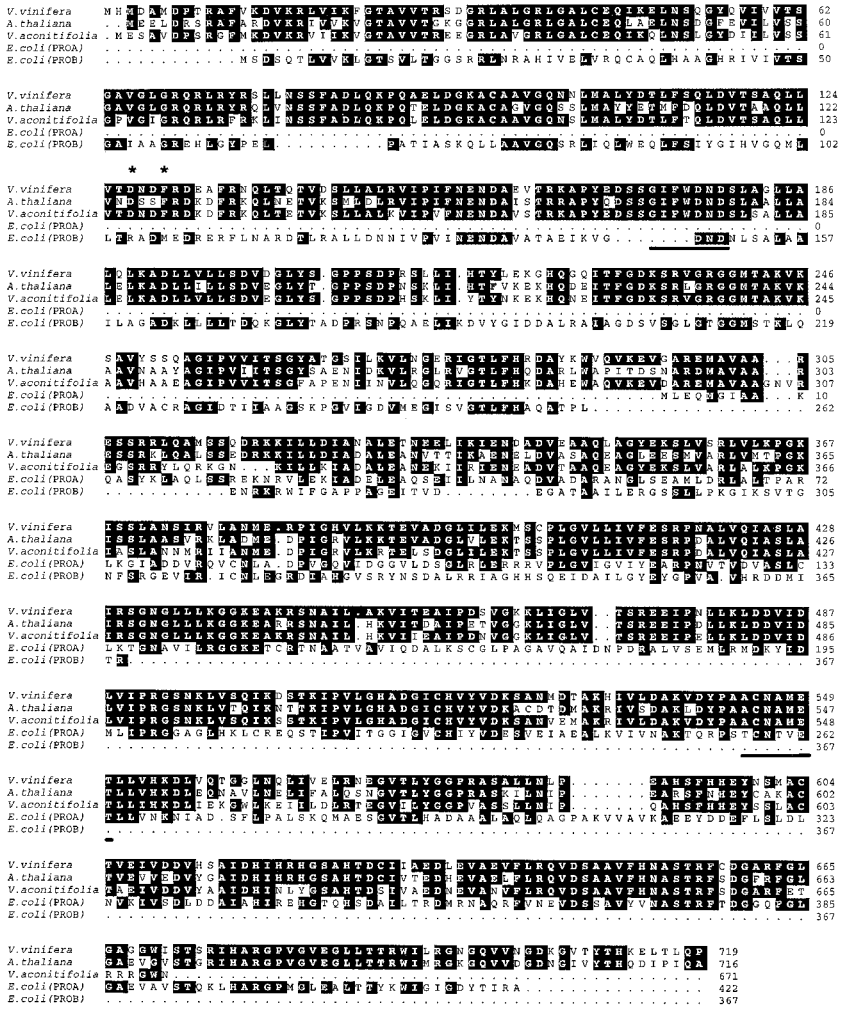

Figure 2. *V. vinifera* P5CS shares a high degree of homology with other P5CS sequences. Multiple alignment of predicted VVP5CS protein sequence with deduced amino acid sequences obtained from nucleotide databases with the following accession numbers: *V. vinifera* (accession no. AJ005686) from this study, Arabidopsis (*A. thaliana*, accession no. AT-P5CS1; SwissProt no. P54887), *V. aconitifolia* (accession no. P32296), and *E. coli* ProA and ProB (accession nos. P07004 and P07005, respectively). Residues identical to the *V. vinifera* sequence are highlighted. Amino acids involved in Pro feedback inhibition of the *V. aconitifolia* P5CS enzyme are indicated with asterisks (Zhang et al., 1995). Degenerate oligonucleotides used in reverse-transcription-PCR were designed based on the sequences that are underlined.

explained if an intron containing a *Pst*I site occurs within the hybridizing region. This is likely considering the large size (1.1 kb) of the cDNA probe and the existence of multiple introns in the homologous region of P5CS clones from Arabidopsis (Savouré et al., 1995; Strizhov et al., 1997). It remains possible that other genes with less homology to P5CS exist, but they are unlikely to interfere in the northern hybridization analyses reported here because of the stringent hybridization conditions used.

VVP5CS mRNA and Protein Levels Remain Relatively Constant throughout Fruit Development

The stress-induced accumulation of Pro in vegetative tissues has been shown in a number of plant species to be correlated with a significant and sustained induction of P5CS gene expression at the level of gene transcription. In contrast, the steady-state levels of *VVP5CS* mRNA remain relatively constant throughout grape berry development, although transient increases are observed at both 4 and 12 weeks postflowering (Fig. 5). These increases in the steady-state levels of mRNA are not translated into significant increases in the steady-state levels of VVP5CS protein (Fig. 6B), although slightly higher levels are observed at 12 and 13 weeks postflowering. These results demonstrate however that the significant increase in free Pro concentration observed late in berry development is not associated with a concurrent increase in steady-state levels of VVP5CS mRNA and protein.

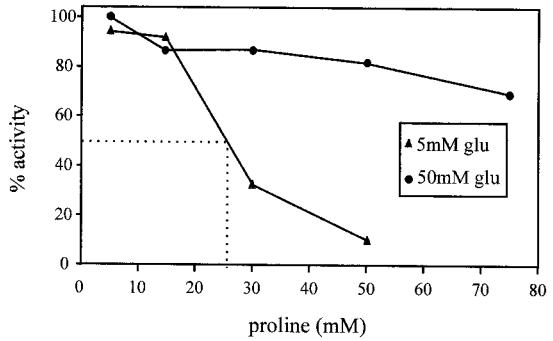

Figure 3. Pro feedback inhibition of VVP5CS expressed in *E. coli* and purified by affinity chromatography. P5CS assays were conducted in the presence of the Pro and Glu concentrations indicated. Results are expressed as the percentages of specific activity in the absence of Pro.

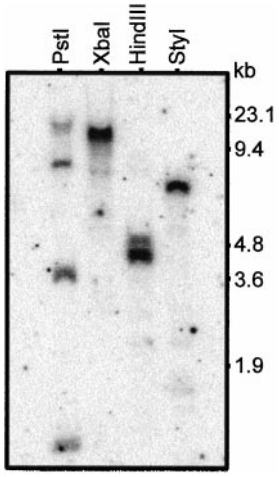

Figure 4. Southern analysis of grapevine genomic DNA indicates that VVP5CS is encoded by a single gene. DNA isolated from *V. vinifera* cv Chardonnay was digested with the restriction enzymes *Pst*I, *Xba*I, *Hin*dIII, and *Sty*I, probed with a 1.1-kb fragment of VVP5CS cDNA (nucleotides 590–1701), and then screened under high- and low-stringency conditions. Since both sets of conditions produced identical results, only those obtained after the high-stringency screen are shown.

PDH Protein Is Present throughout Fruit Development

Incubation of a western blot of grape berry proteins sampled throughout development with polyclonal antibodies raised against AtPDH (N. Verbruggen, personal communication) produced a single band corresponding to a protein of approximately 55 kD (Fig. 6D), which is approximately the size expected for the mitochondrial enzyme PDH (Kiyosue et al., 1996; Peng et al., 1996; Verbruggen et al., 1996). The level of this cross-reactive protein increased steadily until 13 weeks postflowering, after which it remained relatively constant, indicating that Pro accumulation late in berry ripening is not associated with a decrease in PDH protein levels at that time.

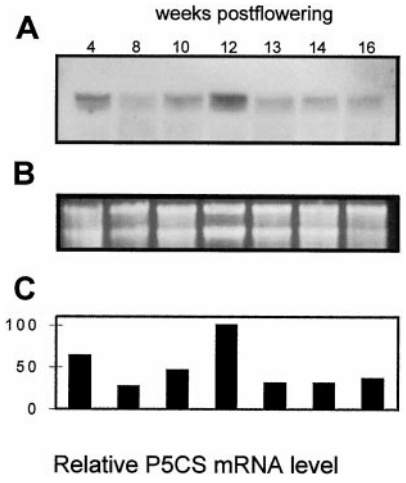

Figure 5. Steady-state levels of VVP5CS mRNA throughout fruit development. A, Total RNA (15 μg) isolated from *V. vinifera* cv Chardonnay berries was electrophoresed, blotted onto a nylon membrane, and probed with a 0.89-kb fragment of VVP5CS cDNA (nucleotides 791–1680). B, A replica gel was stained with ethidium bromide to demonstrate the equivalence of RNA loading in each lane. C, Relative levels of VVP5CS transcript detected.

DISCUSSION

Free Pro accumulates to very high levels in the mature fruit of *V. vinifera* cvs Chardonnay and Cabernet Sauvignon. The 2.8 mg Pro/g fresh weight berry measured in this study was 25- to 80-fold higher than the levels of free Pro found in grapevine leaves or roots (A.P. Stines, P.B. Høj, and R. van Heeswijck, unpublished data). This level of Pro is comparable with that found in other plant organs

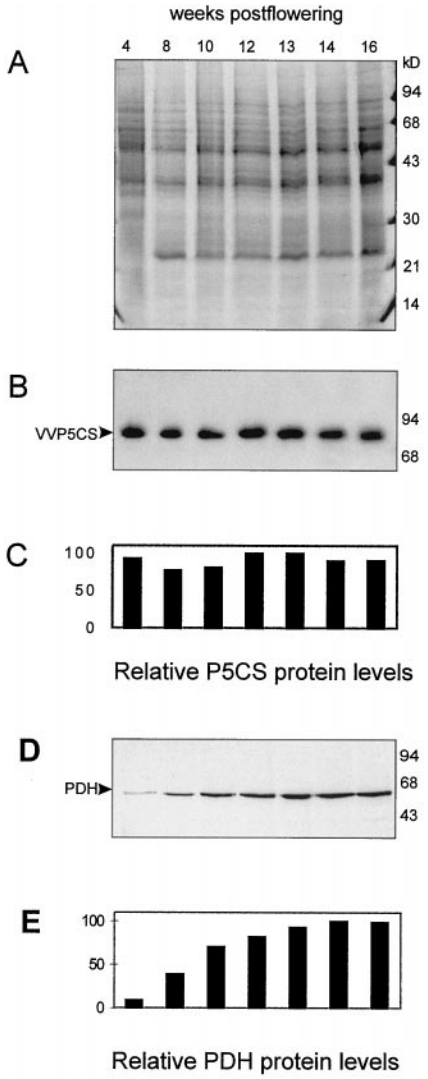

Figure 6. Steady-state levels of VVP5CS and PDH proteins throughout fruit development. A, Protein extracts from whole-berry homogenates of *V. vinifera* cv Chardonnay were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Each lane contained extracts from equivalent amounts of berry homogenate on a fresh weight basis. B and D, Replica gels were transferred to a nitrocellulose membrane and subjected to western analysis using antibodies prepared against VVP5CS (B) or AtPDH (D). C and E, Relative levels of VVP5CS and PDH proteins.

known to accumulate high levels of Pro during normal development, including 0.9 mg Pro/g fresh weight in Arabidopsis flowers (Savouré et al., 1995) and 1.1 mg Pro/g fresh weight in *Vicia faba* ovules (Venekamp and Koot, 1984). It is also similar to the high levels of Pro shown to accumulate in vegetative tissues of some plants under stress, e.g. 0.6 mg Pro/g fresh weight in salt-stressed Arabidopsis seedlings (Savouré et al., 1995) and 3.5 mg Pro/g fresh weight in leaves of water-stressed tobacco plants (Kishor et al., 1995). Because the role of free Pro and the regulation of its accumulation during normal plant development has received little attention compared with the numerous studies of the accumulation of Pro in response to abiotic stress, we examined the expression of key genes in Pro synthesis and degradation during grape berry development.

The accumulation of Pro in grape berries follows a developmental pattern whereby it becomes the predominant free amino acid during the last 4 to 6 weeks of berry ripening (Fig. 1). Steady-state levels of P5CS mRNA remain relatively constant however during the 16 weeks from flowering to fruit maturity, with only transient increases seen at 4 and 12 weeks postflowering (Fig. 5). These transient increases could be a result of the peaks in berry ABA found at flowering and approximately 2 to 3 weeks postveraison (Coombe and Hale, 1973). Treatment with exogenous ABA has been shown to enhance levels of P5CS mRNA in Arabidopsis seedlings (Yoshiba et al., 1995; Igarashi et al., 1997; Savouré et al., 1997; Strizhov et al., 1997). However, the increased P5CS mRNA levels at 4 and 12 weeks postflowering do not appear to be translated into significant changes in the steady-state levels of P5CS protein (Fig. 6). Thus, unlike previous reports of stress-induced Pro accumulation in vegetative tissues (Savouré et al., 1995; Yoshiba et al., 1995; Peng et al., 1996; Igarashi et al., 1997), the primary basis for Pro accumulation late in grape berry development does not appear to be induction of VVP5CS mRNA or protein levels.

Net accumulation of free Pro in the developing grape berry may result from changes in the balance among the transport of metabolites into the berry, the biosynthesis and degradation of Pro, and its incorporation into alternative nitrogen sinks such as cellular protein. Clearly, there are a number of points of regulation other than simple changes in P5CS mRNA and protein levels that could affect this balance. Western analysis of different grapevine tissues has demonstrated that the skin and pulp of grape berries contain relatively high levels of P5CS protein compared with other tissues, such as leaves and seeds (A.P. Stines, P.B. Høj, and R. van Heeswijck, unpublished data), suggesting that the capacity for Pro synthesis, in terms of amount of enzymic protein, remains relatively high in berry tissue throughout development. The lack of Pro accumulation early in berry development could be explained by a deficiency of Glu, the VVP5CS substrate. However, this does not appear to be the case, since the concentrations of both Glu and Gln are relatively high at this stage and decrease later on in berry development (Fig. 1).

Posttranslational modification of P5CS enzyme activity could be involved in regulating Pro accumulation, e.g. by the presence of an inhibitor of P5CS early in grape berry development. We developed buffers specifically for extraction of active enzymes from grapevine tissues that contain high levels of phenolic compounds (Ford and Høj, 1998). However, no P5CS activity has been detectable in berry extracts prepared with these buffers, even with the sensitive TLC-based assay system of Zhang et al. (1995). This may not be surprising given the instability of the recombinant VVP5CS synthesized in *E. coli* and its relatively low specific activity. P5CS activity could not be detected by other workers in extracts of control tobacco or mothbean plants, but could be detected only in extracts of transgenic tobacco plants overexpressing the mothbean P5CS cDNA, and then only after ammonium sulfate fractionation (Kishor et al., 1995; Zhang et al., 1995). We are therefore unable at present to study changes in P5CS activity in situ during berry development.

To investigate further the properties of VVP5CS, we have assayed the activity of the recombinant enzyme produced in *E. coli* and demonstrated that it is subject to feedback inhibition by Pro and that the level of inhibition is influenced by the concentration of Glu, similarly to P5CS from other organisms (Hayzer and Leisinger, 1980; Hu et al., 1992; Zhang et al., 1995; Garcia-Rios et al., 1997). It was estimated that 25 mM Pro was required to achieve 50% inhibition of VVP5CS enzyme activity in the presence of 5 mM Glu, whereas more than 75 mM Pro was required in the presence of 50 mM Glu. This level of feedback inhibition is considerably lower than that observed for the γ-glutamyl kinase activity of *V. aconitifolia* P5CS (50% inhibition of the enzyme activity at 5 mM Pro and 50 mM Glu; Zhang et al., 1995) and orders of magnitude lower than that seen for tomato P5CS encoded by tomPRO1 (50% inhibition of enzyme activity at 0.02 mM Pro and 10 mM Glu; Garcia-Rios et al., 1997). The relative insensitivity of VVP5CS to feedback inhibition by Pro indicates that the capacity for Pro synthesis via P5CS could remain high throughout berry development even when Pro concentrations reach almost 15 mM (Table I).

Enhanced Pro accumulation late in berry development could occur through an activation of Pro biosynthesis from Orn via OAT. However, the steady-state level of OAT mRNA is so low throughout grape berry development that it cannot be detected by northern analysis, but only by reverse-transcription-PCR (A.P. Stines, P.B. Høj, and R. van Heeswijck, unpublished data). This suggests that the level of OAT protein and enzymic activity may also be very low and thus not contribute significantly to Pro levels, but this needs to be confirmed. Enhanced Pro accumulation could also occur through a decrease in the degradation of Pro catalyzed by the mitochondrial enzyme PDH (Elthon and Stewart, 1981). Using antibodies raised against AtPDH (N. Verbruggen, personal communication), we detected by western analysis a protein of approximately 55 kD representing the grapevine PDH homolog. The steady-state levels of grapevine PDH protein increase throughout berry development until 13 weeks postflowering (Fig. 6), possibly in response to the moderate increases in free Pro during this period. PDH mRNA levels in Arabidopsis increase in the presence of high concentrations of Pro, providing that

the plants are not under osmotic stress (Kiyosue et al., 1996; Peng et al., 1996; Verbruggen et al., 1996). The steady-state levels of grapevine PDH protein remain relatively high late in berry development, demonstrating that a decrease in PDH protein levels does not occur at the time of most rapid Pro accumulation. Together with our other observations, this demonstrates that Pro accumulation late in grape berry development is independent of changes in steady-state levels of P5CS mRNA, P5CS protein, and PDH protein. Furthermore, this suggests that the mechanisms of regulation of Pro accumulation during normal plant development are quite different from those operating during the abiotic stress response.

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