# EXPRESSION OF THE KEY PROLINE BIOSYNTHETIC ENZYMES P5CS AND OAT DURING GRAPE BERRY DEVELOPMENT.

Submitted by

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# ABSTRACT

Mature fruit of *Vitis vinifera* L. (grapevine) contains unusually high levels of free proline (up to 24 μmol or 2.8 mg/g fwt). Amino acid analysis of four different grapevine varieties (Chardonnay, Cabernet Sauvignon, Gewurztraminer and Muscat Gordo) revealed that proline accumulation does not occur uniformly throughout berry development, but chiefly during the last 4-6 weeks of ripening. In order to investigate the mechanism underlying the accumulation of proline in grape berries, a full-length cDNA encoding VVP5CS (*V.vinifera* $\Delta^1$-pyrroline-5-carboxylate synthetase), the key regulatory enzyme in the glutamate pathway of proline synthesis, was isolated from post-veraison grape berries. The levels of VVP5CS mRNA and VVP5CS protein were determined in the developing fruit of the four *V.vinifera* cultivars, and were found to be relatively uniform from flowering through to full fruit maturity. This was in contrast to the pattern of proline accumulation, where substantial increases in free proline concentration occurred only late in berry development. Other factors, therefore, which may affect the free proline pool in berries were examined. Proline can also be synthesised from ornithine via the activity of OAT (δ-Ornithine aminotransferase). A full length cDNA encoding VVOAT (*V.vinifera* δ-Ornithine aminotransferase) was cloned from post-veraison berries and the protein it encodes was found to be present throughout berry development. The steady-state levels of VVOAT protein increased in Gewurztraminer and Muscat Gordo berries towards the later stages of ripening. VVOAT enzyme activity was detected throughout development of the ripening fruit, and is, therefore, likely to contribute to free proline levels in the grape berry. The steady-state levels of proline dehydrogenase (PDH) protein, the first enzyme in proline degradation, increased throughout early fruit development but thereafter remained relatively constant. The developmental accumulation of free proline late in grape berry ripening is thus clearly distinct from the osmotic stress-induced accumulation of proline observed in many plants. Its onset is not associated with either an increase in steady-state levels of P5CS mRNA or protein or a decrease in steady-state levels of PDH protein, suggesting that other physiological factors are important in regulating proline accumulation in the grape berry.

# Statement of Authorship

This work contains no material which has been accepted for the award of any other degree or diploma in any institution and, to the best of my knowledge contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Anna P. Stines 12/8/99

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# ABBREVIATIONS

| | |
|---|---|
| A | adenine |
| ABA | abscisic acid (5-[1-Hydroxy-2,6,6-trimethyl-4-oxycyclohex-2-en-yl]-3-methyl-pentadienoic acid) |
| AHA | 4-amino-5-hexynoic acid |
| bp | base pair |
| BSA | bovine serum albumin |
| C | cytosine |
| $^0$C | degrees Celsius |
| cDNA | complementary DNA to an RNA |
| C-terminal | carboxy-terminal |
| cv | cultivar |
| Da | dalton |
| DAP | diammonium phosphate |
| dCTP | 2'-deoxycytidine 5'-triphosphate |
| DNA | deoxyribonucleic acid |
| DTT | 1,4-dithithreitol |
| ECL | enhanced chemiluminescence |
| EDTA | ethylenediamine tetra-acetic acid |
| FMOC-Cl | 9-fluorenylmethylchloroformate |
| fwt | fresh weight |
| g | gram |
| *g* | gravity |
| G | guanine |
| gabaculine | 3-amino-2,3-dihydrobenzoic acid |
| h | hour |
| HPLC | high performance liquid chromatography |
| I | inosine |
| IPTG | isopropyl-β-D-thiogalactaside |
| kb | kilobase |
| kDa | kilodalton |
| L | litre |
| L. | Linnaeus |
| LB | Luria-Bertani |
| µg | microgram |
| mg | milligram |
| min | minute |
| mm | millimetre |
| µl | microlitre |
| ml | millilitre |
| M | molar |
| mM | millimolar |
| µM | micromolar |
| µmol | micromoles |
| MOPS | 3-(N-morpholino) propane sulfonic acid |

# ABBREVIATIONS (continued)

| | |
|---|---|
| mRNA | messenger RNA |
| MW | molecular weight |
| nm | nanometre |
| N-terminal | amino-terminal |
| OAT | ornithine aminotransferase |
| OD | optical density |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| P5CS | $\Delta^1$-pyrroline-5-carboxylate synthetase |
| P5CR | $\Delta^1$-pyrroline-5-carboxylate reductase |
| PDH | proline dehydrogenase |
| PEG | polyethylene glycol |
| pers.comm. | personal communication |
| Ponceau S | 3-hydroxy-4-[2-sulfo-4-(sulfophenylazo) phenylazo]-2,7-naphthalenedisulfonic acid |
| PVPP | polyvinylpolypyrrolidone |
| RACE | rapid amplification of cDNA ends |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase PCR |
| SDS | sodium dodecyl sulfate |
| SSC (20x) | 3M NaCl, 0.3M $Na_3$citrate buffer |
| T | thymine |
| TAE (50x) | 2M tris-HCl buffer, 1M acetic acid, 50mM EDTA, pH 8 |
| TBS | Tris buffered saline |
| TE | 10mM tris-HCl buffer, pH 8, 1mM EDTA |
| Tris | tris(hydroxymethyl)aminomethane |
| Tween-20 | polyoxyethylene-sorbitan monolaurate |
| UV | ultraviolet |
| vol | volume |
| v/v | volume/volume |
| w/v | weight/volume |

One and three letter codes for amino acids

| | | | | | |
|---|---|---|---|---|---|
| A | Ala | alanine | L | Leu | leucine |
| R | Arg | arginine | K | Lys | lysine |
| N | Asn | asparagine | M | Met | methioine |
| D | Asp | aspartic acid | F | Phe | phenylalanine |
| C | Cys | cysteine | P | Pro | proline |
| E | Glu | glutamic acid | S | Ser | serine |
| Q | Gln | glutamine | T | Thr | threonine |
| G | Gly | glycine | W | Trp | tryptophan |
| H | His | histidine | V | Val | valine |
| I | Ile | isoleucine | Y | Tyr | tyrosine |

# PUBLICATIONS

Parts of the work described in this thesis have been published or submitted for publication in the following:

**Stines AP**, Naylor DJ, Høj, PB and van Heeswijck R (1999) Proline accumulation in developing grapevine fruit occurs independently of changes in levels of $\Delta^1$-pyrroline-5-carboxylate synthetase mRNA or protein. Plant Physiol 120: 923-931

**Stines AP**, Grubb J, Gockowiak H, Henschke PA, Høj PB and van Heeswijck R (1999) Proline accumulation in developing berries: influence of vine variety, berry maturity and tissue type. Aust J Grape and Wine Res (submitted)

# Chapter 1
## *General Introduction*

## 1.1 Introduction

The species *Vitis vinifera* L. is the widely cultivated and most economically important form of grapevine (*Vitis* spp.). *V.vinifera* originated from the Indo-European area but is now grown in virtually all temperate areas of the world. The numerous varieties of *V.vinifera* produce white, red or black berries, which are used for dried fruit, table grapes and wine production. This diversity of its use, combined with the total amount of fruit produced and the high commercial value of grapes, make *V.vinifera* one of the most important horticultural crops worldwide. In Australia alone, the total production of wine was valued at approximately $1.8 billion in 1996-97 representing 2.5% of the value of the total world wine production (Source: Australian Wine and Brandy Corporation). Over recent years, the value and competitive nature of the grape and wine industry has led to increasing emphasis on producing quality grape berries, and hence, quality wines. In general terms the 'quality' of a berry is determined by its composition. The research described in this thesis investigates an important aspect of grape composition, that is the accumulation of free amino acids[1], with an emphasis on the molecular biology and biochemistry of the accumulation of free proline.

## 1.2 Amino Acid Composition of Grape Berries

The quality of the grape berry, in terms of wine industry requirements, is affected by its free amino acid content. Free amino acids are comprised of a carbon atom bound on either side by a carboxyl group (COOH) and an amino group ($NH_2$) and are differentiated by the length of the carbon skeleton and the presence of chemically

[1] The term amino acid will be extended to include proline which is an imino acid

distinct side chains. Of the nitrogen (N)[2] in berry juice (60 to 2400 mg N/l (Ough and Amerine, 1988)) 50-90% is in the form of free amino acids (Kliewer, 1969,1970), with the remainder present as ammonium ions (average 96 mg N/l (Ough, 1969)), peptides, proteins and nitrates as well as trace amounts of vitamins, nucleotides and amines. Of the free amino acids, the most predominant are commonly proline[3] (up to 2g/l), arginine (up to 1.6g/l) and to a lesser extent, alanine, aspartic acid and glutamic acid (Castor, 1953; Kliewer, 1969,1970; Huang and Ough, 1991).

The actual content and composition of grape berry amino acids in juice can vary significantly depending on the level of fruit maturity and the particular variety of *V.vinifera* examined (Kliewer, 1969,1970; Ough, 1988; Huang and Ough, 1989; Mullins *et al.*, 1992). A number of other factors including use of rootstocks (Huang and Ough, 1989; Treeby *et al.*, 1998), nitrogen fertilisation (Kliewer, 1971; Goldspink and Gordon, 1991; Spayd *et al.*, 1994), temperature (Buttrose *et al.*, 1971) and crop level (Kliewer and Ough, 1970) have also been reported to have an influence. The mechanisms underlying the effects of these factors are unknown.

The total nitrogen content of berries rises significantly during maturation, primarily due to considerable increases in the amount of free amino acids (Lafon-Lafourcade and Guimberteau, 1962; Kliewer, 1968,1969,1970; Ough, 1969). The concentrations of arginine and proline, in particular, are reported to change by two to six-fold between early and late-harvested (greater maturity) fruit from the same variety (Kliewer, 1968,1969,1970). Most investigations in this area have focused on the later stages of

[2] Nitrogen, refers to nitrogenous compounds such as free amino acids, the ammonium ion, peptides and nucleotides

[3] For the remainder of this thesis the terms proline and arginine etc. will refer to levels or concentrations of the free amino acid, unless otherwise indicated.

berry ripening and have reported that proline and arginine levels undergo the most drastic changes during grape berry maturation (Lafon-Lafourcade and Guimberteau, 1962; Kliewer, 1968,1969,1970; Ough, 1969). Little information, however, exists on the composition of the free amino acid pool in immature berries and what changes occur in the early to middle stages of berry development.

Early studies reported that there were significant variations in the free amino acid composition of different cultivars, however the influence of berry maturity or vineyard location were not taken into account in most cases (Lafon-Lafourcade and Peynaud, 1952; Gallander *et al.*, 1969). In order to compare varietal amino acid compositions, Kliewer (1969, 1970) examined the free amino acid profiles from a large number of grape varieties all grown under the same soil, climatic and cultural conditions in California. Although comparisons were made between varieties at a wide range of $^{0}$Brix[4] levels, it was observed that the amino acid composition was distinct for each variety, and most importantly, that arginine and proline levels in berries varied by 10 to 12 fold between cultivars. A study by Huang and Ough (1991) confirmed that amino acid profiles differed considerably between varieties, although the predominant amino acids in all cases were still proline and/or arginine. Although absolute values for each amino acid varied considerably between these studies, the different ratios of proline:arginine obtained were relatively consistent for each variety. Certain varieties, for example Cabernet Sauvignon and Chardonnay, accumulated extremely high levels of proline, while others, for example Gewurztraminer and Riesling, predominantly accumulated arginine and yet other varieties, for example Merlot, did not accumulate

[4] $^{0}$Brix is a refractive index measure of total soluble solids. In ripe grape berries it is a measure of sucrose concentration (g/100 ml) and is used as an indication of berry maturity.

significant levels of either of these amino acids. Recently, a survey of amino acids in the berry juice from 12 cultivars grown in Washington State, USA was made over a four year period. In this study it was shown that berry amino acid concentrations, and particularly that of arginine, varied from year to year within a variety. Furthermore, it was reported that the proline:arginine ratio for particular varieties was also affected by the season (Spayd and Andersen-Bagge, 1996). Huang and Ough (1991) found that although absolute concentrations of amino acids varied from year to year within a variety, the proline:arginine ratio remained fairly constant. It is clear from the above studies that berry maturity, variety and season can all influence berry amino acid concentrations and these factors need to be taken into account in the design of experiments and interpretation of data.

## *1.3 The Influence of Grape Must Proline on Fermentation*

In order to sustain fermentation, yeast require nitrogen. Amino acids can account for up to 90% of the nitrogen in grape juice and therefore have a significant influence on the rate and extent of a fermentation (Kliewer, 1969,1970; Ough, 1988). Most free amino acids are readily assimilated by *Saccharomyces cerevisae* and are reduced to 10% or less of their original concentration by the end of fermentation (Castor and Archer, 1956; Tercelj, 1965; Huang and Ough, 1991). A large proportion of free proline remains, however, indicating that it is not as easily utilised as other amino acids. This lack of proline assimilation by yeast during fermentation is thought to be due, firstly, to inhibition of the yeast proline uptake system, proline permease, by other amino acids. This enables the yeast to favour the assimilation of more efficient sources of nitrogen, such as arginine (Henschke and Jiranek, 1993). Secondly, the enzyme required for

proline catabolism in yeast, proline oxidase, requires oxygen for catalytic activity (Ingledew *et al.*, 1987; Henschke and Jiranek, 1993). In grape must fermentations oxygen is rapidly consumed, thus reducing proline oxidase activity and the catabolism of proline. Hence, in the absence of aeration, musts with a low total N content and a high proportion of their nitrogen in the form of proline can result in slow rates of fermentation (so-called sluggish ferments). In the extreme case they may have insufficient nitrogen available for the yeast to complete fermentation resulting in a stuck ferment (Ough and Stashak, 1974). Ferments can be supplemented with nitrogen in the form of the chemical diammonium phosphate (DAP) to alleviate this problem. Indeed, this is currently routine procedure in many wineries, however, the addition of nitrogen supplements has a number of drawbacks, including its association with the formation of ethylcarbamate, a suspected carcinogen, in wine (Ough *et al.*, 1988; Ough, 1991). An understanding, therefore, of the mechanisms behind proline accumulation in grape berries and the factors which influence it are of fundamental interest to the grape and wine industries.

## *1.4 Proline Accumulation in Plants*

Kemble and MacPherson (1954) first reported that proline accumulated in wilted perennial rye-grass. Over the next four decades, a large amount of work followed which confirmed that proline accumulation was a widespread response to osmotic stress amongst a number of different plant species (reviewed by Aspinall and Paleg, 1981). The phenomenon is not universal, however, as there are several cases where this relationship has not been observed. Moftah and Michel (1987) found proline accumulation in soybean leaves was not a sensitive indicator of cold or salt stress and

Ashraf (1989) suggested that the level of proline accumulation in blackgram (*Vigna mungo* L.) was too low to have any osmoregulatory effect. Downton and Loveys (1981) made the observation that the level of proline in leaves of salt stressed grapevine was very low, suggesting that the amino acid was of little importance to the plant's osmotic adjustment response. The above examples, however, appear to be an exception, not the rule, and it is now generally accepted that proline accumulation in response to osmotic stress commonly occurs in most plants. Moreover, the demonstration that *Salmonella typhimurium* mutants overproducing proline are more osmotolerant than the wild type suggested that proline accumulation could be directly involved in osmotic protection of cells (Csonka, 1981). In a number of plant studies, correlations have also been observed between the ability of plants to withstand osmotic stress and the degree of proline accumulation in response to that stress (Boggess *et al.*, 1976; Munns *et al.*, 1979; Moftah and Michel, 1987; Songstadt *et al.*, 1990; Chiang and Dandekar, 1991; Szoke *et al.*, 1992; Bohnert *et al.*, 1995; Niu *et al*,. 1995; Martinez *et al.*, 1996; Igarashi *et al.*, 1997).

Proline is believed to protect the plant against the adverse effects of drought, salinity, nutrient deficiencies, temperature, acidity and heavy metals by acting as an osmolyte and as a compatible solute (Stewart and Lee, 1974; Pollard and Wyn Jones, 1979; Paleg *et al.*, 1981; Nash *et al.*, 1982; Delauney and Verma, 1993). There are several classes of compatible solutes which include amino acids, quaternary ammonium compounds, tertiary sulphoniums and certain carbohydrates (Rhodes and Hanson, 1993; Yancy, 1994). Compatible solutes, which are usually uncharged at neutral pH and have high water solubility, are excluded from the hydration sphere of proteins. In contrast,

perturbing solutes interact with and disrupt hydration spheres, favouring protein unfolding (Paleg *et al.*, 1984; Samaras *et al.*, 1995). Alternatively, as an osmolyte, proline accumulation in the cytoplasm may facilitate the uptake of water and hence the osmotic adjustment of the cell (Bohnert *et al.*, 1995). Another possible reason for proline accumulation is that it may be a source of carbon, nitrogen and energy for the cell (Barnard and Oaks, 1970; Yeh and Phang, 1988). The synthesis of proline produces $NADP^+$ and ADP which may then be used as intermediates in pathways which generate cellular energy. Furthermore, it has been proposed that proline may be transported to energy depleted tissue where the degradation of proline results in the production of NADH which would also provide the cell with energy (Hare and Cress, 1997; Kohl *et al.*, 1998; Verslues and Sharp, 1999).

Most of the research on proline metabolism in plants has focused on its accumulation in response to abiotic stress and little research has been carried out on the mechanism and role of free proline accumulation in normal plant development, although high levels of free proline have been shown to occur in particular plant tissues and organs (Ulrich and Thaler, 1955; Clements and Leland, 1962; Zhang *et al.*, 1982; Venekamp and Koot, 1984; Flasinski and Rogozinska, 1985; Walton *et al.*, 1991; Chiang and Dandekar, 1995; Verbruggen *et al.*, 1996; Hua *et al.*, 1997). The high levels of free proline observed in particular plant tissues and organs suggests that this amino acid may have a function in their development. Very high levels of free proline have been reported specifically in the flowers and seeds of *Arabidopsis thaliana* (Chiang and Dandekar, 1995; Verbruggen *et al.*, 1996; Hua *et al.*, 1997), in inflorescences and siliques of *Brassica napus* (Flasinski and Rogozinska, 1985), in ovules of *Vicia faba* (Venekamp

and Koot, 1984), in pollen grains of *Petunia hybrida* (Zhang *et al.*, 1982), in the buds of *Actinidia deliciosa* (Walton *et al.*, 1991) and in the mature fruits of citrus (*Citrus* sp.) (Clements and Leland, 1962), pear (*Pyrus* sp.) (Ulrich and Thaler, 1955) and the grapevine (Lafon-Lafourcade and Guimberteau, 1962; Kliewer, 1968; Ough and Stashak 1974). The role of free proline in the development or function of these organs remains unknown.

## 1.5 Proline Metabolism in Bacteria

Amino acid biosynthesis has been most extensively studied in bacteria where genetic and biochemical techniques can be applied in a relatively simple biological system. The existence of numerous mutants and complementation experiments have allowed the individual steps of particular pathways to be analysed. Information gained from these bacterial studies has been very useful in understanding the corresponding pathways in higher organisms.

### 1.5.1 The Glutamate Pathway of Proline Biosynthesis

The biosynthesis of proline was first characterised in the bacterium *Escherichia coli* where it was shown to involve carbon skeleton rearrangements originating from the amino acid glutamate (Baich, 1969; Hayzer and Moses, 1978; Hayzer and Leisinger, 1980, 1982; Deutch *et al.*, 1982, 1984). In *E.coli* and other bacteria, proline is synthesised from glutamate by three enzymes; γ-glutamyl kinase (GK) (EC 2.7.2.11), γ-glutamyl phosphate reductase (GPR) (EC 1.1.1.41) and $\Delta^1$-pyrroline-5-carboxylate reductase (P5CR) (EC 1.5.1.2) (Figure 1.1) (Vogel and Davis, 1952). The GK reaction

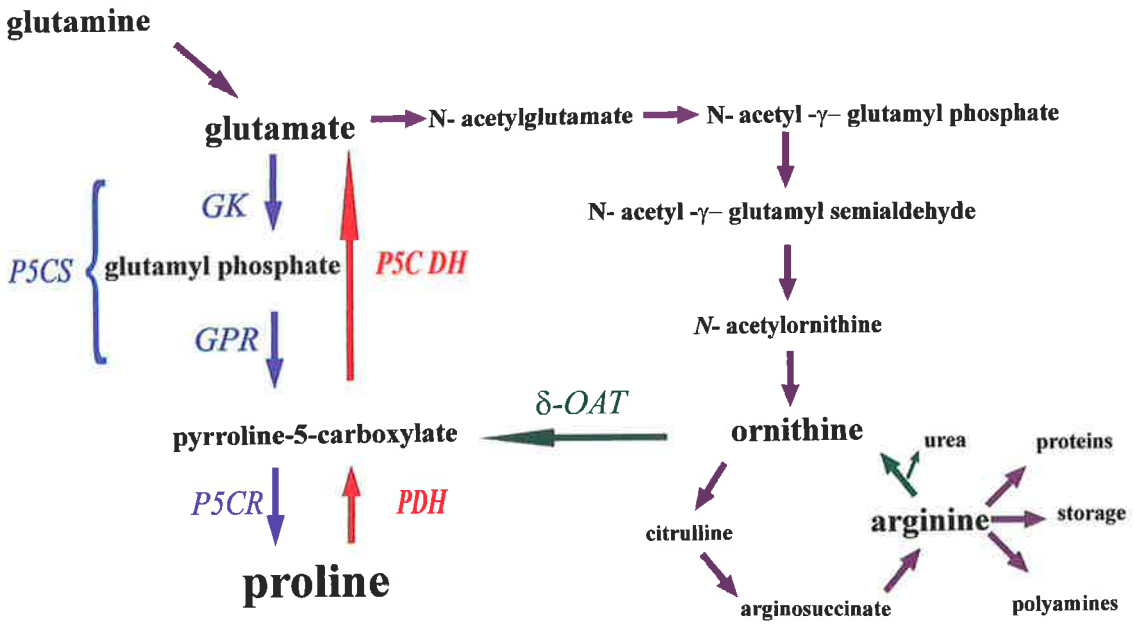



**Figure 1.1** Pathways of proline biosynthesis in higher plants. P5CS: $\Delta^1$-pyrroline-5-carboxylate synthetase (also known as GK: glutamyl kinase; GPR: glutamyl phosphate reductase) P5CR: $\Delta^1$-pyrroline-5-carboxylate reductase; δ-OAT: δ-ornithine aminotransferase; PDH: proline dehydrogenase, P5CDH; $\Delta^1$-pyrroline-5-carboxylate dehydrogenase.

is irreversible and its extremely labile product, γ-glutamyl phosphate, remains bound to GK whilst participating in the second, reversible reaction, performed by GPR to form glutamate semialdehyde (GSA) (Baich, 1969, Hayzer and Leisinger, 1980). The subsequent conversion of GSA to $\Delta^1$-pyrroline-5-carboxylate (P5C) is spontaneous. Finally, P5CR reduces P5C to produce proline and $NADP^+$. The three genes *proA*, *B* and *C* encoding the enzymes GPR, GK and P5CR respectively have been cloned from *E.coli* and characterised (Hayzer and Moses, 1978; Hayzer and Leisinger, 1980).

### 1.5.2 The Ornithine Pathway of Proline Biosynthesis

Proline can also be synthesised from ornithine which links it to the metabolism of arginine. *In vivo* labelling experiments suggest that the synthesis from ornithine may occur via two routes (Figure 1.2). The first involves the transamination of the δ-amino group by the enzyme Ornithine aminotransferase (OAT) (EC 2.6.1.13), resulting in the production of GSA which also cyclises to P5C and is subsequently converted to proline by P5CR (Figures 1.1 and 1.2) (Adams and Frank, 1980; Stewart, 1981). Alternatively the α-amino group of ornithine may be transaminated, resulting in the production of α-keto-δ-aminovalerate which spontaneously cyclises to $\Delta^1$-pyrroline-2-carboxylate (P2C), which is then reduced to proline (Mestichelli *et al*, 1979; Chiang and Dandekar, 1995). A number of OAT cDNA clones believed to be δ-OATs have now been identified and characterised, whilst the enzyme involved in the production of P2C has yet to be isolated. The contribution of this latter pathway therefore remains unknown .

The activity of δ-OAT has been well characterised in the gram positive bacterium *Bacillus subtilis*, where δ-OAT is considered part of the arginine catabolism pathway

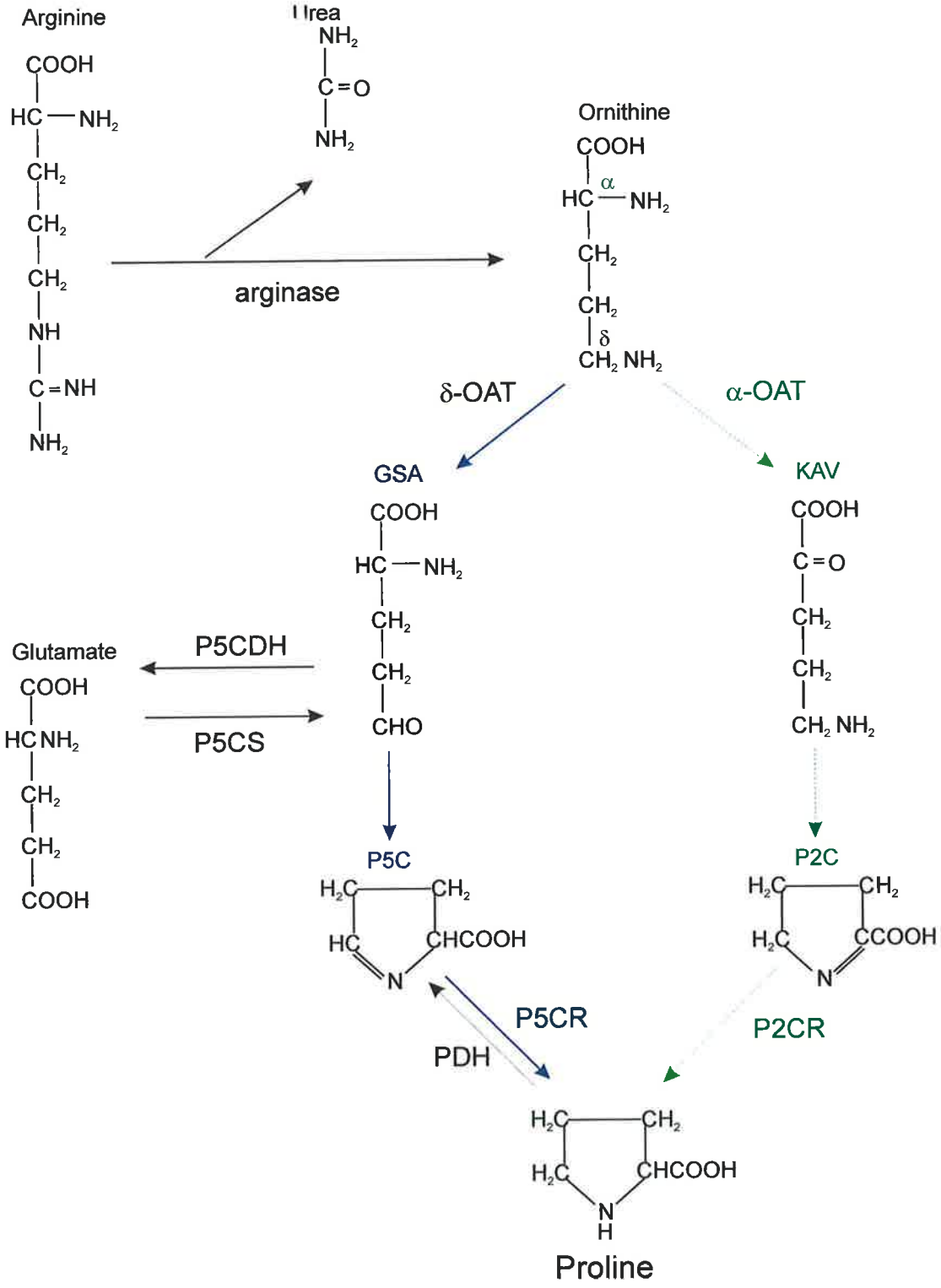



**Figure 1.2.** Pathways of proline sythesis from ornithine. δ-OAT, δ-ornithine aminotransferase; α-OAT, α-ornithine aminotransferase; GSA, glutamate semialdehyde; KAV, α-keto-δ-aminovalerate; P5C, $\Delta^1$-pyrroline-5-carboxylate; P2C, $\Delta^1$-pyrroline-2-carboxylate; P5CR, $\Delta^1$-pyrroline-5-carboxylate reductase; P2CR, $\Delta^1$-pyrroline-2-carboxylate reductase; P5CS, $\Delta^1$-pyrroline-5-carboxylate synthetase; P5CDH, $\Delta^1$-pyrroline-5-carboxylate dehydrogenase.

(Harwood and Baumberg, 1977). The enzyme catalyses transamination of ornithine to α-ketoglutarate, producing glutamate and GSA, the latter spontaneously converting to P5C (Yasuda *et al.*, 1981). The synthesis of δ-OAT has been shown to be regulated in *B.subtilis* in response to varying levels of proline, arginine and ornithine (Baumberg and Harwood, 1977) and the enzyme was also shown to be irreversibly inhibited by gabaculine (GAB, 3-amino-2,3-dihydrobenzoic acid), a suicide substrate (Takechi *et al.*, 1994). Genetic studies *of B. subtilis* have revealed that several arginine catabolising enzymes are encoded in the *rocDEF* operon, including δ-OAT which is encoded by *rocD* (Gardan *et al.*, 1995). A thermostable δ-OAT which can utilise D-ornithine as a substrate has recently been characterised from *Bacillus* sp. YM-2, and has been shown to be most active at $70^0$C (Jhee *et al.*, 1995).

### 1.5.3 Proline Degradation

In bacteria, a single bifunctional enzyme, proline dehydrogenase (PutA), catabolises proline to glutamate (Wood, 1987; DeSpicer and Maloy, 1993). However, the actual enzymatic mechanism behind the conversion was not clearly defined until the fully functional enzyme was purified from *E.coli* (Brown and Wood, 1992). The characterisation of PutA was hampered by the complex nature of its interaction with the inner surface of the bacterial cytoplasmic membrane and its apparent ability to bind DNA. As suggested by earlier genetic studies, the PutA protein has both proline dehydrogenase (EC 1.5.99.8) and P5C dehydrogenase (EC 1.5.1.12) activities, it also acts as its own transcriptional repressor as it disassociates from the membrane and binds

to regulatory sequences in the bacterial genome upstream of the *put* (proline utilisation) operon (Menzel and Roth, 1981; Brown and Wood, 1992).

## 1.6 Proline Metabolism in Higher Plants

Elucidation of the proline biosynthetic pathways in higher plants has proven to be more difficult than in prokaryotes. Evidence for the synthesis of proline from glutamate in plants was provided by the detection of labelled GSA in cell free extracts of sugar beet supplied with radioactively labelled glutamate (Morris *et al.*, 1969). However, efforts to isolate and measure the corresponding enzymatic activities in plant extracts was hampered by the activity of interfering enzymes such as glutamine synthetase (Miflin and Lea, 1977). The molecular cloning of a number of plant cDNAs and genes encoding the enzymes involved in proline biosynthesis has recently been achieved (Delauney and Verma, 1990; Williamson and Slocum, 1992; Hu *et al.*, 1992; Delauney *et al.*, 1993; Savouré *et al.*, 1995).

### 1.6.1 $\Delta^1$-Pyrroline-5-Carboxylate Reductase (P5CR)

Initial attempts to investigate the pathway leading to the production of proline from glutamate were focused on the characterisation of P5CR (Figure 1.1). P5CR has been purified from a variety of plant sources including; barley (*Hordeum vulgare*), tobacco (*Nicotiana* spp.) and *A.thaliana* (Rayapati, *et al.*, 1989, Szoke, 1992, Verbruggen *et al.*, 1993). Genes and/or cDNAs encoding P5CR have been cloned from soybean (*Glycine max*) (Delauney and Verma, 1990), pea (*Pisum sativum* L.) (Williamson and Slocum, 1992), and *A.thaliana* (Verbruggen *et al.*, 1993). Studies of pea, soybean and *A. thaliana* (Rayapati *et al.*, 1989, Williamson and Slocum, 1992; Verbruggen *et al.*, 1993) have

suggested that P5CR may be present in both cytoplasmic and chloroplastic forms, however the subcellular location of proline synthesis in plants is still not clearly established.

In *A. thaliana,* the P5CR mRNA appears to be most abundant in roots and ripening seeds under normal conditions (Verbruggen *et al.*, 1993). Transcription from the *A.thaliana* P5CR gene promoter was measured by creation of a gene fusion with the reporter gene GUS (β-glucuronidase) and was found to most active in root tips, shoot meristems, guard cells, hydathodes, pollen grains, ovules and developing seeds, all of which are rapidly dividing cells or cells undergoing changes in osmotic potential (Hua *et al.*, 1997). The authors suggested that this expression pattern was consist with free proline being a source of nitrogen, carbon and/or energy and an osmoticum (Hua *et al.*, 1997).

The level of P5CR activity and mRNA has also been examined in a number of systems where application of an abiotic stress, such as high salt conditions, leads to enhanced production and accumulation of proline. The level of P5CR transcripts has been shown to be increased five fold in salt treated *A. thaliana* plants (Verbruggen *et al.*, 1993). A similar result was obtained in nodules of osmotically stressed soybean plants (Delauney and Verma 1990). However, Yoshiba *et al.* (1995) were unable to detect significant accumulation of P5CR mRNA in salt, ABA or heat treated *A. thaliana* plants. Moreover, transgenic tobacco plants, expressing enhanced levels of P5CR driven by the constitutive CaMV35S gene promoter, did not contain higher concentrations of proline than control plants (Szoke *et al.*, 1992). Current opinion is, therefore, that P5CR is not the rate-limiting step of proline synthesis in plants.

### 1.6.2 $\Delta^1$-Pyrroline-5-Carboxylate Synthetase (P5CS)

As mentioned in a previous section, the first two steps of proline synthesis from glutamate in *E.coli* are catalysed by two separate enzymes; GK and GPR. Attempts to isolate *Vigna aconitifolia* (mothbean) cDNA clones encoding these enzymes by complementation of *E.coli proA*, *proB* and *proAB* auxotrophic mutants revealed, however, that GK and GPR exist in *V.aconitifolia* in the form of a single bifunctional enzyme, $\Delta^1$-pyrroline-5-carboxylate synthetase (P5CS) (EC number not assigned) (Hu *et al.*, 1992) (Figures 1.1 and 1.3). In the same study it was also observed that P5CS transcript levels increased in the roots of salt treated *V.aconitifolia* plants, coincident with proline accumulation, and the authors suggested that it was likely that the primary control of proline synthesis in plants was exercised at the P5CS step (Hu *et al.*, 1992). Biochemical analysis of the purified *V.aconitifolia* P5CS, using the γ-glutamyl hydroxamate assay method of Hayzer and Leisinger (1980) have shown the GK enzyme activity is feedback inhibited by proline, although it is significantly less sensitive to feedback inhibition than its *E.coli* homologue (Hu *et al.*, 1992, Zhang *et al.*, 1995). Further enzymatic studies also suggested that the GPR activity of *V.aconitifolia* P5CS is insensitive to proline (Zhang *et al.*, 1995).

After the commencement of the work presented in this thesis, cDNAs encoding P5CS have been cloned from cDNA libraries prepared from rice (*Orizya sativa*) (Igarashi *et al.*, 1997), tomato (*Lycopersicum esculentum*) (Garcia Rios *et al.*, 1997), *A. thaliana* (Yoshiba *et al.*, 1995; Strizhov *et al.*, 1997), alfalfa (*Medicago trunculata*) (Ginzberg *et al.*, 1998) and kiwi fruit (*Actinidia deliciosa*) (Walton *et al.*, 1997). Comparisons of the

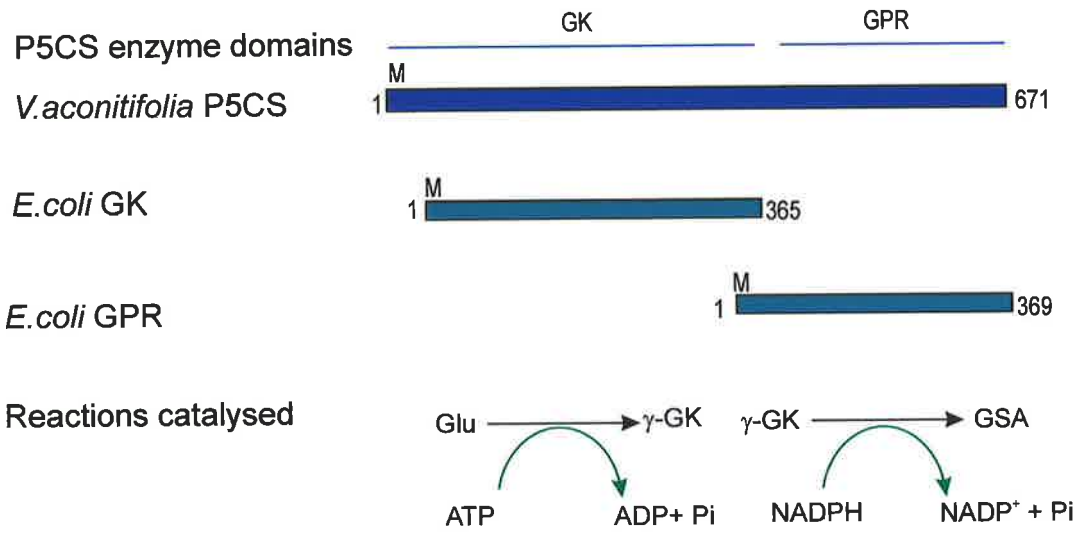



**Figure 1.3.** P5CS is a bifunctional enzyme. The *E.coli* enzymes GK (γ-glutamyl kinase) and GPR (γ-glutamyl phosphate reductase) exist as a single bifunctional enzyme (P5CS; $\Delta^1$-pyrroline-5-carboxylate synthetase) in *V.aconitifolia* (from Zhang *et al.*, 1995).

deduced amino acid sequence for all of these P5CS cDNA clones with *E.coli* GK and GPR show that the plant bifunctional enzyme displays significant homology with both of the bacterial enzymes when they are aligned at the N and C termini respectively. Early work suggested that P5CS was encoded by a single copy gene in *A.thaliana* (Savouré *et al.*, 1995, Yoshiba *et al*, 1995). However, subsequent work demonstrated the presence of two distinct P5CS genes in *A.thaliana* (Strizhov *et al.*, 1997). Multiple P5CS genes have since been reported to be also present in alfalfa (*M.trunculata*) (Ginzberg *et al.*, 1998) and tomato (*L.esculentum*) (Garcia Rios *et al.*, 1997; Fujita *et al.*, 1998).

The role of P5CS in the accumulation of proline during the osmotic stress response has now been relatively well characterised (as described below). Recent attention has turned, therefore, to its activity in developing plant organs. This aspect of P5CS gene expression and its contribution to proline metabolism appears to be quite complex. Analysis of P5CS expression in tomato showed that the steady-state mRNA level for the two P5CS genes in tomato, *tomPRO1* and *tomPRO2,* was not detectable and only slightly induced respectively, in response to salt stress, even though proline accumulated to high levels in the treated plants (Fujita *et al.*, 1998). Furthermore, in the same study, the steady-state level of *tomPRO2* mRNA was examined in several tomato tissues and found to be lowest in pollen, the tissue which contained the highest amount of proline. This lack of correlation between proline levels and P5CS gene expression was not able to be explained, although the authors concluded that transcriptional control of P5CS genes was not important for the osmotic stress or pollen specific regulation of proline synthesis in tomato (Fujita *et al.*, 1998). In contrast, the glutamate pathway of proline

biosynthesis and transcriptional activation of P5CS gene expression was found to play a significant role in the accumulation of proline in kiwifruit buds during bud-break (Walton *et al.*, 1997). Hence, the mechanisms regulating proline accumulation during normal plant development are still not well understood.

### 1.6.3 P5CS, ABA and the Abiotic Stress Response

The regulation of P5CS mRNA levels in response to factors such as salt, dehydration, temperature and abscisic acid (ABA) have been investigated in *A. thaliana* (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995; Savouré *et al.*, 1997) and *V.aconitifolia* (Hu *et al.*, 1992, Zhang *et al.*, 1995). In summary, the levels of P5CS mRNA are rapidly increased when *V.aconitifolia* is exposed to salt and when *A.thaliana* is subjected to dehydration and treatment with ABA. Heat treatments did not affect transcript levels in *A.thaliana*, however some induction was observed after 24 hours at 4°C (Yoshiba *et al.*, 1995). The hypotheses that P5CS is the key step in the regulation of proline synthesis in the plant cell, and that it plays a major role in the plant osmotic stress response were supported by the demonstration that transgenic tobacco plants which expressed high levels of *V.aconitifolia* P5CS accumulated 10-18 times more proline and displayed enhanced root biomass and flower development under osmotic stress conditions compared to control plants (Kishor *et al.*, 1995). The increased proline levels thus conferred some degree of osmotic protection to the plants. Similarly, the major differences between a salt tolerant and a salt sensitive rice cultivar have been shown to be higher levels of P5CS transcripts and proline accumulation in the salt-tolerant cultivar upon exposure to salt stress (Igarashi *et al.*, 1995).

As mentioned above, the levels of P5CS mRNA have been shown to increase rapidly in *A.thaliana* treated with exogenous ABA (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995; Savouré *et al.*, 1997). This suggested that ABA could be a signal mediating abiotic stress and proline accumulation. The relationship between ABA and P5CS gene expression, however, is not clear. More recent work suggests that P5CS gene expression can be controlled by both ABA dependent and independent pathways (Ishitani *et al.*, 1997). The tools to investigate the interaction between ABA, proline and osmotic stress at a fundamental level are now available, however, the relationships are proving to be more complex than initially suspected.

### 1.6.4 δ-Ornithine Aminotransferase

There are contrasting opinions in the literature as to whether the reaction catalysed by OAT in plants occurs via transamination of the α-carbon group or the δ-carbon group of ornithine (Figure 1.2). An *in vivo* radiolabelling study carried out by Mestichelli *et al.* (1979) is often cited as evidence for existence of the α-ornithine aminotransferase. This reaction would involve the transamination of the α-amino group of ornithine resulting in the production of α-keto-δ-aminovalerate which would then spontaneously cyclise to $\Delta^1$-pyrroline-2-carboxylate (P2C). P2C would be subsequently reduced to proline by the action of a putative P2C reductase (P2CR). More recent papers describing the molecular cloning of OAT cDNAs from *V.aconitifolia* and *A.thaliana* have both suggested that the clones encode δ-OATs on the basis of their deduced amino acid sequences which show a high degree of homology to other enzymes which catalyse ω-transaminations (Delauney *et al.*, 1993; Roosens *et al.*, 1998). The ω-transaminases are a group of

enzymes which catalyse the transfer of an amino group from a carbon atom that does not carry a carboxylate group, i.e. the δ-carbon in ornithine (Ohura *et al.*, 1982; Shen *et al.*, 1998). Enzymes belonging to this group include *N*-acetylornithine aminotransferase (EC 2.6.1.11), ω-amino acid pyruvate aminotransferase (EC 2.6.1.18), 4-aminobutyrate aminotransferase (EC 2.1.6.19) and mammalian and bacterial δ-OATs. The designation of OATs as δ-OATs is based on several lines of investigation, as follows: firstly, the stereospecific removal of a labelled hydrogen from the δ-carbon group of ornithine has been demonstrated for OATs from rat liver and *Bacillus* sp. (Williams *et al.*, 1982; Jhee *et al.*, 1996). Further, it was proposed that the α-carbon group is likely to be the site where the substrate binds to the enzyme, hence rendering this group unavailable for transamination (Jhee *et al.*, 1995, 1996). Secondly, all OAT sequences reported thus far, on the basis of amino acid homology, belong to a class of pyridoxal phosphate dependent aminotransferases that are distinct from all known α-aminotransferases. The enzymes within the group all contain a consensus amino acid sequence surrounding the putative pyridoxal phosphate binding site and they also share overall structural similarity (Mehta *et al.*, 1993 and PROSITE from GCG, Madison, Wisconsin, USA). Furthermore, the crystallisation of a human OAT has identified several important structural characteristics which are consistent with its classification as a δ-OAT. These include the identification of the active site residues which interact with the substrates and also create the actual overall structure and charge of the active site itself (Shen *et al.*, 1998). Finally, the cloning of the *V.aconitifolia* OAT was carried out by trans-complementation of an *E.coli* proline auxotroph which indicates that the product it forms is P5C, as the enzyme P5CR would then convert this to proline. If the product of

the reaction catalysed by OAT was P2C the mutant would be unable to produce proline, as no P2CR has yet been identified in *E.coli* (Delauney *et al.*, 1993). In conclusion, there is considerable correlative evidence to suggest that the P5C pathway, via δ-OAT is that which most likely exists in plants, as in mammals and microorganisms, although this remains to be conclusively shown.

The physiological significance of proline production from ornithine in plants is not well understood, although δ-OAT has been well characterised in other eukaryotes including fungi (Scher and Vogel, 1957; Vogel and Kopac, 1960) and mammals (Strecker, 1960; Meuckler and Pitot, 1985; Inaga *et al.*, 1986). The function of δ-OAT in plants has been mainly studied through the use of inhibitors, such as GAB and AHA (4-amino-5-hexynoic acid). Both GAB and AHA inhibit δ-OAT activity by catalysis-dependent formation of a stable covalent adduct with a pyridoxal-phosphate cofactor. *In vitro* studies using radish cotyledons revealed that AHA and GAB inhibition of δ-OAT was dose dependent and irreversible (Hervieu *et al.*,1993).

Although δ-OAT cDNAs had been cloned relatively early on from mammals (Mitchell *et al.*, 1988) and the yeast *S. cerevisiae* (Brandriss, 1979), the only plant δ-OAT cDNA, until recently, was that isolated from a *V.aconitifolia* nodule cDNA expression library by direct complementation of *E.coli proBA* mutants growing on ornithine as the sole carbon source (Delauney *et al.*, 1993). In contrast to P5CS, δ-OAT mRNA levels were shown to be reduced in response to salt stress, and increased in *V.aconitifolia* plants exposed to excess levels of nitrogen. Since the beinning of the work presented in this thesis, the activity and gene expression of an *A.thaliana* δ-OAT has also been examined

(Roosens *et al.*, 1998). Under normal growth conditions δ-OAT activity and mRNA levels were higher in young plantlets than in mature plants. The mRNA was essentially undetectable by northern hybridisation in mature plants. Both OAT activity and mRNA levels appeared to rise in salt stressed 12 day old plantlets (but not four week old plants), and therefore it is possible that δ-OAT contributes to salt stress related proline synthesis in young plants. However the authors suggested that the prime role of δ-OAT in plants is probably involved with the recycling of glutamate under high nitrogen conditions.

The yeast δ-OAT is a cytosolic enzyme, however human δ-OAT has an amino terminal transit sequence which directs it to the mitochondria (Inaga *et al.*, 1986). Based on the high degree of sequence similarity with human δ-OAT and the presence of a putative amino terminal transit sequence, the *V.aconitifolia* and *A.thaliana* enzymes may also be located in the mitochondria (Delauney *et al.*, 1993; Roosens *et al.*, 1998).

### 1.6.5 Proline Degradation

Early experiments using radioactive amino acids showed that certain plant tissues were capable of converting proline to glutamate in an oxidative process (McNamer and Stewart, 1973: Stewart and Boggess, 1978). In plants, as in yeast, there are two separate enzymes (Brandriss and Magasanik, 1979). The first enzyme, which converts proline to P5C in plant mitochondria, is erroneously referred to in the literature as proline oxidase (POX) and is more correctly termed proline dehydrogenase (PDH) (Figure 1.1) (Huang and Cavalieri, 1979, Rayapati and Stewart, 1991). In yeast, the proline oxidase gene is known as *PUT1* (proline utilisation gene) and its product was found to be localised in

the inner matrix of the mitochondria (Brandriss and Magasanik, 1979). The second enzyme, which converts P5C to glutamate is P5C dehydrogenase (P5CDH) (Figure 1.1) (Forlani *et al.*, 1997).

Most studies concerning proline accumulation in plants have focused on the proline biosynthetic enzymes. Increasingly, evidence is showing, however, that the coordinate regulation of the synthesis pathways together with the proline degradation pathway is an important factor in determining final proline concentrations in the cell. Studies of water-stressed barley leaves lead to the first indication that a decrease in proline oxidation may contribute to proline accumulation during osmotic stress (Stewart and Boggess, 1978). Subsequent to the start of research described in this thesis, the first plant cDNA clones encoding PDH were isolated from *A.thaliana* (Kiyosue, *et al*,. 1996; Peng *et al.*, 1996). The clones were used to functionally complement *put*1 yeast mutants to verify their identity, and mitochondrial location. It was demonstrated that the PDH gene was induced by high proline levels, but this induction was inhibited in salt stressed plants. The levels of PDH transcript were also shown to increase significantly in plants recovering after the removal of osmotic stress, coincident with a decrease in P5CS transcript levels. P5CS and PDH were thus shown to be subject to reciprocal regulation, with negative regulation of the PDH gene under osmotic stress conditions (Kiyosue, *et al.*, 1996; Peng *et al.*, 1996; Nakashima *et al.*, 1998). However, PDH mRNA levels were found to be highest in developing *A.thaliana* tissues (pollen, pistils and seeds) which also contained the highest concentrations of proline. It was suggested that the proline was being utilised as a source of energy and nutrients for developing seeds and pollen (Nakashima *et al.*, 1998).

### 1.6.6 Regulation of Flux Through Pathways of Proline Metabolism

The net accumulation of proline in plant tissues occurs as a result of the balance between synthesis and degradation, and incorporation of proline into proteins. Both the glutamate and ornithine based pathways appear to contribute to proline synthesis in plants. The relative levels of δ-OAT and P5CS transcript abundance suggest that the ornithine pathway may play a subsidiary role, although this remains to be confirmed by comparisons at the level of enzyme activity. The major contributing avenue of proline synthesis at a specific time is likely to be influenced by factors such as tissue identity, developmental stage and overall physiological status of the plant. Mechanisms through which one pathway may become dominant over another and how flux between them is controlled remains uncertain. Moreover, the factors affecting P5CS and PDH expression and activity and how the synthesis and degradation pathways are balanced to regulate proline levels during plant growth and development are also still relatively unknown.

## *1.7 Aims of this Study*

The metabolic changes in grape berry composition occurring during development are not well understood at a biochemical or molecular level. It is unclear what functional role free amino acids, and especially proline, have in developmental processes. It is not known whether the high proline levels found in the berries of certain grape cultivars are merely consequential or have a specific function in berry ripening. This study concentrates specifically on the nature and regulation of proline metabolism in *V. vinifera*. Two proline biosynthetic enzymes, $\Delta^1$-pyrroline-5-carboxylate synthetase (P5CS) and δ-ornithine aminotransferase (δ-OAT) are characterised. Other aspects

which could be involved in influencing free proline levels in berry such as proline degradation and changes in metabolic demand for the amino acid are also investigated.

Specifically, in Chapter 2, the pattern of free amino acid accumulation throughout berry development will be examined in four different *V.vinifera* cultivars. In Chapter 3, the cloning and characterisation of cDNAs encoding the two key proline biosynthetic enzymes δ-OAT and P5CS from *V.vinifera* will be reported. In Chapter 4, the tools obtained from the work described in the previous chapter will be used to characterise the gene expression of the *V.vinifera* P5CS during berry development. In Chapter 5, the contribution of δ-OAT to proline synthesis in the berry is examined at a molecular level. Finally, in Chapter 6, other aspects of proline metabolism are also examined, specifically the contributions of proline degradation and protein accumulation to the net accumulation of free proline in the berry.

# Chapter 2
## *Accumulation of Free Amino Acids in Developing Grape Berries of V.vinifera*

## 2.1 Introduction

The ripening of grape berries is non-climacteric and their development is characterised by three distinct growth phases. Two phases of rapid growth, separated by a lag phase of little or no growth, create a double-sigmoid pattern of development (Coombe, 1976). Stage I, which commences immediately postflowering, is characterised by high rates of metabolism and acid accumulation, accompanied by rapid cell division and cell enlargement (Kliewer, 1965; Considine and Knox, 1979). Stage II, the period of little or no growth, occurs approximately 7-10 weeks postflowering (Coombe, 1973). Stage III involves cellular expansion, rapid accumulation of sugars, amino acids, colour and flavour compounds (see Kanellis and Roubelakis-Angelakis, 1993 for review). The transition from stage II to III is termed veraison, and marks the beginning of ripening.

A significant event during berry development is the accumulation of free amino acids, such that they can represent up to 90% of the final nitrogen content in mature berries (Kliewer, 1968,1969,1970). Free amino acid accumulation, in addition to protein synthesis, leads to an overall increase in organic nitrogen during berry development. In contrast, ammonia levels, which are relatively high in immature fruit, steadily decline during maturation (Lafon-Lafourcade and Guimberteau, 1962). The ammonia is assimilated into amino acids through the activities of glutamine synthetase (GS) (EC 6.3.1.2) and glutamate dehydrogenase (GDH) (EC 1.4.1.3) both of which have been detected in extracts of berry tissue (Ghisi *et al.*, 1984; Kanellis and Roubelakis-Angelakis, 1993).

Early studies examining the free amino acid content of mature berries identified nine amino acids (proline, arginine, glutamic acid, alanine, serine, threonine, aspartic acid, valine and γ-amino butyric acid) which accounted for 90% or more of the total free amino acid pool (Castor, 1953; Kliewer, 1968,1969,1970). Significant differences in amino acid profiles between cultivars were observed. However, in almost all varieties, proline, and to a lesser extent, arginine were found in the largest quantities. The proline:arginine ratio varied dramatically between cultivars. Many of the early comparisons between cultivars were made on berries harvested at different stages of maturity and from a range of cultural and climatic conditions. However later studies demonstrated that some of these factors, berry maturity in particular, strongly influence free amino acid content and composition confounding any comparisons made between different cultivars. Very few of the published studies to date have compared different cultivars grown at the same site, during the same season and harvested at the same level of maturity. Such a study was recently carried out on five cultivars in collaboration with the author of this thesis (Grubb, 1997). Whilst the actual amounts of amino acids in berry extracts varied from previous studies, exceptionally high proline concentrations were only found in berries from those cultivars previously reported to have this characteristic (Kliewer, 1970; Huang and Ough, 1991; Spayd and Andersen-Bagge, 1996). This suggests that the basic pattern of amino acid accumulation is determined by genetic factors with environmental and cultural factors only having a modifying effect.

The role of the significant accumulation of free amino acids in the developing berry is unknown. Moreover, the physiological processes leading to very high levels of some of these amino acids in the berries of some grape cultivars are also unexplained. High

levels of free arginine can be found in plant seeds, where it acts as a nitrogen store, released upon germination (Van Etten *et al.*, 1967, Zonia, 1995). High levels of free proline are observed in some reproductive tissues of a number of plants, suggesting that this amino acid may have a role in the development or function of these tissues. For example, the catabolism of stored proline may provide a convenient source of nitrogen and energy for their development.

The temporal patterns of free amino acid accumulation during grape berry development have not been well described, with most studies carried out on mature berries or juice derived therefrom. The analyses described in this chapter document the changes that occur in the free amino acid pool from flowering to maturity for four different *V.vinifera* cultivars. In addition, the amino acid composition of a range of grapevine tissues is presented in order to determine if the accumulation of significant concentrations of proline and arginine is a process unique to the fruit of the grapevine and whether it occurs to a similar extent in all berry components.

## 2.2 Methods

### 2.2.1 Fruit and tissue sampling

Fruit for developmental studies was harvested from 30 *V.vinifera* cv. Chardonnay vines (C.A. Henschke and Co. vineyard, Lenswood, South Australia), 16 cv. Cabernet Sauvignon vines (Waite variety block, Adelaide, South Australia). 32 cv. Muscat Gordo Blanco vines and 10 cv. Gewurztraminer vines (Alverstoke vineyard, Adelaide, South Australia). Sampling of synchronously developing bunches of fruit was ensured by identifying and tagging them 2 weeks postflowering. Ten 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 50 berry subsample was used to determine average berry weight, deformability, juice pH and $^{0}$Brix. Deformability was measured as described previously by Coombe and Bishop (1980). $^{0}$Brix was determined with a digital refractometer (Erma). The remaining berries were stored at -70°C prior to further analyses. Berries were dissected into skin, seeds and pulp whilst still frozen and the skin and seeds rinsed with distilled water. Flower buds (two weeks preflowering), flowers (at anthesis), and leaves were collected from the same cv. Chardonnay vines from which the fruit was obtained. Roots were harvested from glasshouse grown cv. Chardonnay vines.

### 2.2.2 Amino Acid Analysis

All tissues were weighed and frozen in liquid nitrogen before being ground to a fine powder with either a mortar and pestle or a coffee grinder. For whole berry analyses,

each sample consisted of 25 powdered berries, from which a 0.5 g subsample was taken. For berry tissue analyses (pulp, skin and seed) 6 berries were dissected, pulp represented up to 75-80% of the berry weight, skin represented 10-15% and seeds up to 5% of the berry weight. Subsamples of approximately 0.1-0.75 g of powdered tissue, depending on tissue type, were used in the analysis of other grapevine tissues. Free amino acids were extracted from powdered tissue in 1 ml of 12:5:3 (v/v/v) methanol:chloroform:water and mixed on a rotating wheel at room temperature for 20 min, using a modification of the method of Bieleski and Turner (1966). After centrifugation at 12000 *g* for 5 min, the supernatant was diluted 1:5 (v/v) with 0.25 M borate buffer pH 8.5, then derivatised with FMOC-Cl (9-fluorenylmethylchloroformate) before separation on a reverse-phase $C_{18}$ column (Hypersil 150x 4.6 mm ID) using a GBC HPLC (according to the manufacturer's instructions). Data were analysed using the WinChrom™ 1.2 chromatography management system.

## 2.3 Results

### 2.3.1 Free amino acid composition of the ripe fruit of four *V. vinifera* cultivars

Analysis of free amino acids in the ripe fruit of four different cultivars of *V.vinifera* (Table 2.1) demonstrated notable differences in the concentrations of proline and other members of the glutamate family of amino acids (glutamine, glutamate, arginine). Consistent with previous studies, berries of all of the cultivars had concentrations of proline and arginine which were significantly higher than that of the other amino acids, and the proline:arginine ratios differed between cultivars (Lafon-Lafourcade and Guimberteau, 1962; Kliewer, 1968,1969,1970; Ough and Stashak, 1974). Very high levels of proline were found in both Chardonnay and Cabernet Sauvignon (16.03 μmoles/g fwt berry and 23.69 μmoles/g fwt berry respectively) and therefore these cultivars, in particular, have been used for later studies described in this thesis. Interestingly, the total free amino acid concentration (μmol/g fwt) for each cultivar was relatively similar (Table 2.1). The percentage of the total amino acid concentration represented by the sum of the proline and arginine concentrations ranged between 56 to 69% (Table 2.1).

### 2.3.2 Berry growth parameters

In order to determine the changes in free amino acid pools of the grape berry during development and, in particular, to determine when significant accumulation of proline takes place, the development of fruit from *V.vinifera* cvs Chardonnay, Cabernet Sauvignon, Gewurztraminer and Muscat Gordo was followed from flowering through to

**Table 2.1** Free proline is present at high concentrations in ripe fruit from several cultivars of *V.vinifera*. Amino acids were extracted from mature Chardonnay (25°Brix) (CH), Cabernet Sauvignon (25°Brix) (CS), Gewurztraminer (24°Brix) (GW) and Muscat Gordo (22°Brix) (MG) berries.

| amino acid(µmol/g fwt) | CH | CS | GW | MG |
|---|---|---|---|---|
| aspartic acid | 0.47 | 0.44 | 0.45 | 0.23 |
| glutamate | 1.48 | 0.44 | 0.76 | 1.12 |
| hydroxyproline | 0.09 | 0.31 | 0.16 | 0.14 |
| asparagine | 0.21 | 0.11 | 0.01 | 0.03 |
| glutamine | 1.21 | 1.35 | 2.47 | 2.27 |
| serine | 0.49 | 1.21 | 0.11 | 0.45 |
| histidine | 0.67 | 0.24 | 0.11 | 0.36 |
| glycine | 0.21 | 0.87 | 1.37 | 0.02 |
| threonine | 1.49 | 0.96 | 0.01 | 0.41 |
| alanine | 1.67 | 0.95 | 1.92 | 0.82 |
| GABA | 1.62 | 1.32 | 1.91 | 0.68 |
| proline | 16.03 | 23.69 | 10.66 | 6.34 |
| tyrosine | 0.56 | 0.57 | 0.46 | 0.15 |
| arginine | 2.11 | 2.14 | 8.97 | 8.01 |
| isoleucine | 0.71 | 0.64 | 0.28 | 0.14 |
| leucine | 0.26 | 0.34 | 0.11 | 0.18 |
| valine | 1.02 | 0.55 | 0.08 | 0.26 |
| methionine | 0.71 | 0.44 | 0.35 | 0.04 |
| phenylalanine | 0.83 | 0.29 | 0.08 | 0.01 |
| ornithine | 0.07 | 0.04 | 0.04 | 0.02 |
| lysine | 0.27 | 0.05 | 0.12 | 0.14 |
| total | 32.18 | 36.95 | 30.43 | 21.82 |
| %pro + arg | 56% | 69% | 64% | 68% |

full berry maturity. These cultivars were chosen because they represented both high and medium level proline accumulators. Measured parameters of berry weight, deformability, juice pH, and $^{0}$Brix are shown in Figures 2.1 to 2.4. The rapid increase in accumulation of soluble solids observed between eight and ten weeks postflowering for each variety indicates that veraison, which marks the beginning of fruit ripening, occurred at approximately nine weeks postflowering (Coombe, 1973).

### 2.3.3 Changes in concentrations of berry amino acids during development

Figures 2.5 to 2.8 and Tables 2.2, 2.3, 2.4 and 2.5 present data for the changes in the concentrations of the glutamate family of amino acids during berry development. All other amino acids remained at relatively low levels, similar to those reported in Table 2.1. The data presented illustrate that significant changes in the concentrations of the glutamate family of amino acids occur throughout fruit development in all of the cultivars of *V.vinifera* studied. In Chardonnay and Cabernet Sauvignon berries the concentration of free proline in particular changes dramatically, although only during the later stages of fruit ripening when its accumulation parallels the increasing sugar concentration. In these cultivars the concentration of free arginine increases slightly preveraison, then remains somewhat stable, while the concentration of glutamate, and glutamine in particular, both decrease throughout fruit development. In comparison, Gewurztraminer and Muscat Gordo berries also accumulate free proline post-veraison, although to a lesser extent. In these varieties, however, the free arginine concentration increases during ripening to levels similar to that of the free proline. Furthermore, in

contrast to the accumulation of proline, this accumulation of arginine appears to begin before veraison.

### 2.3.4 Amino acid content of other *V. vinifera* tissues

Free amino acids were extracted from skin, pulp[6] and seeds of *V.vinifera* cv. Chardonnay berries harvested at 16 weeks postflowering, flower buds, flowers, young and mature leaves. Comparison of the concentrations of free proline in the tissue samples (Figure 2.9 and Table 2.6), confirms the disproportionate accumulation of this amino acid within berry tissues, compared to other grapevine tissues. In berry pulp, the percentage of total amino acids present as proline, on a molar basis reached 59.4%. As pulp accounts for approximately 80% of the berry weight, the whole berry homogenate results presented previously (Figures 2.5-2.8 and Table 2.1), predominantly reflect the amino acid composition of berry pulp. Interestingly, the molar ratio of proline:arginine in mature berry pulp (8.25:1) was significantly different to that in berry skin (1.2:1). To investigate this further the ratio of proline to arginine in Chardonnay berries was determined for both pulp and skin throughout development (Figure 2.10 and Tables 2.7 and 2.8). In the berry pulp the proline:arginine ratio changes dramatically in the later stages of berry ripening due to a large increase in proline. In contrast the proline:arginine ratio in the skin tissue remains relatively constant throughout berry development with significant accumulation of both amino acids. Although berries subsampled for use in these analyses were the same as those used in analysis of whole berry homogenates (Figure 2.6 and Tables 2.1 and 2.3), the free amino acid concentrations obtained for skin and pulp vary from those obtained for the whole berry

[6] pulp refers to all the berry tissue remaining after the skin and seeds have been removed.

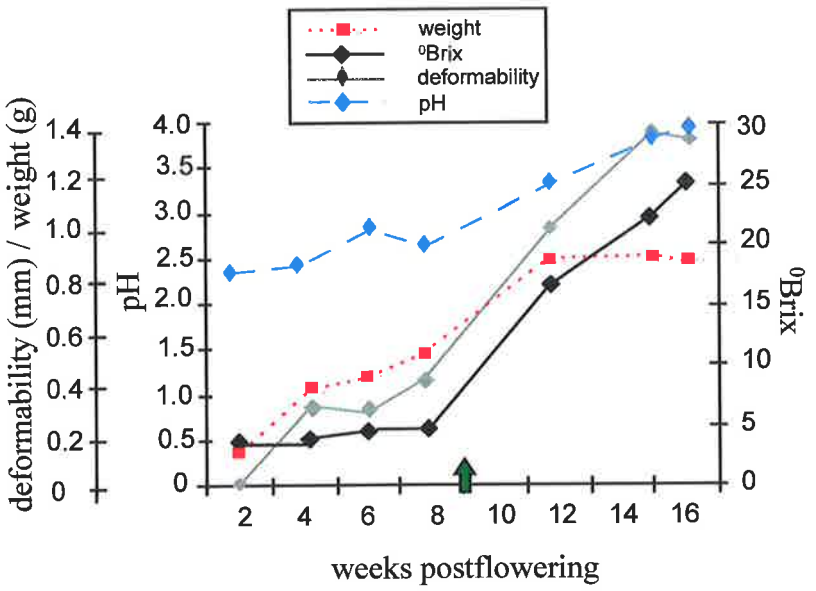



**Figure 2.1** Growth and maturity parameters for Cabernet Sauvignon berries harvested in the 1996/97 season. The arrow indicates time of veraison.

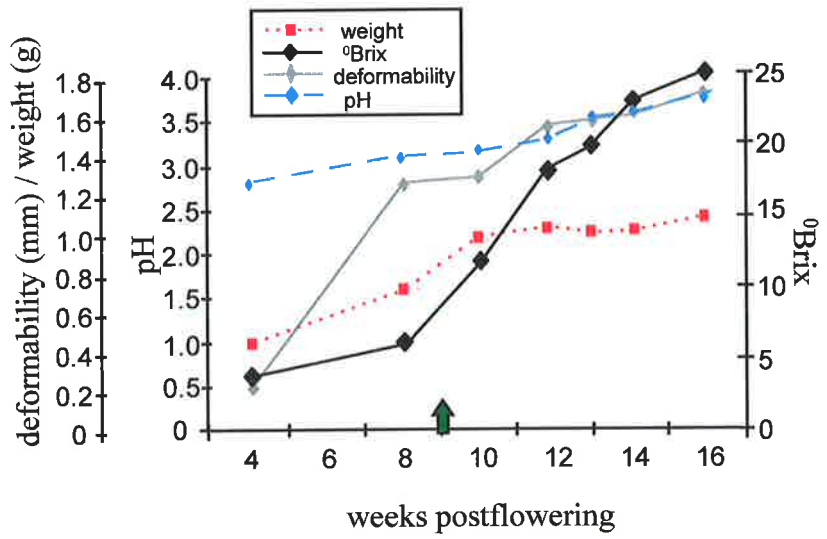



**Figure 2.2** Growth and maturity parameters for Chardonnay berries harvested in the 1996/97 season. The arrow indicates time of veraison.

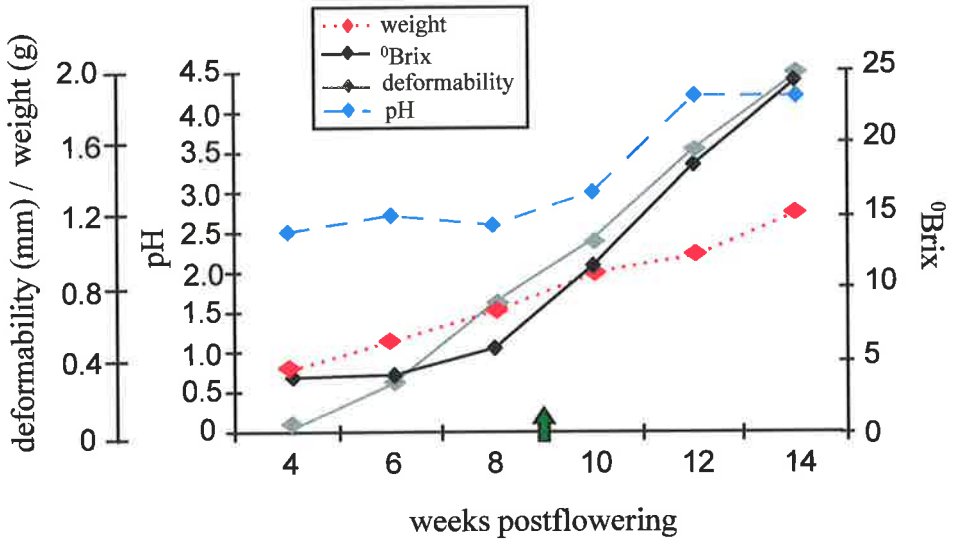



**Figure 2.3** Growth and maturity parameters for Gewurztraminer berries harvested in the 1996/97 season. The arrow indicates time of veraison.

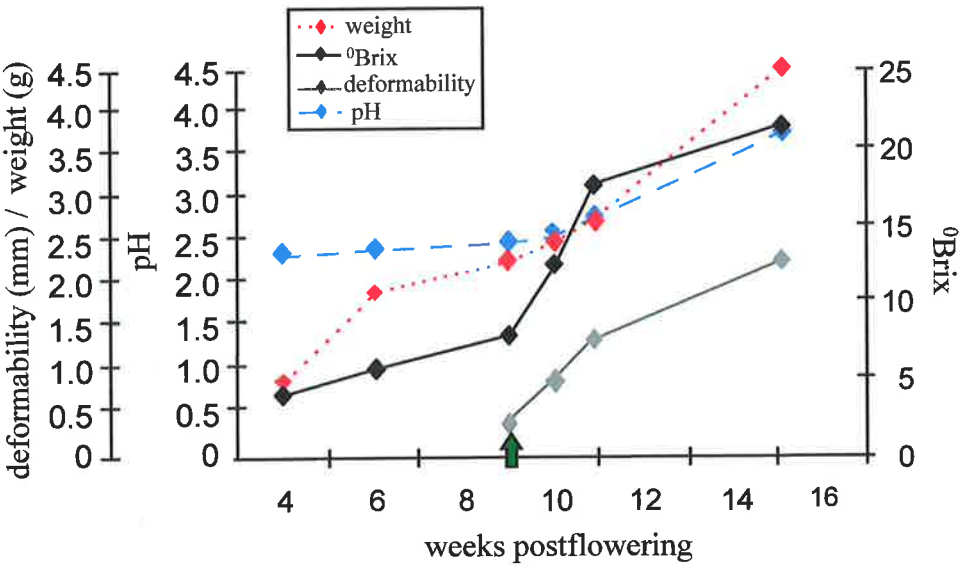



**Figure 2.4** Growth and maturity parameters for Muscat Gordo berries harvested in the 1995/96 season. The arrow indicates time of veraison.

**Table 2.2** Glutamate, glutamine, proline and arginine concentrations in whole berry homogenates of Cabernet Sauvignon at different stages of development.

| | amino acid (μmol/g fwt) | | | |
|---|---|---|---|---|
| [a]wpf | glutamate | glutamine | proline | arginine |
| 2 | 1.16 | 5.71 | 0.36 | 0.17 |
| 4 | 1.32 | 6.39 | 1.06 | 1.10 |
| 6 | 1.03 | 3.68 | 1.10 | 4.47 |
| 8 | 0.72 | 2.78 | 0.79 | 3.70 |
| 12 | 0.92 | 1.30 | 6.76 | 3.20 |
| 15 | 0.45 | 1.52 | 19.99 | 4.12 |
| 16 | 0.44 | 1.35 | 23.69 | 2.14 |

[a]wpf, weeks post flowering

**Table 2.3** Glutamate, glutamine, proline and arginine concentrations in whole berry homogenates of Chardonnay at different stages of development.

| | amino acid (μmol/g fwt) | | | |
|---|---|---|---|---|
| [a]wpf | glutamate | glutamine | proline | arginine |
| 4 | 1.20 | 7.19 | 0.43 | 1.50 |
| 8 | 0.98 | 5.61 | 1.09 | 3.77 |
| 10 | 0.66 | 3.97 | 3.30 | 2.80 |
| 12 | 0.43 | 2.75 | 5.36 | 2.37 |
| 13 | 0.40 | 1.77 | 8.00 | 2.57 |
| 14 | 0.39 | 1.78 | 12.60 | 3.03 |
| 16 | 1.48 | 1.21 | 16.03 | 2.11 |

[a]wpf, weeks post flowering.

**Table 2.4** Glutamate, glutamine, proline and arginine concentrations in whole berry homogenates of Gewurztraminer at different stages of development.

| | amino acid (µmol/g fwt) | | | |
|---|---|---|---|---|
| [a]wpf | glutamate | glutamine | proline | arginine |
| 4 | 1.22 | 4.99 | 0.47 | 0.24 |
| 6 | 1.81 | 4.57 | 0.41 | 1.70 |
| 8 | 1.06 | 2.53 | 0.47 | 2.14 |
| 10 | 0.66 | 2.10 | 1.07 | 3.66 |
| 12 | 0.89 | 2.65 | 7.79 | 8.12 |
| 14 | 0.76 | 2.47 | 10.66 | 8.97 |

[a]wpf, weeks post flowering

**Table 2.5** Glutamate, glutamine, proline and arginine concentrations in whole berry homogenates of Muscat Gordo at different stages of development.

| | amino acid (µmol/g fwt) | | | |
|---|---|---|---|---|
| [a]wpf | glutamate | glutamine | proline | arginine |
| 4 | 0.60 | 5.46 | 0.28 | 0.12 |
| 6 | 0.78 | 3.08 | 0.21 | 1.57 |
| 9 | 0.86 | 1.45 | 0.36 | 3.36 |
| 10 | 0.95 | 2.75 | 0.91 | 4.17 |
| 11 | 0.83 | 2.04 | 1.56 | 5.01 |
| 15 | 1.12 | 2.27 | 6.34 | 8.00 |

[a]wpf, weeks post flowering

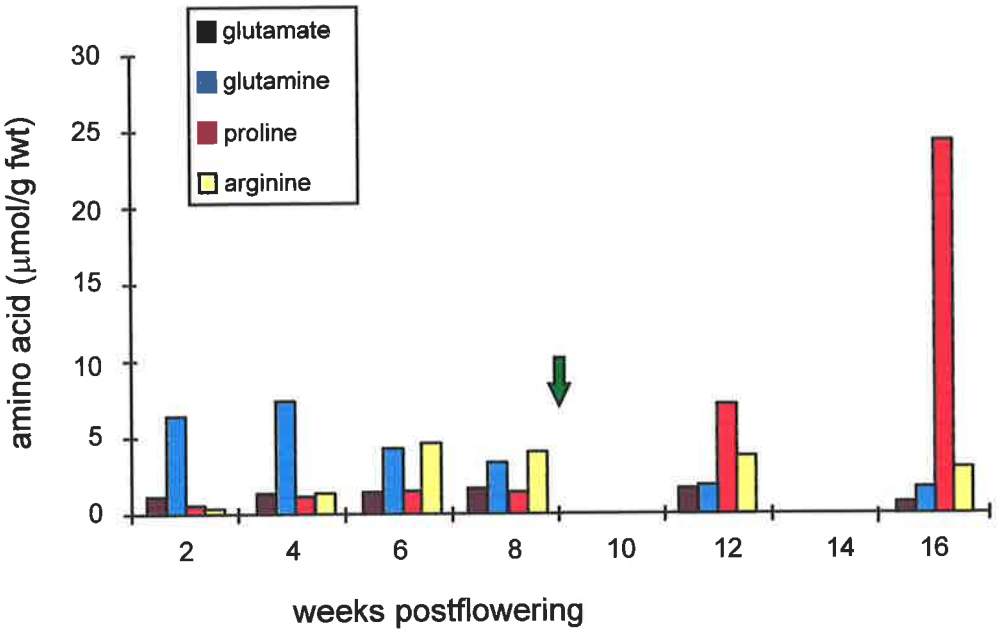



**Figure 2.5** Glutamine, glutamate, proline and arginine concentrations in whole berry homogenates of Cabernet Sauvignon. The arrow indicates time of veraison. (Note: to keep X axis linear, the data from 15 weeks postflowering has been omitted, see Table 2.2)

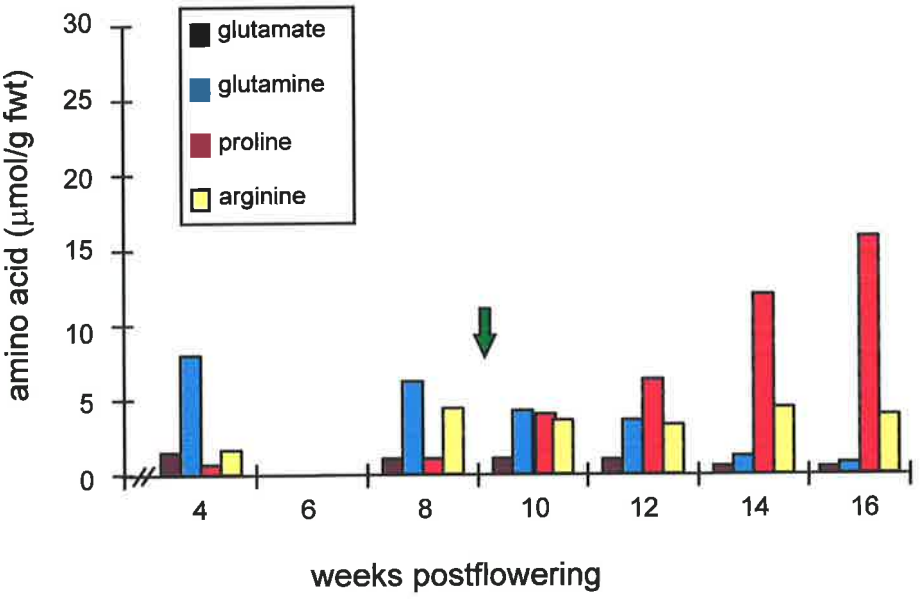



**Figure 2.6** Proline, arginine, glutamine and glutamate concentrations in whole berry homogenates of Chardonnay. The arrow indicates time of veraison. (Note: to keep X axis linear, the data from 13 weeks postflowering has been omitted, see Table 2.3)

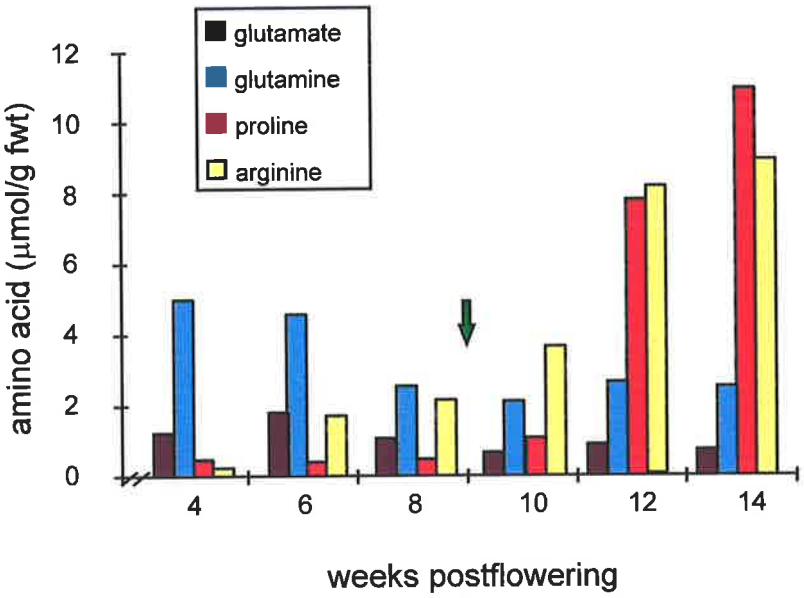



**Figure 2.7** Glutamate, glutamine, proline and arginine concentrations in whole berry homogenates of Gewurztraminer. The arrow indicates time of veraison.

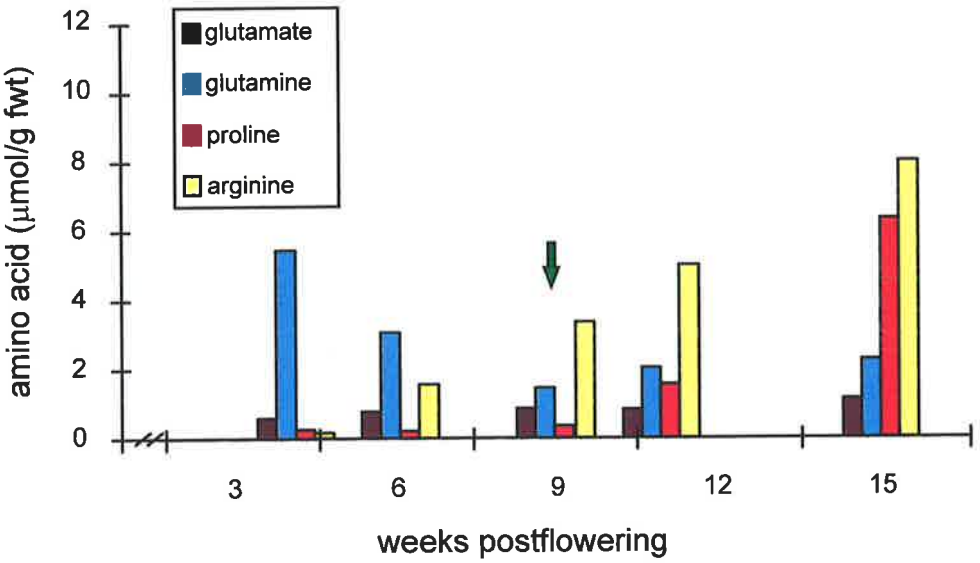



**Figure 2.8** Glutamine, glutamate, proline and arginine concentrations in whole berry homogenates of Muscat Gordo. The arrow indicates time of veraison. (Note: to keep X axis linear, the data from 11 weeks postflowering has been omitted, see Table 2.5)

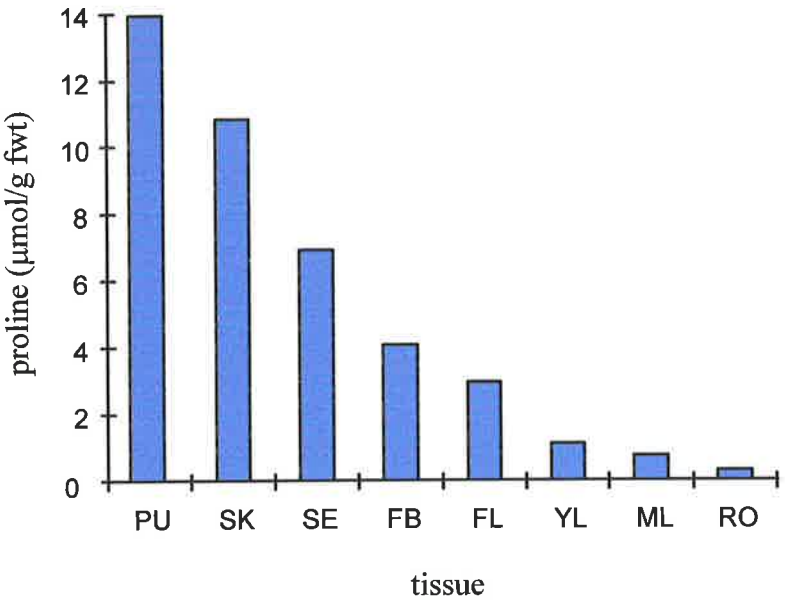



**Figure 2.9** Relative concentrations of free proline, on a per g fwt tissue basis, in a range of *V.vinifera* cv. Chardonnay tissues. Berry pulp (PU), berry skin (SK), berry seeds (SE), flower buds (FB), flowers (FL), young leaves (YL), mature leaves (ML) and roots (RO).

**Table 2.6** Proline,arginine, glutamate and glutamine concentrations in a range of *V.vinifera* cv. Chardonnay tissues.

| tissue | amino acid (µmol/g fwt) | | | | |
|---|---|---|---|---|---|
| | proline | arginine | glutamate | glutamine | % proline[a] |
| berry pulp | 14.06 | 1.70 | 0.26 | 1.60 | 59.4 |
| berry skin | 11.02 | 9.29 | 1.29 | 2.04 | 25 |
| berry seed | 6.94 | 3.41 | 5.04 | 4.05 | 15 |
| flower buds | 4.19 | 0.92 | 6.42 | 29.23 | 5.8 |
| flowers | 3.00 | 0.48 | 3.47 | 10.03 | 6.2 |
| young leaves | 1.31 | 1.43 | 5.20 | 7.17 | 3.5 |
| mature leaves | 0.88 | 0.31 | 4.05 | 0.96 | 4.6 |
| roots | 0.36 | 0.64 | 2.53 | 1.29 | 2.2 |

[a]The molar percentage of total free amino acids present as proline is indicated as %proline.

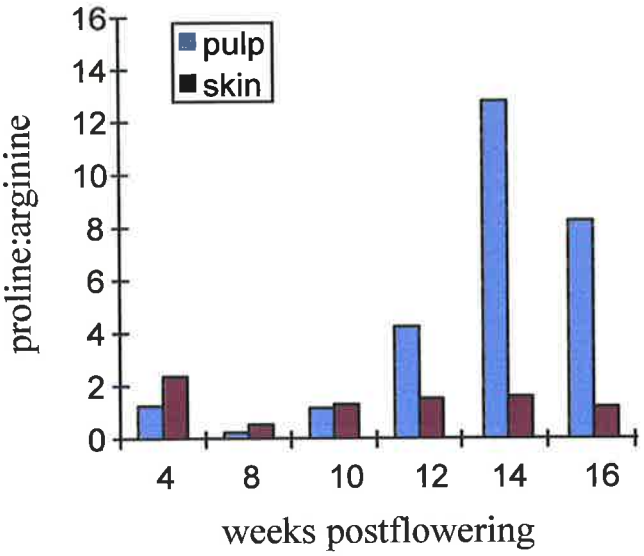



**Figure 2.10** The ratio of proline:arginine in the skin and pulp of *V.vinifera* cv. Chardonnay berries over development. The ratios were calculated based on the concentrations of proline and arginine (μmol/g fwt tissue).

**Table 2.7** Proline, arginine, glutamate and glutamine concentrations in *V.vinifera* cv.Chardonnay skin homogenates at different stages of development.

| skin | amino acid (μmol/g fwt) | | | |
|---|---|---|---|---|
| wpf[a] | proline | arginine | glutamate | glutamine |
| 4 | 0.43 | 0.18 | 1.22 | 7.40 |
| 8 | 1.15 | 2.17 | 1.26 | 4.81 |
| 10 | 7.80 | 5.91 | 0.71 | 3.25 |
| 12 | 5.70 | 3.74 | 0.85 | 2.20 |
| 14 | 7.69 | 4.80 | 1.20 | 1.47 |
| 16 | 11.02 | 9.29 | 1.29 | 2.04 |

[a]wpf, weeks postflowering. Note: analyses carried out on tissue extracted from six berries.

**Table 2.8** Proline, arginine, glutamate and glutamine concentrations in *V.vinifera* cv. Chardonnay pulp homogenates at different stages of development.

| pulp | amino acid (μmol/g fwt) | | | |
|---|---|---|---|---|
| wpf[a] | proline | arginine | glutamate | glutamine |
| 4 | 0.25 | 0.195 | 0.53 | 5.62 |
| 8 | 0.72 | 3.1 | 0.4 | 3.53 |
| 10 | 3.13 | 2.66 | 0.69 | 3.78 |
| 12 | 4.98 | 1.18 | 0.18 | 1.91 |
| 14 | 10.78 | 0.85 | 0.34 | 0.61 |
| 16 | 14.06 | 1.7 | 0.26 | 1.6 |

[a]wpf, weeks postflowering. Note: analyses carried out on tissue extracted from six berries.

homogenates at various stages of development. The subsample used for the skin and pulp analyses consisted of only six berries, compared with 25 berries for the whole berry homogenates. It is possible that such a small subsample produces results that are not truly representative of the whole sample.

## 2.4 Discussion

Analysis of the content of free amino acids in the ripe fruit of four different cultivars of *V.vinifera* demonstrated notable differences in the concentrations of proline and other members of the glutamate family of amino acids (glutamine, glutamate, arginine), consistent with previous studies (Lafon-Lafourcade and Gimberteau, 1962; Kliewer, 1968,1969,1970; Ough and Stashak, 1974, Spayd and Andersen-Bagge, 1996). In all the varieties examined, proline and arginine were the predominant amino acids present in the ripe fruit and proline accumulated to particularly high levels in berries of both Chardonnay and Cabernet Sauvignon. Such levels of free proline are similar to the high levels previously reported in other plant species for particular tissues and organs during normal development, or in vegetative tissues during exposure to abiotic stress (Venekamp and Koot, 1984; Kishor *et al.*, 1995; Savouré *et al.*, 1995). Interestingly, the final ratio of arginine and proline in the mature fruit is characteristic for each cultivar, suggesting that the metabolism of these two amino acids is determined by genetic factors. The nature of the genetic differences between cultivars leading to the differences in proline and arginine accumulation are unknown, however they may be manifested as the presence or absence of particular alleles that contribute to the operation and regulation of metabolic pathways.

To date, most of the research concerned with amino acid accumulation in grape berries has been confined to the study of mature berries or their juice (Kliewer, 1968,1969,1970; Kliewer and Ough, 1970; Huang and Ough, 1989; Krueger and Kliewer, 1995; Spayd *et al.*, 1995). These studies have shown that the degree of berry

maturity has a strong influence on its amino acid composition and this aspect must be taken into account when comparing berries from different cultivars. Little was known, however, about the changes in the free amino acid pool which occur from early berry development and how these changes contribute to the final amino acid profile observed in the ripe fruit. The amino acid analyses in this study provides a unique picture of amino acid accumulation during development of the fruit from flowering to maturity.

Glutamine has been shown to be a major form of transportable nitrogen present in the phloem sap of grapevine (Gholami, 1996). The demonstration that glutamine is the predominant amino acid present early in berry development (Figures 2.5-2.8), suggests that it is supplied to the berry via the phloem and there acts as the precursor for the subsequent biosynthesis of amino acids, including arginine and proline via glutamate. The concentration of glutamine declines as fruit development continues, suggesting that either its transport to the berry decreases during development, or, more likely, that it is converted to other amino acids (such as proline and arginine) at a rate which is faster than its import or synthesis. However, prior to this study, it was not known whether the enzymes which synthesise proline from glutamate are present in grape berries. The evidence for this will be presented in the following chapters.

In all four cultivars examined, the accumulation of proline was not uniform throughout berry development but clearly confined to the later stages of maturation. Furthermore, in the two varieties with the lower molar ratio of proline:arginine (Gewurztraminer and Muscat Gordo), the accumulation of free arginine followed a markedly different pattern during development than in Chardonnay and Cabernet Sauvignon. These results suggest

that, in grape berries, proline and arginine metabolism may be linked such that the final concentration of one is influenced by the other through an as yet undefined mechanism, with arginine possibly acting as a precursor for at least some of the proline accumulated (Kliewer, 1968). It is known that in other plants, proline and arginine metabolism are linked via the amino acid ornithine (for review see Verma and Zhang, 1999). Ornithine, which was detected at low levels in berry extracts, is an intermediate in the synthesis of arginine from glutamate and also a product of arginine degradation via arginase (EC 3.5.3.1). Ornithine can also participate in the synthesis of P5C, a precursor of proline, via the activity of OAT. Arginase activity has been detected in developing grape berries (Roubelakis-Angelakis and Kliewer, 1981). However, prior to this study, it was not known whether OAT, the enzyme linking proline and arginine metabolism, was present in grape berries. The evidence for this will be presented in the following chapters.

The results presented in this study, together with those reported previously, demonstrate that the concentration of free proline and other amino acids in the berry is strongly influenced by the degree berry of maturity (Coombe and Monk, 1979, Kliewer, 1968,1969,1970). ABA levels also increase in berries with increasing maturity, reaching a peak at the middle of the third stage of development after which they subsequently decline (Coombe and Monk, 1979, Downton and Loveys, 1978). Proline accumulation is linked to ABA levels in certain plant species during the osmotic stress response (see Chapter 1 section 1.6.3). During the later stages of maturation, berry tissue cells may be affected by an increase in osmotic pressure, due to the increased concentration of sugar (Downton and Loveys, 1978). It is possible, therefore, that proline accumulation occurs in response to a developmentally-imposed osmotic stress, and it may have an

osmoprotective function in developing berry cells. Although this possible role for proline in berries has not yet been fully investigated. Some studies have, however, examined the influences of vine osmotic stress on berry development. Coombe and Monk (1979) observed that ABA levels in berries were sensitive to the water status of the plant, whereas the proline concentration appeared unaffected. Downton and Loveys (1978) demonstrated that, although the timing of events during berry development is affected by water stress, the overall sequence remains unchanged, as do the final concentrations of proline and ABA.

Analysis of the amino acid profiles of berry skin, pulp and seeds, together with measurement of the amino acid composition of other *V.vinifera* tissues, reveals that the degree of proline accumulation which occurs in the berry skin and pulp is significantly different from that of other tissues. The pattern and degree of arginine accumulation differs between skin and pulp leading to a higher proline:arginine ratio in the pulp of ripe fruit, compared to that of the skin. This is consistent with results obtained for Cabernet Sauvignon in a study performed in parallel to the work conducted in this thesis (Grubb, 1997). Interestingly in that study it was also shown that in berries of cv. Riesling, there is a moderate accumulation of proline, and the proline:arginine ratio remained essentially constant throughout the later stages of ripening in both the skin and the pulp. Together with the results presented in this chapter, this suggests that particular physiological events occur during berry development which lead to the accumulation of both proline and arginine specifically in skin and pulp of some cultivars (classified as moderate proline accumulators), whilst in other cultivars (high proline accumulators) they lead to higher levels of proline in the pulp in association with lower levels of

arginine. The following chapter describes the isolation, from grape berries, of cDNAs encoding P5CS and OAT, two key proline biosynthetic enzymes. In subsequent chapters, the patterns of P5CS and OAT gene expression are examined and the possible role of these and other factors in proline accumulation during grape berry development is discussed.

# Chapter 3
## *Cloning and Characterisation of cDNAs Encoding VVP5CS and VVOAT*

## 3.1 Introduction

Initial research on proline biosynthesis in plants involved the feeding of radioactive precursors under various conditions with subsequent monitoring of the radiolabelled products which were produced (Morris *et al.*, 1969; Boggess *et al.*, 1976; Dierks-Ventling and Tonelli, 1982; Kueh *et al.*, 1984). These experiments indicated that proline was synthesised from glutamate by a pathway similar to that already identified in *E.coli* (Vogel and Davis, 1952; Hayzer and Leisinger, 1980, 1982). This was confirmed by the cloning of cDNAs encoding the enzymes P5CS and P5CR from plants (Delauney and Verma, 1990; Verbruggen *et al.*, 1991; Hu *et al.*, 1992; Williamson and Slocum, 1992). Two important differences between proline biosynthesis in plants and *E.coli* were observed. Surprisingly, isolation of a cDNA encoding P5CS from *V.aconitifolia*, the first from a eukaryote, revealed that it encoded a bifunctional enzyme which incorporated the activities of the first two enzymes in the bacterial biosynthetic pathway, namely γ-glutamyl kinase and γ-glutamyl phosphate reductase, into one large protein (Hu *et al.*, 1992). Secondly, although the glutamate pathway appeared to be the only route of proline synthesis in *E.coli*, it was evident that the additional pathway of proline synthesis from ornithine was operating in plants (Delauney *et al.*, 1993). *In vivo* labelling experiments suggest that the synthesis of proline from ornithine may occur through two routes, via the intermediates P5C or P2C (reviewed in Chapter 1, Section 1.5.2). The cloning of a cDNA encoding a δ-ornithine aminotransferase from *V.aconitifolia* confirmed the existence of the route via P5C in plants (Delauney *et al.*, 1993). However, an α-ornithine aminotransferase has not yet been isolated from any source. This chapter reports the isolation of full-length cDNA clones encoding P5CS

and δ-OAT from a cDNA library prepared from *V.vinifera* berry mRNA. This is the first evidence that enzymes from both of the proline synthesis pathways are present in berry tissue.

The first plant cDNAs encoding P5CS, P5CR and δ-OAT were isolated by functional complementation of auxotrophic *E.coli* mutants with a *V.aconitifolia* root nodule cDNA library (Delauney and Verma, 1990; Hu *et al.*, 1992; Delauney *et al.*, 1993). Subsequently, most of the cDNAs encoding these enzymes have been cloned from other plant species by virtue of their sequence homology with these original cDNA clones. At the beginning of the work described in this thesis, cDNAs encoding P5CS had been isolated from *V.aconitifolia* (Hu *et al.*, 1992) and *A.thaliana* (Savouré *et al.*, 1995). Therefore, these sequences and those of the *E.coli* GK and GPR were used as a basis for designing primers that would enable the isolation of a *V. vinifera* berry P5CS cDNA clone. Subsequently P5CS cDNAs have been cloned from several other plant species (see Chapter 1 for review).

At the commencement of the research described in this thesis, the *V.aconitifolia* δ-OAT cDNA was the only one characterised from plants, although δ-OAT cDNAs had also been isolated from bacteria (*B. subtilis*), *Plasmodium falciparum*, yeast and mammalian species (Mueckler and Pitot, 1985; Inaga *et al.*, 1986; Degols, 1987; Schmid *et al.*, 1993; Gardan *et al.*, 1995). Hence, to isolate the *V. vinifera* OAT cDNA, a range of sequences were employed to design PCR primers. During the final stages of this research the isolation of a plant δ-OAT cDNA clone from *A.thaliana* was also reported (Roosens *et al.*, 1998).

## 3.2 Materials and Methods

### 3.2.1 RNA isolation

Total RNA was extracted from grapevine tissues essentially as described by Levi *et al.* (1992), except that the extraction buffer contained 500 mM Tris-Cl, pH 8.0 whilst thiourea and aurintricarboxylic acid were excluded. Frozen tissue was ground to a powder in liquid $N_2$ then buffer was added at a rate of 2 ml buffer to 1g fwt tissue. RNA concentration and purity were determined by scanning UV spectroscopy (Beckman). RNA samples were stored at -70$^0$C.

### 3.2.2 Cloning of VVP5CS cDNA

#### 3.2.2.1 cDNA synthesis

First strand cDNAs were generated with RNase H reverse transcriptase (Superscript II, Gibco BRL) according to the manufacturer's instructions using 2 µg RNA isolated from 10 weeks postflowering cv. Muscat Gordo berries as template and a $(dT)_{15}$ primer in a volume of 20 µl. Based on the deduced amino acid sequences of P5CS from *V. aconitifolia*, *A.thaliana* and *E.coli* ProA/ProB (Figure 3.4), degenerate oligonucleotide primers were designed in order to amplify by PCR a partial *V. vinifera* P5CS (VVP5CS) cDNA clone. The oligonucleotides were:

3BP5CS: 5'AARCARAARCAYCARRAYGAYAT 3'

7P5CS: 5' GTYTCCATIGCRTTRCAIGC 3'.

The location of the amino acid sequences corresponding to these primers are illustrated in Figure 3.4. PCR reaction mixtures were buffered according to the manufacturer's

instructions and contained 1 unit of *Taq* polymerase (Gibco-BRL), 50 pmol of each primer and 2 µl of the first-strand cDNA reaction mix as template in a final volume of 25 µl. A 1.1 kb PCR product was generated by incubating the reaction mix at $94^0$C for 4 min, followed by 30 cycles of $94^0$C for 1 min, $55^0$C for 1 min 30 s, $72^0$C for 1 min 30 s with a final extension step of $72^0$C for 7 min. The PCR fragment was purified from TAE (40 mM Tris-acetate, pH 8.0 and 1 mM $Na_2EDTA$) buffered agarose gels using Bresaclean (Bresatec), ligated into the pGEM-T vector (Promega) and transformed into *E.coli* JM109 cells (Promega) after which its DNA sequence was determined according to Sanger *et al.* (1977).

### 3.2.2.2 cDNA library screening

A *V. vinifera* cv. Shiraz cDNA library, constructed from 10 weeks postflowering berry RNA in the Lambda-ZAPII vector (Stratagene) was kindly provided by Dr. C. Davies (CSIRO Plant Industry). The 1.1 kb partial cDNA clone of VVP5CS, labelled with [$^{32}$P]dCTP (Bresatec) using a Megaprime kit (Amersham) was used to screen this library after its transfer to Hybond $N^+$ (Amersham) according to the manufacturer's instructions. All hybridisations were carried out at $65^0$C. Approximately $5x10^5$ plaques were screened in the first round. Plaques which appeared to give positive hybridisation signals were rescreened through a further two rounds of plating and hybridisation. *In vivo* excision (Stratagene), which excises the phagemid pBluescript SK(-) from the Lambda Zap II vector, was performed on phage isolated from four positively hybridising plaques obtained from the tertiary screen. The phagemid preparations of two clones (3-1-1, 5-1-1) were transfected into *E.coli* XL1 Blue cells (Stratagene) and plated on LB

media containing ampicillin (50 μg/ml). Ampicillin resistant colonies were transferred to Hybond $N^+$ membrane (Amersham) according to the manufacturer's instructions. The colonies were screened with the radioactively labelled partial VVP5CS cDNA clone, synthesised as described above, in order to confirm their authenticity. The pBluescript phagemids obtained from positively hybridising colonies were purified using a Midi Prep Kit (Talent, Italy). The complete DNA sequence of one full length P5CS cDNA clone, named 3-1-1-12, was determined by sequencing both strands using the method of Sanger *et al.* (1977).

### 3.2.3 Cloning of VVOAT cDNA

#### 3.2.3.1 cDNA synthesis

First strand cDNAs were generated with RNase H reverse transcriptase (Superscript II, Gibco BRL) according to the manufacturer's instructions using 2 μg RNA isolated from *V. vinifera* cv. Sultana leaf as template and random hexamer primers. Based on deduced amino acid sequences of OAT sequences from *V. aconitifolia*, *Rattus norvegicus, Homo sapiens and P. falciparum* (Figure 3.6), degenerate oligonucleotides were designed in order to amplify by PCR a partial *V. vinifera* OAT (VVOAT) cDNA clone. The oligonucleotides were:

1OAT: 5'AARTAYGGIGCICAYAAYTA 3'

5ROAT: 5' ACICCIGCYTCICCYTG 3'.

The location of the amino acid sequences corresponding to these primers is illustrated in Figure 3.6. PCR reaction mixtures were as described previously (section 3.2.2.1). A 580 bp PCR product was generated by incubating the reaction mix at $94^0$C for 4 min, followed by 30 cycles of $94^0$C for 1 min, $45^0$C for 1 min 30 s, $72^0$C for 1 min 30 s with

a final extension step of 72$^0$C for 7 min. The PCR fragment was purified from TAE (40 mM Tris-acetate, pH 8.0 and 1 mM $Na_2EDTA$) buffered agarose gels using Bresaclean (Bresatec), ligated into the pGEM-T vector (Promega) and transformed into *E.coli* JM109 cells (Promega) after which its DNA sequence was determined according to Sanger *et al.* (1977).

### 3.2.3.2 cDNA library screening

The 580bp partial cDNA clone of VVOAT, labelled with [$^{32}$P]dCTP (Bresatec) using a Megaprime kit (Amersham) was used to screen the *V. vinifera* cv. Shiraz cDNA library, constructed from 10 weeks postflowering berry RNA, essentially as described in section 3.2.2.2. *In vivo* excision (Stratagene), was performed on phage isolated from 4 positively hybridising plaques obtained after the tertiary screen. The phagemid preparations of three clones (2-1-1, 4-2-1 and 6-2-1) were transformed into *E.coli* XL1 Blue cells (Stratagene) and grown as described previously. The colonies were transferred to Hybond $N^+$ membrane (Amersham) according to the manufacturer's instructions. The colonies were screened with the radioactively labelled partial VVOAT cDNA clone, synthesised as described above. The pBluescript phagemids obtained from positively hybridising colonies were purified using a Midi Prep Kit (Talent, Italy). The complete DNA sequence of a full-length VVOAT cDNA clone, named 4-2-1, was determined in both directions using the method of Sanger *et al.* (1977).

### 3.2.4 Southern analyses

Genomic DNA was extracted from grapevine leaves according to the method of Steenkamp *et al.* (1994). For Southern analyses, 10 µg of DNA was digested to

completion with the specified restriction enzymes, according to the manufacturer's instructions (Promega). The digested DNA was resolved in 0.8% (w/v) agarose gels (buffered in TAE), blotted onto Hybond $N^+$ membrane (Amersham) and fixed in a UV crosslinker according to the manufacturer's recommendations. The blots were incubated at $60^0$C for 4 h in prehybridisation solution (5 X SSC, 0.5% (v/v) SDS, 5 X Denhardt's reagent (Sambrook *et al.*, 1989), and 100 µg/ml denatured and sheared salmon-sperm DNA). Denatured DNA probes, labelled with $^{32}$P-dCTP as described above were incubated with the blots for 17 h at $60^0$C. The membranes were washed at $60^0$C at low stringency (1 X SSC, 0.1% (w/v) SDS) and high stringency (0.1X SSC, 0.1% (w/v) SDS) according to Meinkoth and Wahl (1984). Hybridising DNA was detected with a phosphorimaging screen (Kodak) and analysed using a phosphorimager (Storm 860, Molecular Dynamics) and ImageQuant software (Molecular Dynamics).

### 3.2.5 Construction of prokaryotic expression vectors

In order to produce recombinant VVP5CS and VVOAT in *E.coli*, the coding regions of the cDNAs described in sections 3.2.2 and 3.2.3 were inserted into the prokaryotic expression vector pET14-b (Novagen) to create VVP5CS pET-14b (Figure 3.1) and VVOAT pET-14b (Figure 3.2). The first step in the construction of VVP5CS pET-14b was the introduction of *Nde*I and *Bam*HI restriction sites into the 5' and 3' ends respectively of the VVP5CS cDNA sequence through PCR with Vent$^R$ DNA polymerase (New England Biolabs) and the primers:

VVP5CSN: 5'-GTTAACATATGGACGCCATGGACCCAACTCGA-3'

VVP5CSC: 5'-AGCCGGATCCTTAGGGCTGCAAAGTAAGCTCCTT-3'.

Due to the existence of a *Bam*HI site at position +2bp from the third ATG in the

VVP5CS sequence (Figure 3.3), the 'N terminal primer' (VVP5CSN) was designed to introduce a single base pair change during PCR which would eliminate the *Bam*HI site, but encode the same amino acid sequence as the original clone. An additional *Nde*I site is also located at position +550bp from the third ATG (Figure 3.3). The PCR product was blunt-end cloned into pCRScript (Stratagene) using the PCRscript cloning kit (Stratagene) and *E.coli* JM109 supercompetent cells (Promega), after which the orientation of the insert was determined by restriction fragment analysis. The fidelity of the PCR was determined by sequencing the 5' and 3' ends of the cloned insert. The subsequent strategy for the construction of VVP5CS pET14-b is illustrated in figure 3.1. The pCRscript clone was digested to completion with *Bam*HI (Promega), according to the manufacturer's instructions, and the linearised plasmid gel purified (Bresaclean, Bresatec). This was followed by partial digestion with the enzyme *Nde*I (Promega), 10 units/μg DNA for 3.5 min at $37^0$C and the reaction was terminated by the addition of EDTA to a final concentration of 25 mM. The partial digest produced a 2.3 kb, full length VVP5CS fragment with 5' *Nde*I and 3' *Bam*HI overhangs, for ligation with *Nde*I and *Bam*HI cut pET-14b vector. After transformation into *E.coli* JM109 cells (Promega) the success of the ligation was assessed by PCR using the vector primers T7 and T7 terminator. For expression of the protein encoded by the insert, pET 14-b VVP5CS #6, was transformed into *E.coli* BL21 (DE3) cells (Novagen) co-harbouring the pLysS plasmid (Sambrook *et al.*, 1989).

The VVOAT pET-14b expression construct was created by introducing *Nde*I and *Bam*HI restriction sites into the 5' and 3' ends, respectively, of the VVOAT cDNA through PCR with Vent$^R$ DNA polymerase (New England Biolabs) and the primers

VVOATN: 5'-GTTAACATATGGCACTGGCTACCAGGAGATTC -3'

VVOATC: 5'-AGCCGGATCCTTAAGAATCATCCATATTTCGACCACAACGATCACAGAT-3'

Due to the existence of an *Nde*I site 39bp from the translation termination codon the primer VVOATC was designed so that a single base pair change would be introduced, that would eliminate the *Nde*I site from the 3' end during PCR without altering the encoded amino acid sequence. Subsequent analysis of cloned products (see below) demonstrated however, that this strategy failed to remove the *Nde*I site. A *Bam*HI site was also located at +190bp from the ATG in the VVOAT sequence (Figure 3.5). The PCR product was blunt-end cloned into pCRScript (Stratagene) as described previously. The orientation of the insert was determined by restriction fragment analysis. The fidelity of the PCR was determined by sequencing the 5' and 3' ends of the cloned insert. This revealed that the 3' *Nde*I site had been retained within the VVOAT ORF sequence. The subsequent strategy for construction of VVOAT pET14-b is illustrated in figure 3.2. A clone was digested to completion with *Bam*HI (as described above) and the resulting 1.1kb partial VVOAT *Bam*HI/*Bam*HI fragment and the linearised plasmid were gel purified (Bresaclean, Bresatec). The linearised plasmid was digested to completion with *Nde*I (Promega), according to the manufacturer's directions, after which the resulting 200bp partial VVOAT *Nde*I/*Bam*HI fragment, representing the 5' end of VVOAT, was gel purified and ligated into *Nde*I and *Bam*HI cut pET-14b vector. The pET14-b plasmid containing 200bp of VVOAT was then digested to completion (as described above) with BamHI (Promega) and the 1.1 kb VVOAT *Bam*HI/*Bam*HI fragment, obtained earlier was cloned into the resulting site. Restriction fragment analysis of the putative clones revealed 12 contained the insert, with one clone having the fragment in the correct orientation. For expression of the protein encoded by the

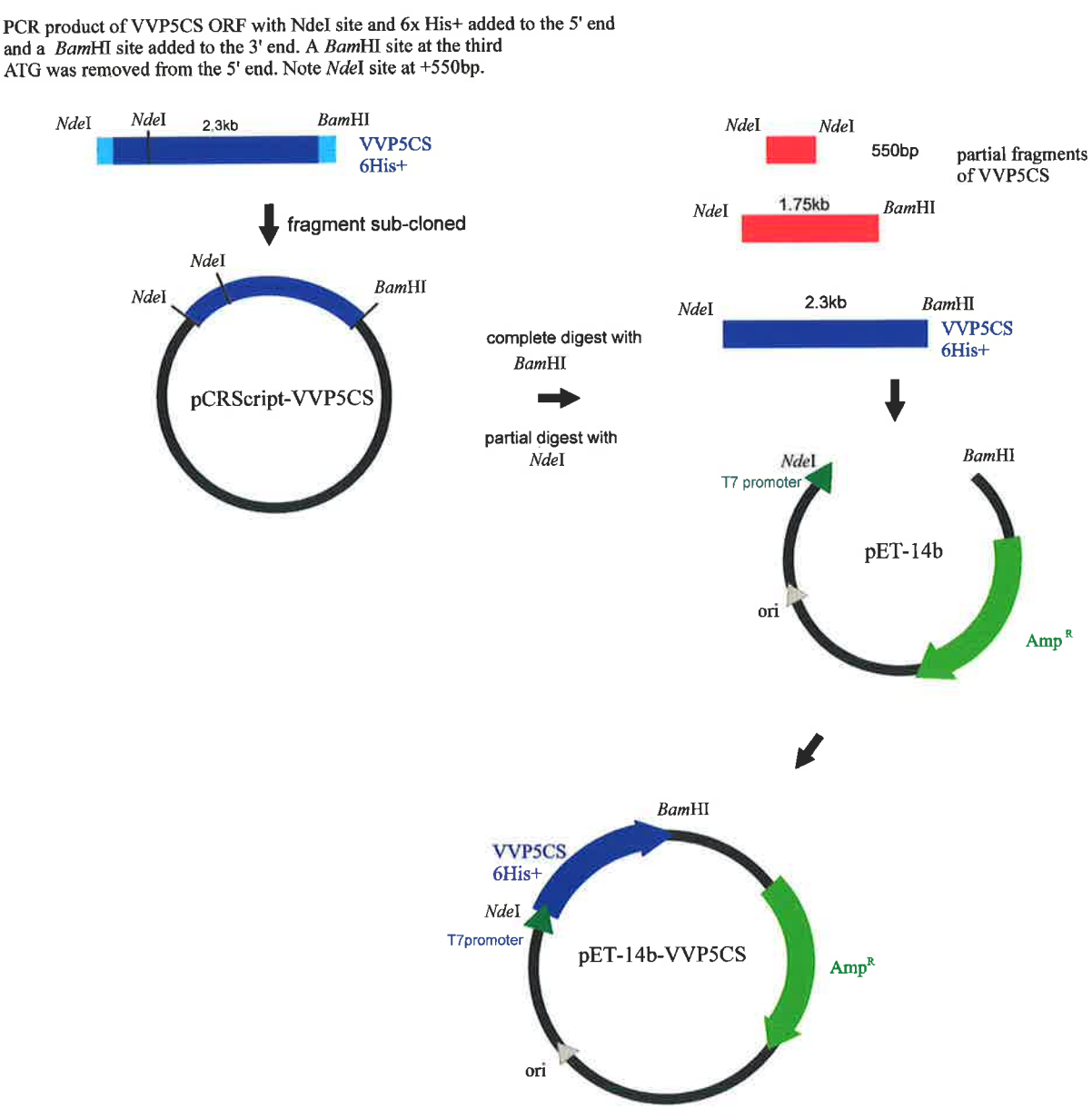



**Figure 3.1** Diagrammatic representation of the cloning strategy for the construction of pET-14b-VVP5CS. A 2.3kb PCR product containing the open reading frame encoding VVP5CS with an N terminal hexa-histidine tag was cloned into the vector pCRscript. Due to the presence of an internal *Nde*I site the fragment was digested with *Bam*HI followed by partial digestion with *Nde*I. Three different fragments were produced, however only the 2.3kb fragment contained the entire VVP5CS open reading frame. The 2.3kb fragment was cloned into the expression vector pET-14b which had been pre-digested with *Nde*I and *Bam*HI and then transformed into *E. coli* BL21 pLys S .

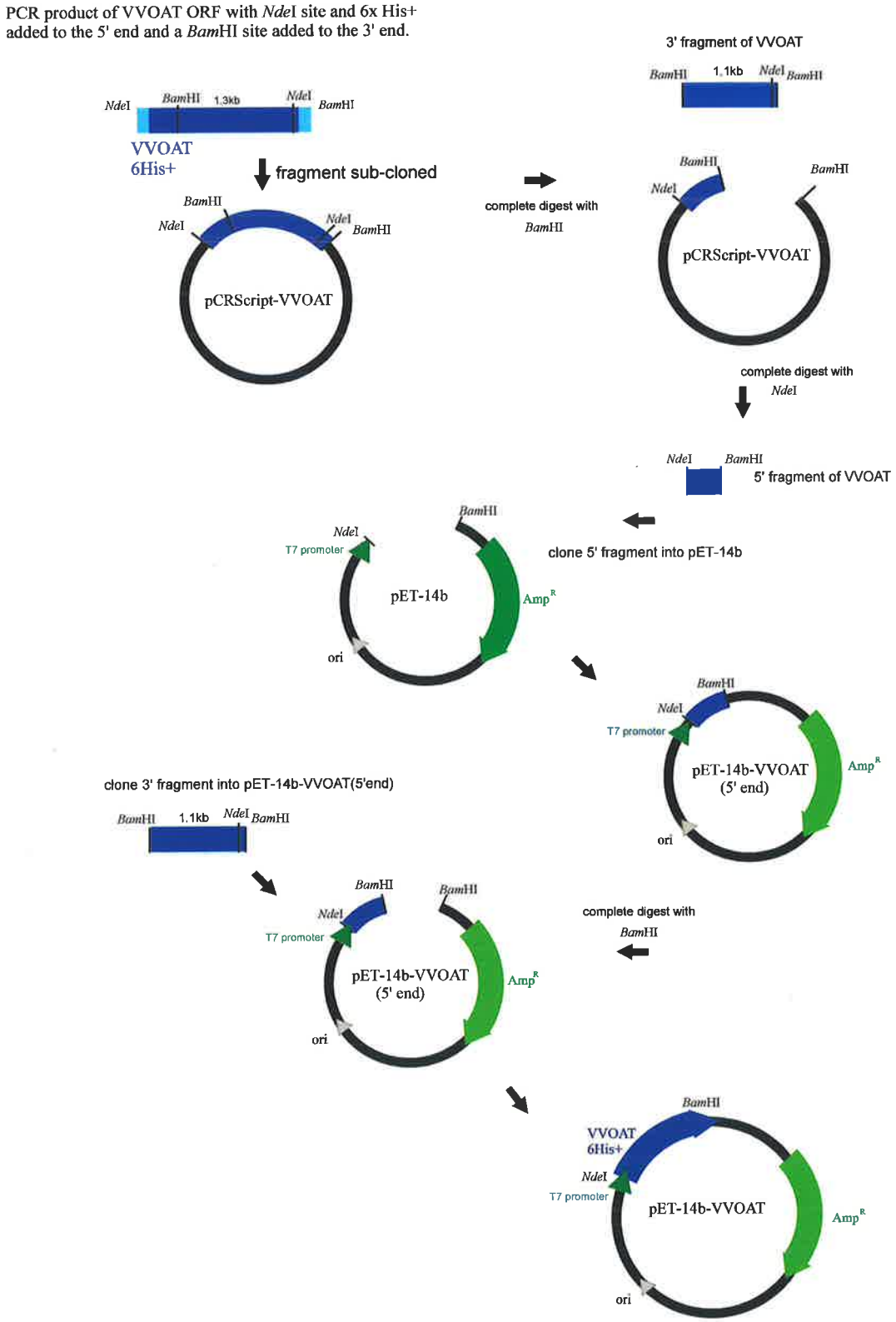



**Figure 3.2** Diagrammatic representation of the cloning strategy for the construction of pET-14b-VVOAT. A 1.3kb PCR product containing the ORF encoding OAT with an N-terminal hexahistidine tag was cloned into the vector pCRscript. Due to the presence of internal *Bam*HI and *Nde*I sites, the fragment was first digested with *Bam*HI and then digested with *Nde*I to divide the VVOAT fragment into two separate fragments. The 5' fragment was ligated with the expression vector pET-14b, which had been predigested with *Bam*HI and *Nde*I. This plasmid was then recut with *Bam*HI and the 3' fragment of VVOAT was ligated into the *Bam*HI site. This created a full-length VVOAT fragment within the pET-14b vector. The expression vector was then transformed into *E.coli* BL21 pLysS.

insert, pET 14-b VVOAT #59, was transformed into *E.coli* BL21 (DE3) cells (Novagen) co-harbouring the pLysS plasmid (Sambrook *et al.*, 1989).

### 3.2.6 SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed using 10-12.5% Tris-Glycine gels (Fling and Gregerson, 1986). Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in ethanol/acetic acid/water (40:7:53, by vol.) and destained with a solution containing 20% (v/v) ethanol and 7% (v/v) acetic acid.

### 3.2.7 Production of recombinant VVP5CS and VVOAT in *E.coli*

Recombinant VVP5CS or VVOAT bearing an N-terminal hexahistidine tag was typically produced by inoculating one litre of LB (100 μg/ml ampicillin, 40 μg/ml chloramphenicol) with a transformed *E.coli* colony followed by incubation with shaking at $37^0$C until $OD_{600nm}$ equalled 0.5. Expression of the recombinant protein was initiated by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.4 mM. At hourly time intervals, 1 ml aliquots of the culture were harvested. Following centrifugation, cell pellets were resuspended in 80 μl of SDS-sample buffer followed by boiling for 5 min and electrophoresis of a 10 μl aliquot in a 10% SDS-Polyacrylamide gel (as described in section 3.2.6). To purify the recombinant proteins, cells from the remaining culture were harvested after a 5 h incubation post-induction by 5 min centrifugation at $4^0$C and 5000 *g* the pellet was resuspended in (3 ml/g wet cells) lysis buffer (25 mM Tris-Cl buffer, pH 8.0, 200 mM NaCl) and then processed by the lysozyme method of Sambrook *et al.* (1989). Almost all of the recombinant VVP5CS

and VVOAT produced by *E.coli* at 37$^0$C was recovered in the insoluble fraction after cell lysis. In order to obtain a greater recovery of soluble protein, IPTG induction of the *E.coli* BL21 cells was carried out at 16$^0$C for up to 20 hours. Using this method, most of the recombinant protein was still insoluble, however, a minor soluble fraction was obtained after cell lysis. Insoluble protein was resolubilised by addition of urea to a final concentration of 8 M to purification buffers. Both soluble and resolubilised denatured recombinant protein was then purified using Talon$^{TM}$ Metal Affinity Resin (Clontech) according to the manufacturer's instructions. Protein concentration was determined using Coomassie-Brilliant Blue binding using BSA as a protein standard (Sedmak and Grossberg, 1977).

### 3.2.8 Production of polyclonal antibodies to VVP5CS and VVOAT

Antigen for polyclonal antibody production was prepared by excising insoluble recombinant VVP5CS or VVOAT from 10 or 12.5% SDS-PAGE gels, and developing the gel pieces into a slurry as described by Harlow and Lane (1988). For the initial immunisations, approximately 350 µg of antigen was mixed with an equal volume of Freund's complete adjuvant (Gibco BRL) and injected subcutaneously into New Zealand white rabbits. The rabbits were boosted three times, at six weekly intervals, using approximately 150 µg of the antigen mixed with an equal volume of Freund's incomplete adjuvant (Gibco BRL). Ear bleeds were performed ten days after the third boost and the sera containing polyclonal antibodies to VVP5CS or VVOAT were collected. The sera were supplemented with 0.02% (w/v) sodium azide and stored at -70$^0$C.

### 3.2.9 Western analysis

Proteins were resolved by SDS-PAGE in 10% or 12.5% Tris-Glycine gels (Fling and Gregerson, 1986). Proteins were blotted onto nitrocellulose membranes (MSI Laboratories, Westboro, MA) using a semidry transfer unit (LKB, Broma, Sweden) as described by Harlow and Lane (1988). The filter was blocked overnight in 5% (w/v) skim milk powder in TBS-Tween (25 mM Tris-Cl, pH 8.0, 200 mM NaCl, 2.5 mM KCl, 0.5% (v/v) Tween-20). The blot was probed with rabbit anti-VVP5CS serum (diluted 1/10000 in TBS-Tween) or anti-VVOAT (diluted 1/1000 in TBS-Tween and 1/250 in TBS-Tween for affinity purified antibodies) serum and/or affinity purified antibodies (see section 3.2.10) and HRP-labelled goat anti-rabbit IgG. The blot was incubated with enhanced chemiluminescence (ECL) detection reagents (Amersham) and exposed to Hyperfilm$^{TM}$-MP (Amersham).

### 3.2.10 Affinity purification of anti-VVOAT antibodies

The VVOAT antibody was affinity purified according to the method described by Harlow and Lane (1988). Approximately 50 µg of recombinant VVOAT was run on a 12.5% SDS PAGE gel, across 10 lanes. The gel was blotted onto nitrocellulose as described above (section 3.2.9). After transfer, the membrane was stained with Ponceau Red stain to locate the position of VVOAT, and this strip was excised. The strip was blocked for 2 hours at 4$^0$C in 10% (v/w) BSA TBS-Tween and then subjected to four 15 min washes in TBS-Tween. The strip was incubated with 150 µl of VVOAT antigen in 4% (w/v) BSA TBS-Tween for 2 h and then washed two times in a high salt wash buffer (0.2 M Tris-Cl (pH 7.6), 0.5 M NaCl), two times in a low salt wash buffer (10 mM Tris-

Cl (pH 7.6)) and rinsed in distilled water before the bound antibody was eluted by a 10 min incubation in 1.5 ml 100 mM glycine (pH 2.5). The solution was removed from the membrane and immediately neutralised with 1/10th volume 1 M Tris-Cl (pH 8.0). The purified antibody was supplemented with 0.01% sodium azide and stored at $4^0$C. For western blots, a 1 in 250 dilution of purified anti-VVOAT was used as described.

### 3.2.11 P5CS enzyme assay

The combined activities of the GK and GPR activities of P5CS were assayed essentially as described by Garcia-Rios *et al.* (1997). The assay is based on the ATP and glutamate dependent conversion of NADPH to NADP as measured by a decrease in absorbance at 340 nm. Soluble, recombinant VVP5CS was produced for assay as described in section 3.2.7. Typical assay mixes contained 50 mM glutamate, 100 mM Tris-Cl (pH 7.4), 25 mM $MgCl_2$, 5 mM ATP, 0.4 mM NADPH and approximately 20 µg of protein in a 1 ml volume. The reaction rate was measured as the decrease in absorbance over time using a Pharmacia Biotech Novaspec II Spectrophotmeter and a Kipp and Zonen Chart Recorder. The specific activity was calculated based on the amount of NADPH converted to NADP/ min/ mg protein.

### 3.2.12 OAT enzyme assay

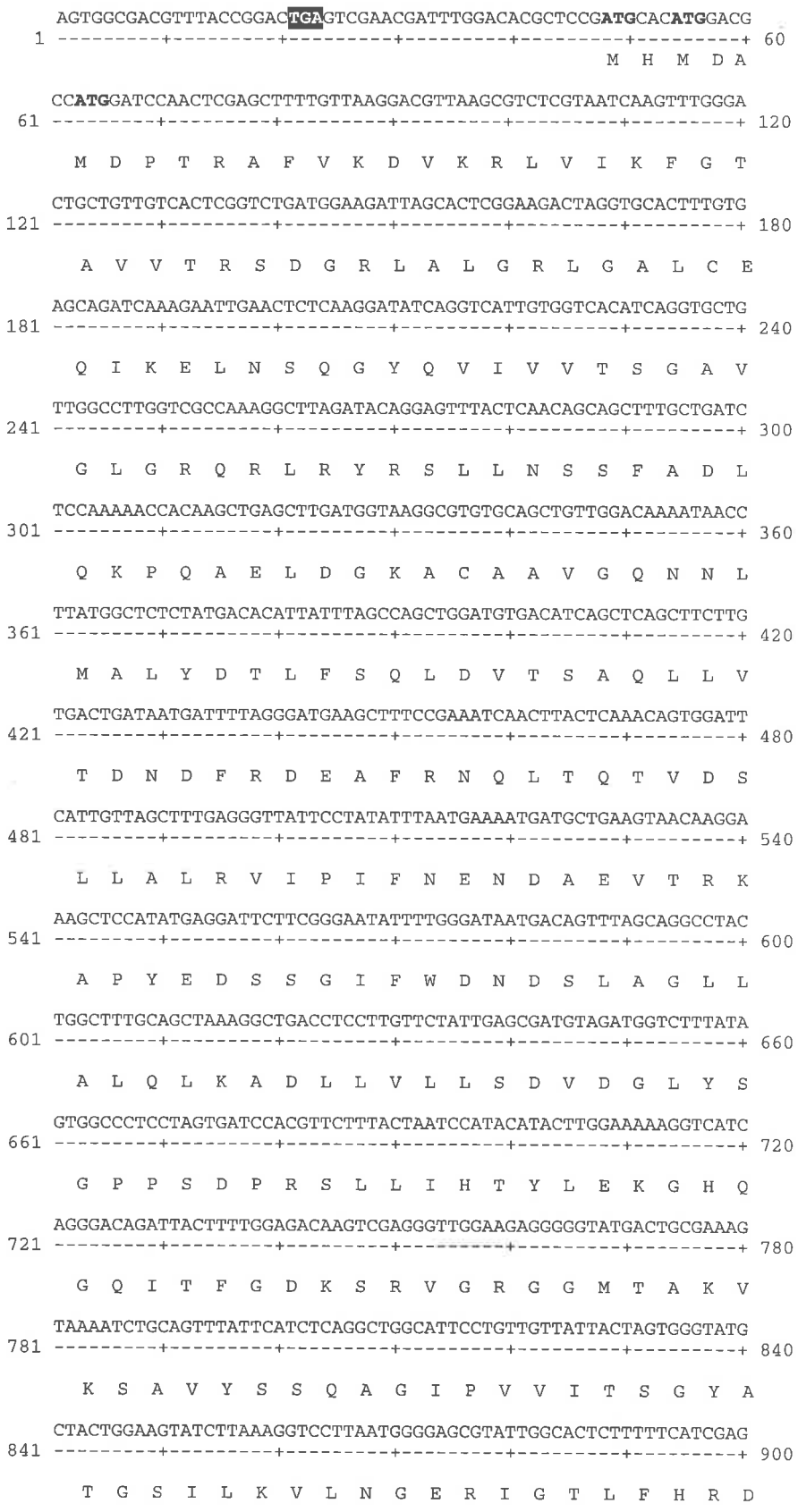
The activity of OAT was assayed as described by Kim *et al.* (1994). The assay was based on the production of P5C and its reaction with ninhydrin under hot acidic conditions. A red pigment is formed, the absorbance of which can be measured at 510 nm. Assay mixes were composed of the following; 5 mM α-ketoglutarate, 30 mM L-

ornithine, 0.5 mM pyridoxal-5-phosphate, 20 µg BSA and approximately 2 µg enzyme in a 1 ml volume. Reactions were allowed to proceed for 30 min after which they were terminated with 300 µl ice cold 3N perchloric acid. To measure OAT activity in the presence of gabaculine, it was added to a final concentration of 50 µM and preincubated with the protein for two hours before the assay was conducted. To denature the enzyme, protein was boiled in a water bath at for ten minutes. A soluble protein extract from untransformed *E.coli* cells, prepared as described by Sambrook *et al.* (1989) was used as a negative control. To measure P5C production, 200 µl of 2% (w/v) ninhydrin was added to the mixture. The mixture was boiled for 5 min and centrifuged for 5 min at 12,000 *g*. The resulting pellet was resuspended in 1 ml absolute ethanol and recentrifuged to remove insoluble debris. The absorbance of the supernatant at 510 nm was measured using a spectrophotometer as described in the previous section. The specific activity was calculated based on the amount of P5C produced/ h/ mg protein.

## 3.3 Results

### 3.3.1 Cloning of VVP5CS cDNA

In order to obtain a P5CS probe for screening of a cDNA library, RT-PCR was employed. At the beginning of this study, there was no information on the timing or tissue specificity of P5CS gene expression in *V.vinifera*. As we were chiefly interested in proline biosynthesis during berry development, we decided to use berry RNA as the initial template for RT-PCR. Total RNA was isolated from 10 weeks postflowering Muscat Gordo berries. Following oligo dT-primed, first-strand cDNA synthesis and PCR with degenerate oligonucleotide primers 3BP5CS and 7P5CS at an annealing temperature of $55^0$C, a DNA fragment of 1.1kb was generated which corresponded to the size expected, based on other published sequences (Figure 3.4) The PCR product was cloned and sequence analysis confirmed its identity as a P5CS cDNA homologue. comprising the region 565bp to 1691bp (Figure 3.3) The entire DNA fragment was subsequently used to screen a Shiraz ten weeks postflowering berry cDNA library. The phagemids from four positively hybridising plaques were isolated and the insert of one cDNA clone (3-1-2-12) was sequenced fully, using both strands as templates. The 2376bp insert encodes an 82.6 kDa protein with significant homology to both γ-glutamyl phosphate reductase and γ-glutamyl kinase domains (found in ProB and ProA in *E. coli*) as well as P5CS sequences from a number of other plant species, for example, *V.aconitifolia* P5CS (76% amino acid identity) and *A. thaliana* AtP5CS-1 (79% amino acid identity) (Figure 3.4). The identity of the translation iniation codon in VVP5CS is uncertain due to the presence of three in-frame start codons within close proximity of


AGTGGCGACGTTTACCGGACTGAGTCGAACGATTTGGACACGCTCCGATGCACATGGACG
1 ---------+---------+---------+---------+---------+---------+ 60
M H M D A
CCATGGATCCAACTCGAGCTTTTGTTAAGGACGTTAAGCGTCTCGTAATCAAGTTTGGGA
61 ---------+---------+---------+---------+---------+---------+ 120
M D P T R A F V K D V K R L V I K F G T
CTGCTGTTGTCACTCGGTCTGATGGAAGATTAGCACTCGGAAGACTAGGTGCACTTTGTG
121 ---------+---------+---------+---------+---------+---------+ 180
A V V T R S D G R L A L G R L G A L C E
AGCAGATCAAAGAATTGAACTCTCAAGGATATCAGGTCATTGTGGTCACATCAGGTGCTG
181 ---------+---------+---------+---------+---------+---------+ 240
Q I K E L N S Q G Y Q V I V V T S G A V
TTGGCCTTGGTCGCCAAAGGCTTAGATACAGGAGTTTACTCAACAGCAGCTTTGCTGATC
241 ---------+---------+---------+---------+---------+---------+ 300
G L G R Q R L R Y R S L L N S S F A D L
TCCAAAAACCACAAGCTGAGCTTGATGGTAAGGCGTGTGCAGCTGTTGGACAAAATAACC
301 ---------+---------+---------+---------+---------+---------+ 360
Q K P Q A E L D G K A C A A V G Q N N L
TTATGGCTCTCTATGACACATTATTTAGCCAGCTGGATGTGACATCAGCTCAGCTTCTTG
361 ---------+---------+---------+---------+---------+---------+ 420
M A L Y D T L F S Q L D V T S A Q L L V
TGACTGATAATGATTTTAGGGATGAAGCTTTCCGAAATCAACTTACTCAAACAGTGGATT
421 ---------+---------+---------+---------+---------+---------+ 480
T D N D F R D E A F R N Q L T Q T V D S
CATTGTTAGCTTTGAGGGTTATTCCTATATTTAATGAAAATGATGCTGAAGTAACAAGGA
481 ---------+---------+---------+---------+---------+---------+ 540
L L A L R V I P I F N E N D A E V T R K
AAGCTCCATATGAGGATTCTTCGGGAATATTTTGGGATAATGACAGTTTAGCAGGCCTAC
541 ---------+---------+---------+---------+---------+---------+ 600
A P Y E D S S G I F W D N D S L A G L L
TGGCTTTGCAGCTAAAGGCTGACCTCCTTGTTCTATTGAGCGATGTAGATGGTCTTTATA
601 ---------+---------+---------+---------+---------+---------+ 660
A L Q L K A D L L V L L S D V D G L Y S
GTGGCCCTCCTAGTGATCCACGTTCTTTACTAATCCATACATACTTGGAAAAAGGTCATC
661 ---------+---------+---------+---------+---------+---------+ 720
G P P S D P R S L L I H T Y L E K G H Q
AGGGACAGATTACTTTTGGAGACAAGTCGAGGGTTGGAAGAGGGGGTATGACTGCGAAAG
721 ---------+---------+---------+---------+---------+---------+ 780
G Q I T F G D K S R V G R G G M T A K V
TAAAATCTGCAGTTTATTCATCTCAGGCTGGCATTCCTGTTGTTATTACTAGTGGGTATG
781 ---------+---------+---------+---------+---------+---------+ 840
K S A V Y S S Q A G I P V V I T S G Y A
CTACTGGAAGTATCTTAAAGGTCCTTAATGGGGAGCGTATTGGCACTCTTTTTCATCGAG
841 ---------+---------+---------+---------+---------+---------+ 900
T G S I L K V L N G E R I G T L F H R D

```
    ATGCCTATAAATGGGTACAAGTTAAAGAAGTTGGCGCACGTGAAATGGCAGTTGCAGCAA
901 ---------+---------+---------+---------+---------+---------+ 960

      A  Y  K  W  V  Q  V  K  E  V  G  A  R  E  M  A  V  A  A  R

    GGGAAAGTTCCAGGCGGCTTCAGGCAATGTCTTCACAAGACAGGAAGAAGATTTTACTGG
961 ---------+---------+---------+---------+---------+---------+ 1020

      E  S  S  R  R  L  Q  A  M  S  S  Q  D  R  K  K  I  L  L  D

    ACATAGCCAATGCACTGGAAACAAATGAAGAACTGATTAAAATTGAAAATGATGCTGATG
1021 ---------+---------+---------+---------+---------+---------+ 1080

      I  A  N  A  L  E  T  N  E  E  L  I  K  I  E  N  D  A  D  V

    TTGAGGCAGCACAACTGGCTGGATATGAAAAATCATTGGTTTCAAGGCTGGTTCTAAAGC
1081 ---------+---------+---------+---------+---------+---------+ 1140

      E  A  A  Q  L  A  G  Y  E  K  S  L  V  S  R  L  V  L  K  P

    CTGGAAAGATTTCAAGCCTTGCAAACTCAATTCGTGTGCTTGCAAACATGGAAAGACCAA
1141 ---------+---------+---------+---------+---------+---------+ 1200

      G  K  I  S  S  L  A  N  S  I  R  V  L  A  N  M  E  R  P  I

    TTGGTCATGTTTTGAAGAAAACTGAAGTTGCAGATGGACTTATCCTAGAAAAGATGTCAT
1201 ---------+---------+---------+---------+---------+---------+ 1260

      G  H  V  L  K  K  T  E  V  A  D  G  L  I  L  E  K  M  S  C

    GCCCATTAGGTGTGCTCCTAATTGTTTTTGAGTCTCGACCAAATGCTCTGGTACAGATAG
1261 ---------+---------+---------+---------+---------+---------+ 1320

      P  L  G  V  L  L  I  V  F  E  S  R  P  N  A  L  V  Q  I  A

    CTTCATTAGCAATCCGAAGTGGGAATGGACTTCTCTTGAAAGGTGGAAAAGAGGCCAAGC
1321 ---------+---------+---------+---------+---------+---------+ 1380

      S  L  A  I  R  S  G  N  G  L  L  L  K  G  G  K  E  A  K  R

    GATCTAATGCAATTCTTGCAAAGGTTATTACTGAAGCCATTCCAGACAGTGTTGGGAAGA
1381 ---------+---------+---------+---------+---------+---------+ 1440

      S  N  A  I  L  A  K  V  I  T  E  A  I  P  D  S  V  G  K  K

    AGCTAATTGGACTTGTGACTTCAAGAGAGGAGATCCCTAATCTCCTCAAGCTTGATGATG
1441 ---------+---------+---------+---------+---------+---------+ 1500

      L  I  G  L  V  T  S  R  E  E  I  P  N  L  L  K  L  D  D  V

    TGATCGACCTTGTAATCCCAAGAGGCAGCAATAAACTTGTTTCTCAAATAAAGGATTCAA
1501 ---------+---------+---------+---------+---------+---------+ 1560

      I  D  L  V  I  P  R  G  S  N  K  L  V  S  Q  I  K  D  S  T

    CAAAAATTCCTGTTCTGGGCCATGCTGATGGCATCTGCCACGTTTATGTCGACAAGTCTG
1561 ---------+---------+---------+---------+---------+---------+ 1620

      K  I  P  V  L  G  H  A  D  G  I  C  H  V  Y  V  D  K  S  A

    CTAATATGGATACTGCAAAGCACATCGTATTGGATGCAAAAGTAGATTATCCTGCAGCCT
1621 ---------+---------+---------+---------+---------+---------+ 1680

      N  M  D  T  A  K  H  I  V  L  D  A  K  V  D  Y  P  A  A  C

    GTAATGCAATGGAAACACTTCTTGTACACAAGGATTTAGTGCAGACTGGTGGCCTCAATC
1681 ---------+---------+---------+---------+---------+---------+ 1740

      N  A  M  E  T  L  L  V  H  K  D  L  V  Q  T  G  G  L  N  Q

    AGCTTATCGTAGAGCTCCGCAATGAAGGGGTTACTTTATATGGTGGACCAAAGGCAAGTG
1741 ---------+---------+---------+---------+---------+---------+ 1800

      L  I  V  E  L  R  N  E  G  V  T  L  Y  G  G  P  K  A  S  A
```

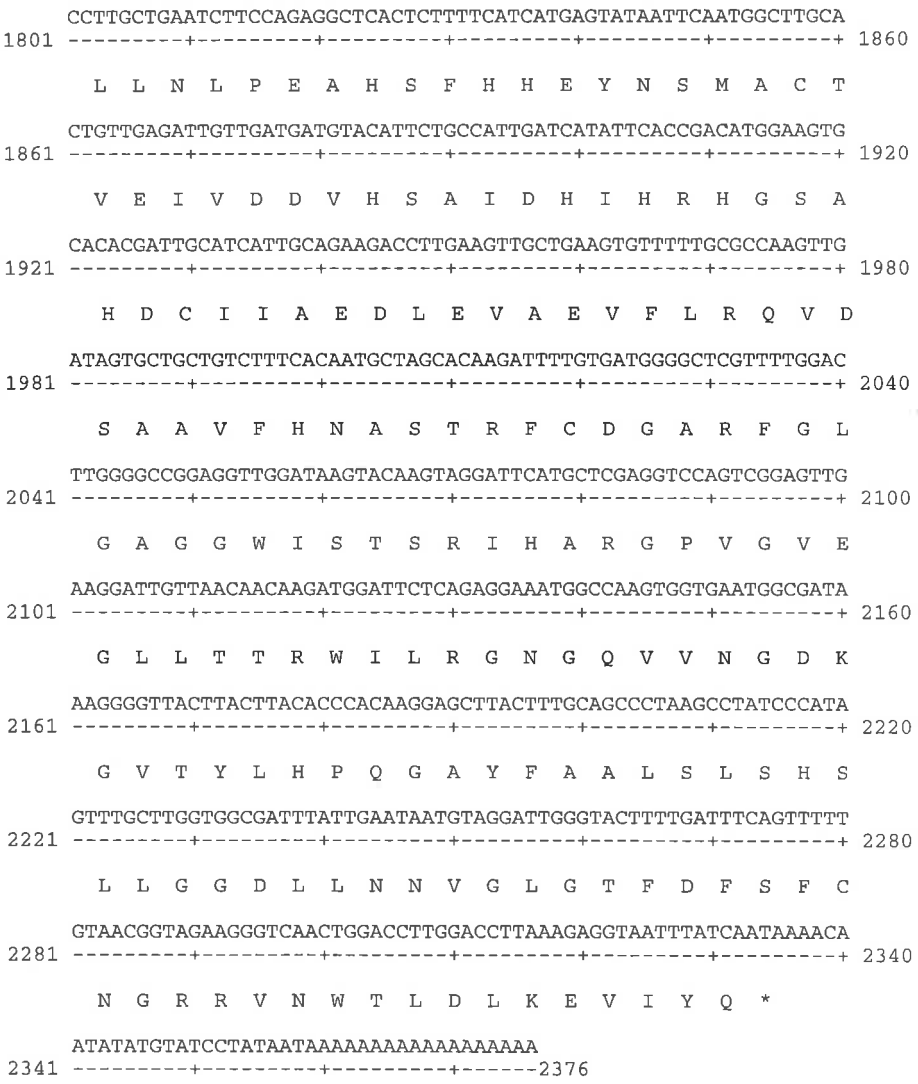
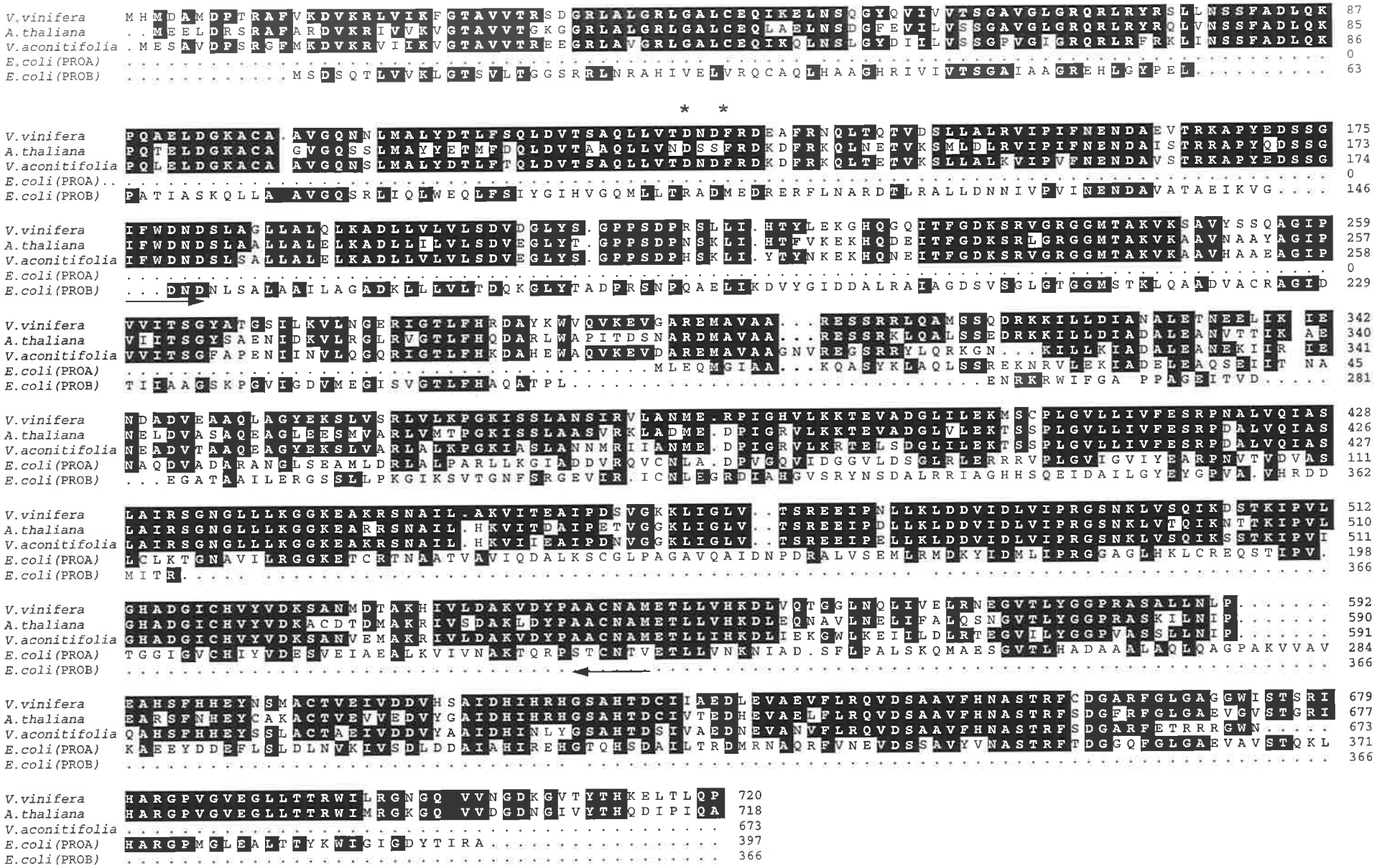



**Figure 3.3** The complete nucleotide and deduced amino acid sequence of the VVP5CS cDNA obtained from a *V.vinifera* cv Shiraz 10 weeks postflowering berry cDNA library. The translational stop codon is denoted by an asterisk (*) and an in-frame stopcodon upstream of the iniation codon is shaded in black.

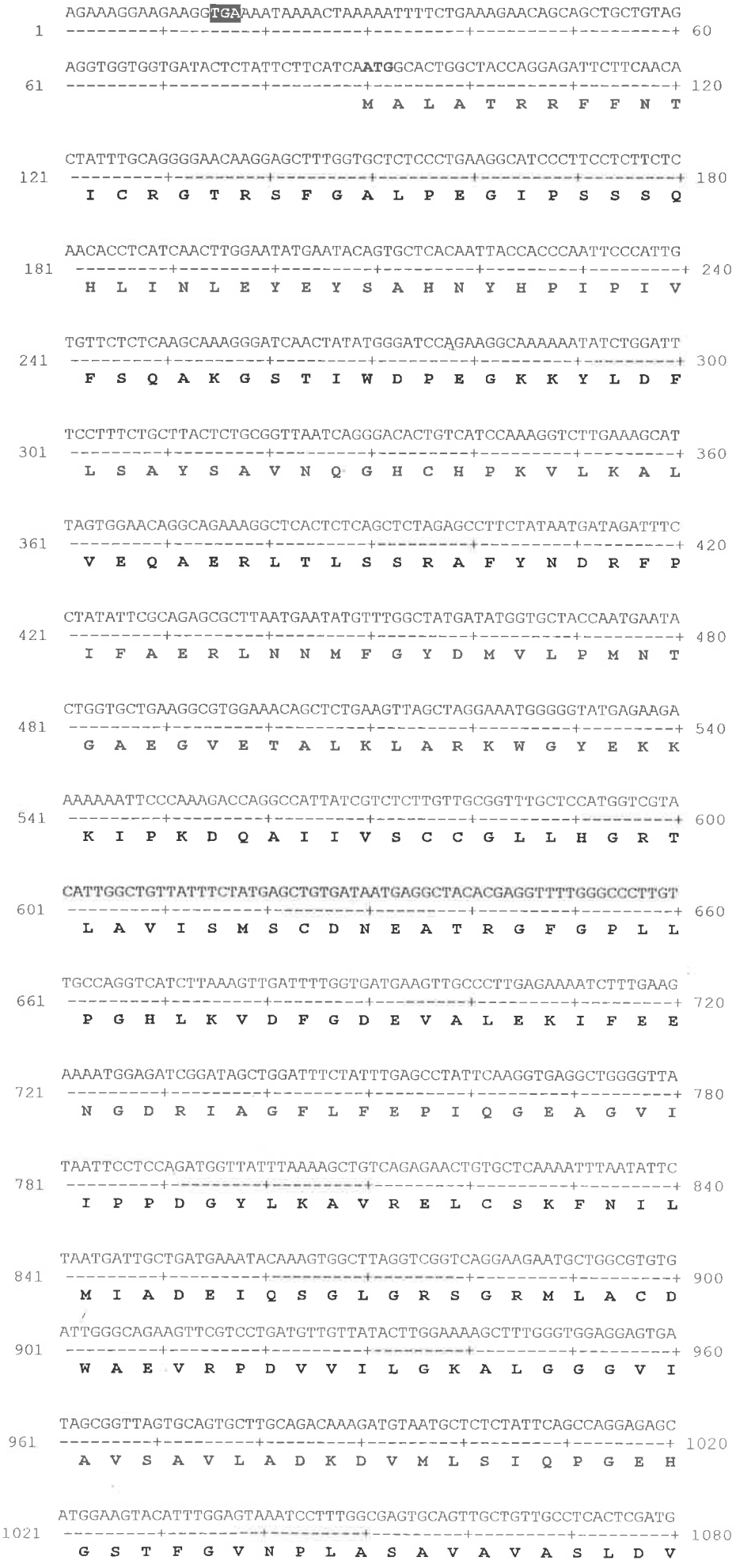
**Figure 3.4** Multiple alignment of deduced amino acid sequences of P5CS proteins using the PILEUP program. Residues identical to the *V.vinifera* P5CS sequence are highlighted. Deduced amino acid sequences were obtained from SWISSPROT and GenBank. The location and direction of degenerate oligonucleotide primers used to generate the original partial cDNA clone of VVP5CS are indicated with arrows. The amino acid residues believed to be involved in the feedback regulation of the *V.aconitifolia* P5CS by proline are denoted by asterisks (*).


V.vinifera
A.thaliana
V.aconitifolia
E.coli(PROA)
E.coli(PROB)

each other. The deduced amino acid sequence shown in figures 3.3 and 3.4 contains all three of these codons. Analysis of the N-terminal sequence does not indicate the presence of any protein targeting signals.

### 3.3.2 Cloning of the VVOAT cDNA

Synthesis of a partial OAT cDNA clone for screening of a cDNA library was carried out by RT-PCR, as described in the previous section, except that Sultana leaf RNA was used as the template. Following PCR with the degenerate oligonucleotide primers 1OAT and 5ROAT at a relatively low annealing temperature (45°C) a DNA fragment of 580bp was detected which was of the expected size, based on published sequences (Figure 3.6). Subsequent cloning and sequencing of the DNA fragment confirmed its identity as an OAT cDNA homologue. The DNA fragment encoded the region of 200bp to 780bp (Figure 3.5). Four positively hybridising plaques were subsequently isolated from the Shiraz berry cDNA library and one cDNA clone (4-2-1), was fully sequenced using both DNA strands as templates. The 1724bp cDNA insert encodes a 48kDa primary translation product with significant homology to δ-OATs from several organisms (Figure 3.6). VVOAT most closely resembles the *A.thaliana* δ-OAT (57% amino acid identity), however a high degree of homology is shared by all OAT sequences isolated examined (Figure 3.6). These comparisons based on amino acid sequence homology suggest that the *V.aconitifolia* OAT sequence is more similar to non-plant OAT sequences than it is to the *A.thaliana* and *V.vinifera* OAT sequences. Analysis of the N-terminal region of VVOAT, a region which is not highly conserved between the published OAT sequences, suggests the existence of a basic targeting-signal, characteristic of proteins targeted to the mitochondrial matrix (von Heijne, 1986)



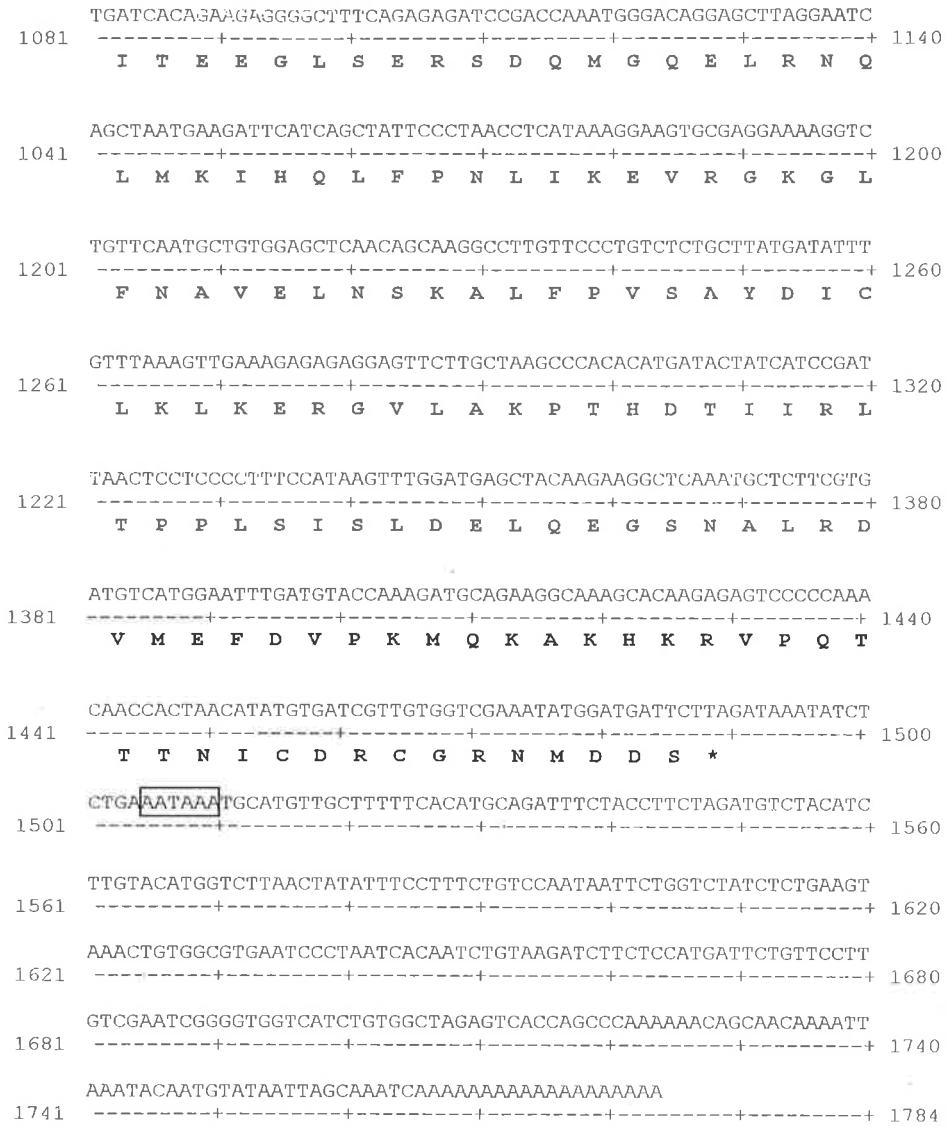
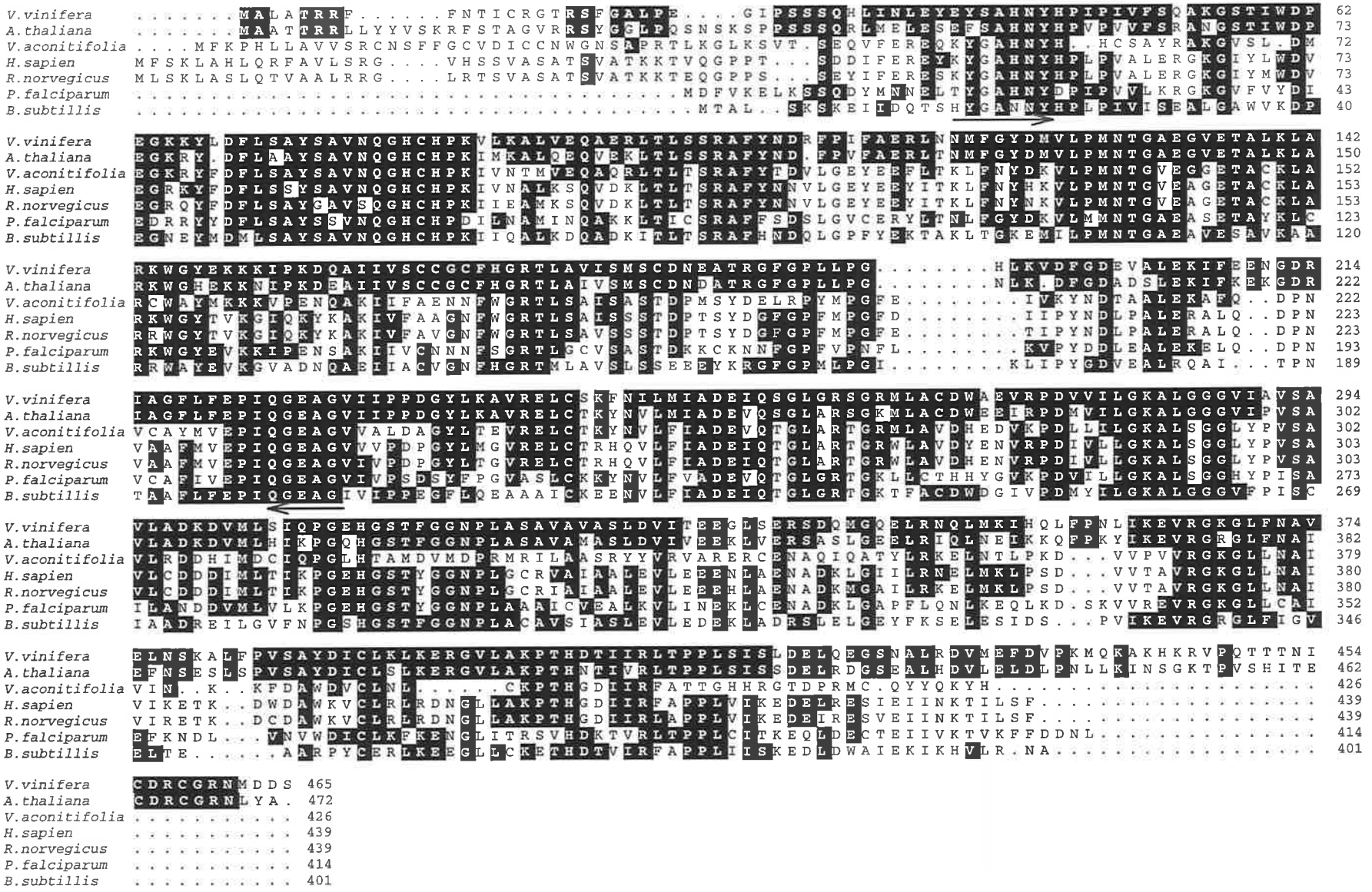



**Figure 3.5** The complete nucleotide and deduced amino acid sequence of the VVOAT cDNA obtained from a *V.vinifera* cv Shiraz 10 weeks postflowering berry cDNA library. The translational stop codon is denoted by an asterisk (*) and an in-frame stop codon upstream of the iniation codon is shaded black. A putative polyadenylation signal is boxed.

**Figure 3.6** Multiple alignment of deduced amino acid sequences of OAT proteins using the PILEUP program. Residues that are identical to the *V.vinifera* OAT sequence are highlighted. Deduced amino acid sequences were obtained from SWISSPROT and GenBank. The location and direction of degenerate oligonucleotide primers used to generate the original partial cDNA clone of VVOAT are indicated with arrows.


V.vinifera
A.thaliana
V.aconitifolia
H.sapien
R.norvegicus
P.falciparum
B.subtillis

(Figures 3.5 and 3.6).

### 3.3.3 VVP5CS and VVOAT are both encoded by single genes in the grapevine genome

Southern hybridisation analyses were performed to determine whether VVP5CS and VVOAT are encoded by single genes or if other closely related genes exist in the grapevine genome. High stringency Southern analysis with the 1.1kb VVP5CS partial cDNA clone (section 3.2.2.1) gave rise to single bands when genomic DNA was digested with *Sty*I and *Xba*I, and two bands detected after digestion with *Hind*III (Figure 3.8). This is consistent with VVP5CS being encoded by a single gene, as the partial cDNA clone used as the probe contained no *Xba*I or *Sty*I sites, and only one *Hind*III site. The three bands detected when the DNA was digested with *Pst*I (in addition to the higher band of undigested DNA) are one more than might be expected considering the partial cDNA clone contained only one *Pst*I site. However, the extra band could be explained if an intron containing a *Pst*I site occurs within the hybridising region. This is likely considering the large size (1.1kb) of the cDNA probe and the existence of multiple introns in the homologous region of P5CS clones from *A.thaliana* (Savouré *et al.*, 1995; Strizhov *et al.*, 1997). Washing the Southern blot at two different levels of stringency for detection of nucleotide sequences with greater than 65% or 95% identity, respectively, (Meinkoth and Wahl, 1984) produced identical patterns of hybridisation so only the high stringency blot is presented here (Figure 3.8). It remains possible that other genes with lower homology to P5CS exist, but they were not detected using the methods employed here.

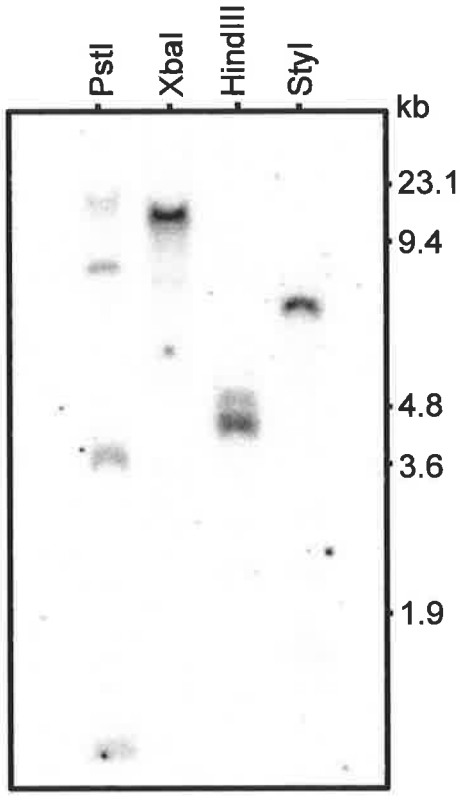



**Figure 3.8** Southern analysis of grapevine genomic DNA indicates that VVP5CS is encoded by a single gene. DNA isolated from *V.vinifera* cv. Chardonnay was digested with the restriction enzymes *Pst*I, *Xba*I, *Hind*III, and *Sty*I, probed with a 1.1kb fragment of VVP5CS cDNA, then screened under high and low stringencyconditions. Since both sets of conditions produced identical results, only those obtained after the high stringency screen are shown.

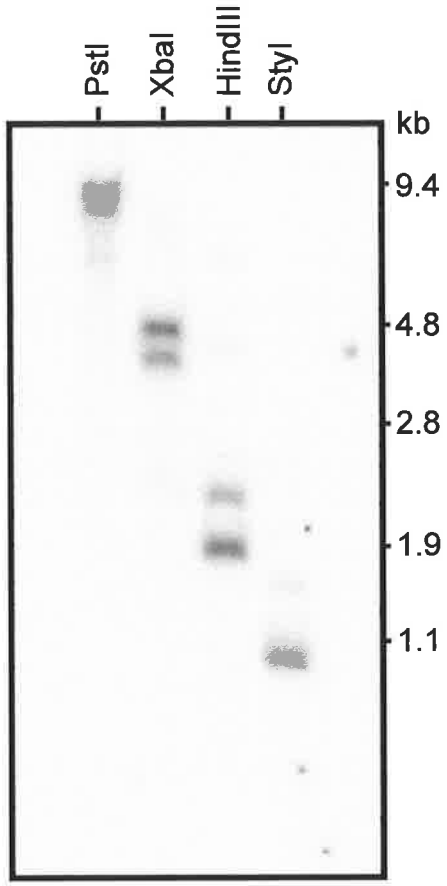



**Figure 3.9** Southern analysis of grapevine genomic DNA indicates that VVOAT is encoded by a single gene. DNA isolated from *V.vinifera* cv. Muscat Gordo was digested with the restriction enzymes *Pst*I, *Xba*I, *Hind*III and *Sty*I, probed with the partial OAT cDNA clone (580bp), then screened under high and low stringency conditions. Both conditions produced identical results, therefore only those obtained after the high stringency screen are shown.

The Southern hybridisation pattern obtained for VVOAT (Figure 3.9) also indicated that it is encoded by a single gene in the *V.vinifera* genome. An identical pattern of hybridisation was observed when the blot was washed at both high and low stringencies as described above. The partial cDNA clone (section 3.2.3.1) used as a probe contained both a *Sty*I site and an *Xba*I site, which explains the detection of two hybridising bands when the DNA was digested with these enzymes. There are no *Pst*I or *Hind*III sites in the cDNA probe, therefore the detection of two bands when the DNA was digested with these suggests that the probed region spanned an intron containing a *Pst*I site and a *Hind*III site. Indeed partial sequencing of a genomic clone for VVOAT isolated from a Shiraz genomic library (data not shown), indicates that an intron of unknown size is present in this region.

### 3.3.4 Expression of VVP5CS (6-His+) in *E.coli* and production of polyclonal antibodies

IPTG induction of VVP5CS pET14-b (Figure 3.1) in *E.coli* grown at $37^0$C resulted in expression of VVP5CS (6-His+) as a predominantly insoluble 82.6kDa protein (Figure 3.10A). The insoluble VVP5CS (6-His+) was purified by affinity chromatography and SDS-PAGE and used to produce polyclonal antibodies. The specificity of the anti-VVP5CS antibodies was verified by immunoblot analysis of the recombinant protein preparation (Figure 3.10B). A small amount of soluble VVP5CS (6-His+) was recovered from extracts of *E.coli* containing VVP5CS pET14b when the cells were grown post- induction at $16^0$C (Figure 3.10C).

### 3.3.5 Enzymatic analysis of recombinant VVP5CS

VVP5CS (6-His+), when expressed as soluble protein in *E.coli* and purified by affinity chromatography had P5CS activity as demonstrated by the ATP and glutamate dependent consumption of NADPH according to Garcia-Rios *et al.* (1997). The production of active enzyme was not straightforward because of its insolubility and instability, but could be enhanced by post-induction incubation of the *E.coli* cultures at $16^0$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/mg (at $37^0$C, 50 mM glutamate) which is similar to that reported for the two component activities of *V.aconitifolia* P5CS (Zhang *et al.*, 1995). In the presence of 5 mM glutamate, VVP5CS was sensitive to feedback inhibition by proline, with a 50% reduction in activity at 25 mM proline (Figure 3.11). In the presence of 50 mM glutamate, less inhibition was seen, with only a 33% reduction in activity at 75 mM proline.

### 3.3.6 Expression of VVOAT (6xHis+) in *E.coli* and production of polyclonal antibodies

IPTG induction of VVOAT pET14b (Figure 3.2) in *E.coli* grown at $37^0$C resulted in expression of VVOAT (6-His+) as a predominantly insoluble 48kDa protein (Figure 3.12A). The insoluble VVOAT protein was purified by affinity chromatography and SDS-PAGE and used to produce polyclonal antibodies. The specificity of these antibodies was demonstrated by immunoblot analysis of the recombinant protein preparation (Figure 3.12B). A small quantity of soluble protein was recovered when bacterial cells were grown after induction at $16^0$C.

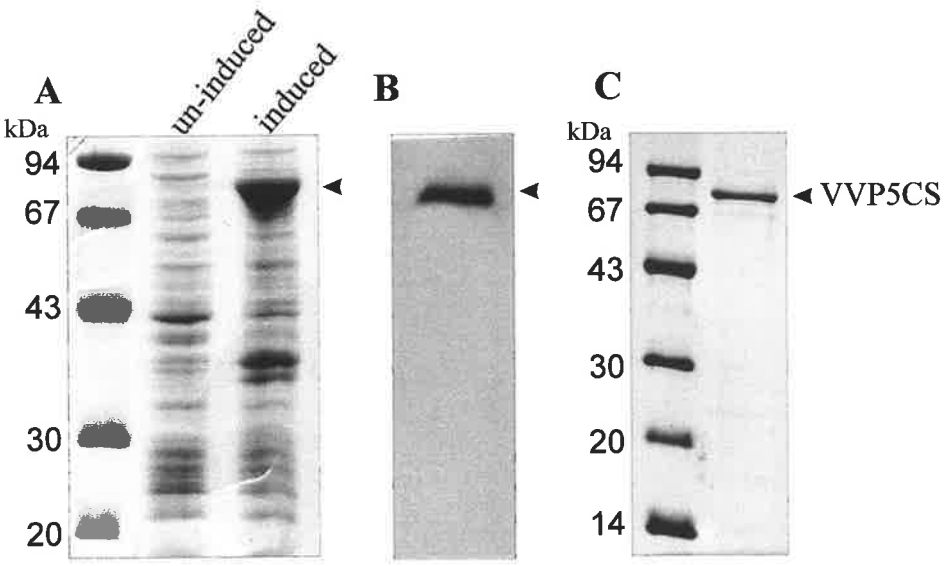



**Figure 3.10** **A.** Expression of recombinant VVP5CS in *E.coli* grown and induced at 37°C resulted in predominantly insoluble VVP5CS protein which was purified by affinity chromatography and SDS-PAGE used to produce polyclonal antibodies. **B.** The specificity of the polyclonal antibodies was verified by western blot analysis of the recombinant protein preparation. **C.** Soluble VVP5CS was purified from *E.coli* cells induced and grown at 16°C. This material was used in VVP5CS enzyme assays.

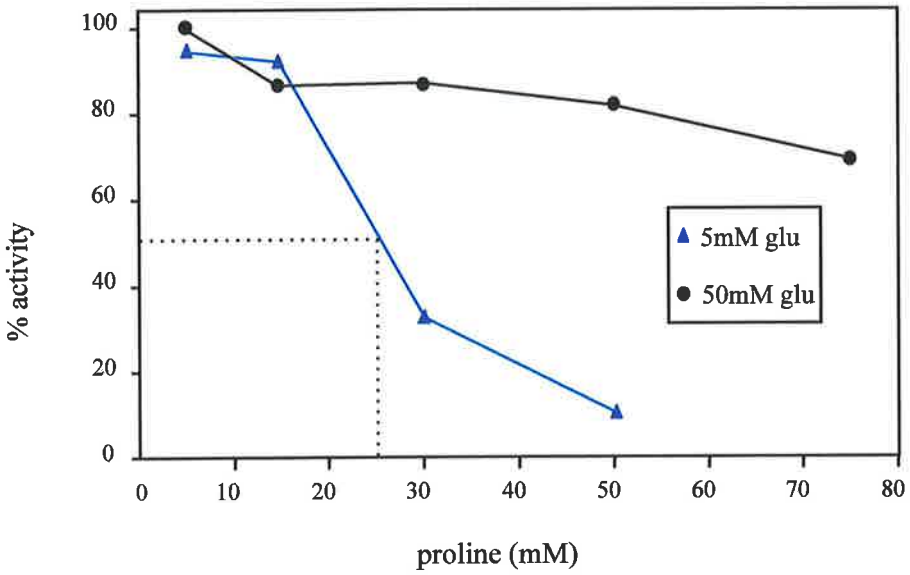



**Figure 3.11** Proline feed-back inhibition of VVP5CS expressed in *E.coli* and purified by affinity chromatography. P5CS assays were conducted in the presence of the proline and glutamate concentrations indicated. Results are expressed as % of specific activity in the absence of proline.

### 3.3.7 Enzymatic analysis of recombinant VVOAT

Soluble recombinant VVOAT (6-His+) produced in *E.coli* grown after induction at $16^0$C and purified by affinity chromatography possessed ornithine aminotransferase activity when assayed according to the method of Kim *et al.* (1994). This activity was dependent on the presence of α-ketoglutarate and L-ornithine and was inhibited by pre-incubation of the enzyme with the suicide substrate gabaculine and also pre-incubation of the enzyme in boiling water (Table 3.1).

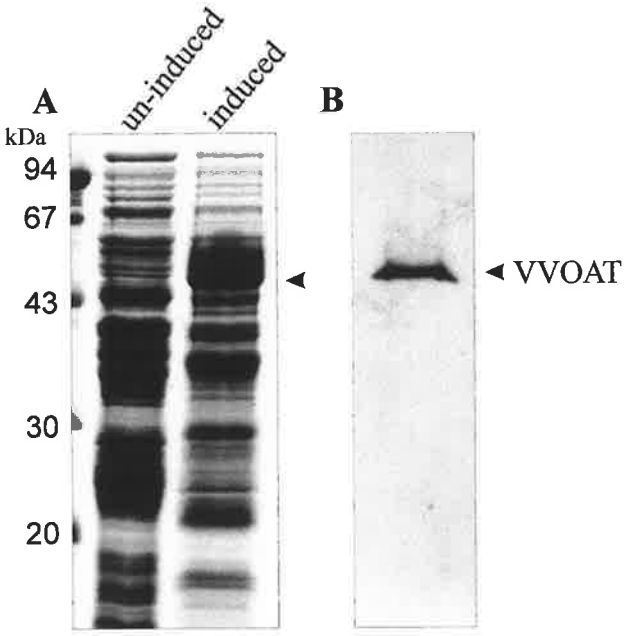



**Figure 3.12 A**. Expression of recombinant VVOAT in *E.coli* grown and induced at 37°C resulted in predominantly insoluble VVOAT protein which was purified by affinity chromatography and SDS-PAGE and used to produce polyclonal antibodies. **B**.The specificity of these antibodies was verified by western blot analysis of the crude recombinant protein preparation.

**Table 3.1** The activity of soluble recombinant VVOAT is dependent on α-ketoglutarate and ornithine, is inhibited by preincubation of the enzyme with 50μM gabaculine (two hours) and also by preincubation of the enzyme in boiling water for 10 min. Approximately 30μg of total soluble extract from *E.coli* cells not harbouring pET14b-VVOAT was used as a negative control.

| treatment | % of maximum VVOAT activity |
|---|---|
| no α-ketoglutarate | 2.0 |
| no ornithine | 0.2 |
| 50μM gabaculine | 0.1 |
| 10 min at $100^0$C | 0.1 |
| *E.coli* extract | 2.7 |

## 3.4. Discussion

Whilst the presence of significant concentrations of free amino acids in ripe grape berries has been known for some time, there is relatively little work information on the capacity of the berry to synthesise these amino acids *in situ*. Glutamine synthetase (GS) (EC 6.3.1.2) and glutamate dehydrogenase (GDH) (EC 1.4.1.2) activities were detected in *V.vinifera* cv. merlot berries, suggesting that berries have the ability to assimilate ammonium by the GS pathway and reductive amination (Ghisi *et al.*, 1984). The activity of certain members of the Krebs-Henseleit pathway, namely arginase and ornithine transcarbamylase (OTC) (EC 2.1.3.3), have been detected throughout berry development in *V.vinifera* (Roubelakis-Angelakis and Kliewer, 1981). It was demonstrated that both enzymes reached a peak of activity at veraison and that OTC activity increased in extracts of berries from vines supplied with exogenous nitrogen. In addition, aspartate aminotransferase (AspAT) (EC 2.6.1.1), an α-aminotransferase which synthesises aspartate and α-ketoglutarate was reported to be highly active in berries of *V.vinifera* cv. Carignane (Sauvage *et al.*, 1991). The isolation of cDNAs encoding the key proline biosynthetic enzymes P5CS and OAT from *V. vinifera* berries as described in this thesis, suggests that proline synthesis also occurs *de novo* in berry tissue, potentially via both the glutamate and ornithine pathways. OAT activity, but not P5CS activity, has now also been detected in crude berry extracts (discussed in Chapters 4 and 5).

Analysis of the predicted amino acid sequence of VVP5CS reveals a very high degree of homology with P5CS clones isolated from other plant species as well as GK and GPR from bacteria. This indicates that the enzyme has been well conserved throughout

evolution plays a vital role in cellular metabolism. The bifunctional enzyme in plants and mammals appears to have evolved through fusion of two genes, otherwise organised as an operon in bacteria. This idea is supported by the recent discovery of a P5CS cDNA from tomato (*tomPRO1*) which encodes a single bifunctional protein in tomato, but when expressed in *E.coli*, encodes two smaller proteins (Garcia-Rios *et al.*, 1997). This cDNA has both a stop codon and a shift in reading frame within the region where the two enzyme domains overlap and hence, appears to function as a polycistronic gene in bacteria. The mechanism by which a single large transcript is produced from *tomPRO1* in plants is not yet known.

Site directed mutagenesis of the *V. aconitifolia* P5CS revealed that changes in a particular group of amino acids appeared to reduce the sensitivity of the enzyme to feedback inhibition by proline (Zhang *et al.*, 1995). VVP5CS has an identical sequence to the wild-type *V.aconitifolia* P5CS in this region (Figure 3.4), suggesting that it is sensitive to feedback inhibition in a manner similar to the *V.aconitifolia* enzyme. To investigate the properties of VVP5CS further, the recombinant enzyme produced in *E.coli* was assayed and it was demonstrated that it is subject to feedback inhibition by proline, and that the level of inhibition is influenced by the concentration of glutamate, similar 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 proline was required to achieve 50% inhibition of VVP5CS activity in the presence of 5 mM glutamate, while more than 75 mM proline was required in the presence of 50 mM glutamate. This level of feedback inhibition is considerably lower than that observed for the γ-glutamyl kinase activity of *V.aconitifolia* (50% inhibition of the enzyme activity at

5 mM proline, 50 mM glutamate) (Zhang *et al.*, 1995) and orders of magnitude lower than that reported for the tomato P5CS encoded by *tomPRO1* (50% inhibition of enzyme activity at 0.02 mM proline, 10 mM glutamate) (Garcia-Rios *et al.*, 1997). The relative insensitivity of VVP5CS to feedback inhibition by proline indicates that the capacity for proline synthesis via P5CS could remain high throughout berry development even when proline concentrations reach almost 15 mM (Table 2.1).

VVP5CS has three in-frame methionines in its N terminal region, therefore the actual iniation codon has not yet been determined. Interestingly, investigation of the *A. thaliana* P5CS (At-PCS) promoter revealed that it had two functional transcriptional start sites, the significance of which is unknown. These observations suggest that there may be some form of transcriptional or translational regulation of P5CS that is yet to be identified.

Southern analyses suggest that VVP5CS is encoded by a single gene in the *V.vinifera* genome. The VVP5CS cDNA, therefore, can be used as a probe to effectively analyse the relationship between P5CS expression and proline accumulation in *V.vinifera* berries. It cannot be fully discounted however, that other P5CS-like genes are present, although these would have to have less than ~ 65% homology to the VVP5CS cDNA identified here. Initially, P5CS was reported to be encoded by a single gene in *A.thaliana* by two separate groups (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995), and it was only recently that a second P5CS gene was shown to exist in the *A. thaliana* genome (Strizhov *et al.*, 1997). The second gene is 90% identical at the nucleotide level to At-PCS1 and it is not clear why this additional P5CS from *A.thaliana* was not identified in earlier studies. Two P5CS genes, *tomPRO1* and *tomPRO2* have also been

identified in tomato, however as discussed above, the *tomPRO1* gene has unusual structural characteristics and has very low homology (<35%) to other published P5CS sequences (Garcia-Rios *et al.*, 1997; Fujita *et al.*, 1998). The functional significance of *tomPRO1* has not yet been determined.

The OAT cDNA was relatively difficult to amplify by RT-PCR from *V.vinifera* RNA and did not appear to be highly represented in the *V.vinifera* berry cDNA library (data not shown). This suggested that the VVOAT gene is not very highly expressed in the grapevine tissues examined (discussed further in Chapter 5). A low level of expression may explain why, at the time of the cloning of VVOAT, only one other plant OAT cDNA, that of *V.aconitifolia*, had been isolated. The latter was cloned by functional complementation of a proline auxotroph of *E.coli*. Based on homologies between the *V. aconitifolia* OAT sequence and that from rat, mouse, human and *P.falciparum*, degenerate primers were designed and a RT-PCR product generated from *V. vinifera* leaf RNA, which had a high degree of sequence similarity at the amino acid level with the other OAT sequences. Interestingly the deduced amino acid sequence of VVOAT showed less homology with the *V.aconitifolia* OAT than with other published OAT sequences, and greatest homology with the *A.thaliana* OAT sequence. The N-terminal regions of all known OAT sequences are not well conserved although all, except the yeast and bacterial proteins, are thought to encode mitochondrial targeting peptides (Mueckler and Pitot, 1985; Inaga *et al.*, 1986; Degols, 1987; Delauney *et al.*, 1993; Gardan *et al.*, 1995, Roosens *et al,.* 1998). The three dimensional structure of the human OAT protein suggests that the N-terminal region is exposed and highly susceptible to protease attack (Ohura *et al.*, 1982). This may be the reason why the actual N terminal

amino acid of the mature protein has yet to be determined in mammalian species (Shen *et al.*, 1997).

The results from Southern analyses indicate that VVOAT is encoded by a single gene in the *V.vinifera* genome. In *A. thaliana* and *V.aconitifolia*, OAT also appears to be encoded by a single gene. However, as for P5CS, there remains the possibility that distantly related homologues are present in grapevine or indeed other plant species.

The VVOAT protein expressed in *E.coli* possessed OAT activity which was inhibited by the suicide substrate gabaculine. As a δ-OAT protein has not yet been purified to homogeneity from any plant source, any other information currently available on the kinetic properties of plant δ-OATs is predominantly derived from enzymic measurements using crude plant extracts (discussed further in Chapter 5). The one exception is the *V.aconitifolia* δ-OAT which was expressed in an *E.coli* auxotroph and partially purified from bacterial cell extracts (Delauney *et al.*, 1993). Using this preparation the $K_m$ values for ornithine and α-ketoglutarate were reported to be 2 mM and 0.75 mM respectively, although no data to support these kinetic measurements was shown.

The information and tools gained from the isolation and characterisation of the VVP5CS and VVOAT cDNAs and recombinant proteins provides an excellent basis for investigating their characteristics *in vivo*. In the following chapters the patterns of VVP5CS and VVOAT expression during grape berry development and in other *V.vinifera* tissues are examined.

# Chapter 4
# *Expression of VVP5CS in* V.vinifera

## 4.1 Introduction

Osmotic stress induces proline accumulation in the vegetative tissues of many plant species. In such a situation it appears that proline acts as a compatible solute, protecting the cell from the various deleterious effects of osmotic stress (see Chapter 1 section 1.4 for a review). To better understand the mechanisms of proline accumulation in plants, cDNAs encoding the key enzymes thought to be involved have been cloned and their expression in plants under various conditions analysed. The majority of studies have focused on the expression of P5CS and P5CR in *A.thaliana* and *V.aconitifolia* and have demonstrated that P5CS is the key regulatory enzyme in the glutamate pathway of proline synthesis and that enhanced proline biosynthesis during osmotic stress is due to transcriptional induction of the P5CS gene (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, Roosens *et al.*, 1998). For example, increases in P5CS mRNA levels precede proline accumulation in osmotically stressed *A. thaliana* tissue (Hu *et al.*, 1992). Further studies have indicated that treatment of plants with moderately high levels of ABA or NaCl also induces P5CS gene expression and leads to an enhanced synthesis and accumulation of proline (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995; Peng *et al.*, 1996; Savouré *et al.*, 1997). The role of P5CR in the osmotic stress response was not as readily established due to conflicting reports of whether P5CR gene expression was affected by osmotic stress conditions (Verbruggen *et al.*, 1993; Yoshiba *et al.*, 1995). However, the demonstration that overexpression of P5CR in tobacco plants did not lead to an increase in proline concentration has provided compelling evidence that P5CR does not catalyse the regulatory step in proline biosynthesis or

accumulation (Szoke *et al.*, 1992). A subsequent report demonstrating that overexpression of the *V.aconitifolia* P5CS in transgenic tobacco plants leads to accumulation of ten fold more proline than in wild type controls (Kishor *et al.*, 1995) confirmed that P5CS is indeed the rate limiting and key regulatory enzyme in the glutamate pathway of proline synthesis. This chapter describes the characterisation of P5CS gene expression in *V.vinifera*, with an emphasis on its expression during grape berry development. The aim of this study was to determine if the high level of proline accumulation which occurs in developing grape berries (Chapter 2) could be due to transcriptional activation of the VVP5CS gene, similar to the induction observed in osmotically stressed plant tissues.

## 4.2 Materials and Methods

### 4.2.1 RNA isolation

RNA was extracted from grapevine tissues as described in section 3.2.1.

### 4.2.2 Northern analysis of P5CS mRNA levels

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 Hybond N membrane (Amersham) and fixed using a UV crosslinker according to the manufacturer's instructions (Amersham). After prehybridisation at 65$^0$C for 5 h in prehybridisation solution (section 3.2.4), the membrane was incubated with radioactively labelled probe (synthesised as described in section 3.2.2.2) at 65°C for 16 h in the same solution. The membrane was washed at 65°C in 0.1 x SSC, 0.1%(w/v) SDS and the hybridising bands were detected as described for Southern analysis (section 3.2.4).

### 4.2.3 Protein extraction from plant tissue and analysis by SDS-PAGE and western blotting

Tissue frozen in liquid nitrogen was homogenised in a coffee grinder or mortar and pestle and 1-5 g was added to 2-10 ml of protein extraction buffer (500 mM Tris-Cl, pH 8.0, 5% (w/v) SDS, 10 mM DTT and 10 mM sodium diethyldithiocarbamate), followed by incubation at 95$^0$C for 5 min, then centrifuged at 3,000 *g* for 3 min. The supernatant was stored at -20°C until analysis by SDS-PAGE and western blotting (sections 3.2.6

and 3.2.9). For the isolation of proteins under non-denaturing conditions the method of Ford *et al.* (1998) was used. Briefly, frozen tissue was homogenised in ice-cold extraction buffer (100 mM Tris-Cl (pH8.0), 10% glycerol, 1 mM DTT, 10% PVPP) at a ratio of 1:5 (w/v). Homogenate was filtered through two layers of 0.45 μm nylon cloth and centrifuged at 10000 *g* for 60 min. The supernatant containing the crude extract was collected and stored at -70$^0$C until required.

## 4.3 Results

### 4.3.1 Analysis of VVP5CS mRNA levels throughout grape berry development

The stress-induced accumulation of proline in vegetative tissues has been shown in a number of plant species to correlate with the induction of P5CS gene expression resulting in enhanced levels of P5CS mRNA. In order to examine the expression of P5CS during grape berry development, RNA was prepared from *V.vinifera* cv. Chardonnay fruit sampled from four weeks postflowering through to full maturity. Northern analysis indicates that the steady-state levels of P5CS mRNA remain relatively constant throughout grape berry development, although transient increases are observed at both 4 and 12 weeks postflowering (Figure 4.1). There were no significant changes in steady-state P5CS mRNA levels which would parallel the significant increase in proline accumulation late in berry development. Thus, unlike the numerous reports on stress-induced proline accumulation in vegetative tissues, the primary basis for proline accumulation in the case of grape berry development, does not appear to be a dramatic induction of P5CS mRNA levels.

### 4.3.2 Analysis of VVP5CS protein levels throughout grape berry development

To investigate the role of post-transcriptional control of VVP5CS in the regulation of proline synthesis and accumulation, Western blot analyses were performed on protein extracts derived from the fruit of four different *V.vinifera* cultivars at different stages of development (Figures 4.2, 4.3, 4.4, 4.5). VVP5CS is clearly present throughout fruit development and does not appear to be induced post-veraison in any of the four varieties

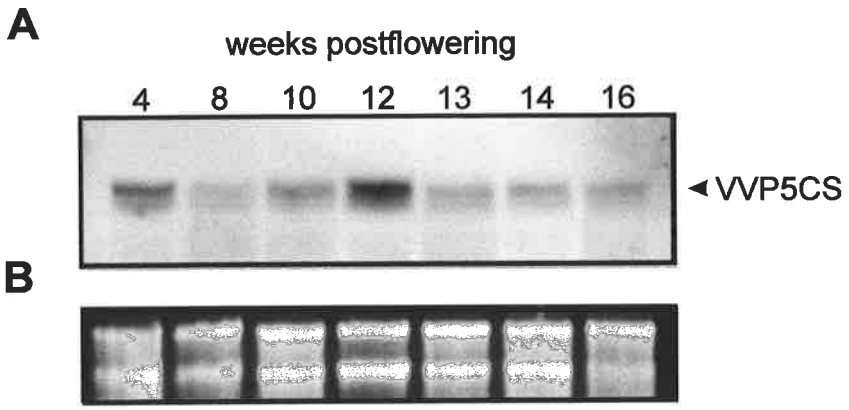



**Figure 4.1** Steady-state levels of VVP5CS mRNA throughout fruit development. **A.** Total RNA (15μg) isolated from *V.vinifera* cv. Chardonnay berries was electrophoresed, blotted onto a nylon membrane and probed with a 0.89kb fragment of VVP5CS cDNA (nucleotides 791-1680, Figure 3.3) **B.** A replica gel was stained with ethidium bromide to demonstrate the equivalence of RNA loading in each lane.

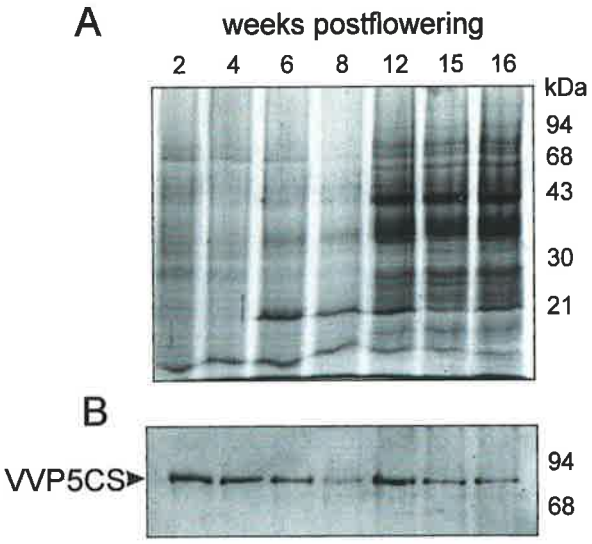



**Figure 4.2** Steady-state level of VVP5CS protein throughout development of *V.vinifera* cv. Cabernet Sauvignon berries. **A.** Proteins extracted from whole berry homogenates were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Each lane contained equivalent amounts of berry homogenate on a fresh weight basis. **B.** A replica gel was transferred to nitrocellulose membrane and subjected to western analysis using antibodies raised against VVP5CS.

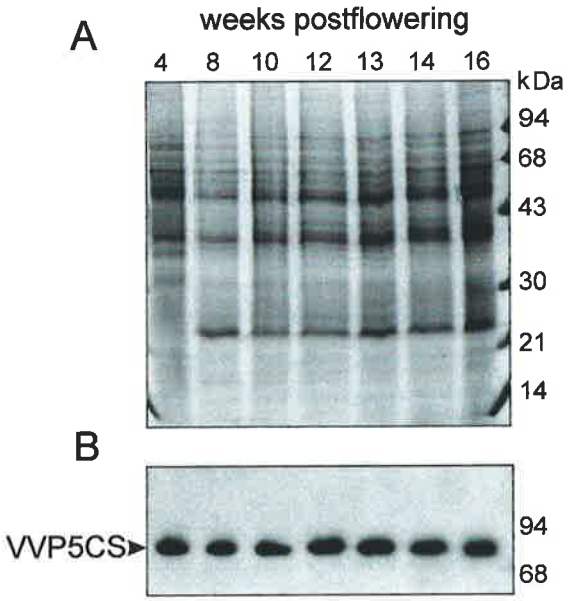



**Figure 4.3** Steady-state level of VVP5CS protein throughout development of *V.vinifera* cv. Chardonnay berries. **A.** Proteins extracted from whole berry homogenates were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Each lane contained equivalent amounts of berry homogenate on a fresh weight basis. **B.** A replica gel was transferred to nitrocellulose membrane and subjected to western analysis using antibodies raised against VVP5CS.

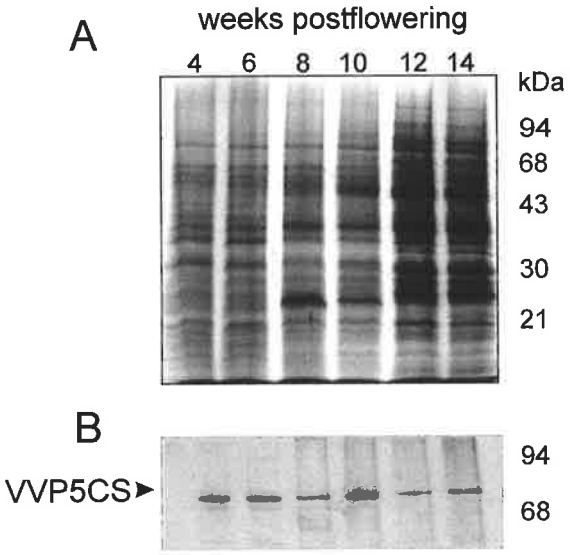



**Figure 4.4** Steady-state level of VVP5CS protein throughout development of *V.vinifera* cv. Gewurztraminer berries. **A.** Proteins extracted from whole berry homogenates were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Each lane contained equivalent amounts of berry homogenate on a fresh weight basis. **B.** A replica gel was transferred to nitrocellulose membrane and subjected to western analysis using antibodies raised against VVP5CS.

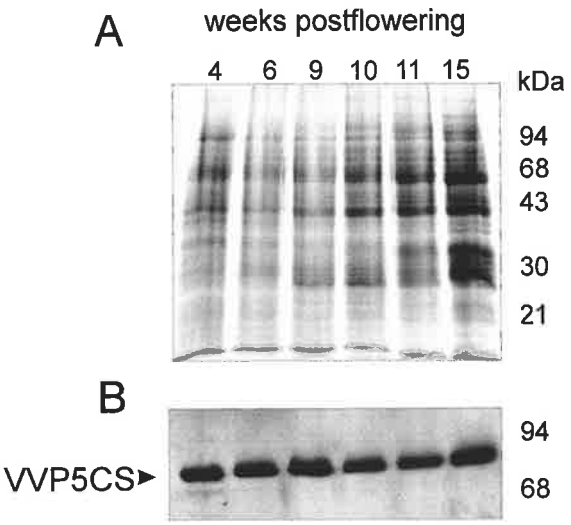



**Figure 4.5** Steady-state level of VVP5CS protein throughout development of *V.vinifera* cv. Muscat Gordo berries. **A.** Proteins extracted from whole berry homogenates were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Each lane contained equivalent amounts of berry homogenate on a fresh weight basis. **B.** A replica gel was transferred to nitrocellulose membrane and subjected to western analysis using antibodies raised against VVP5CS.

examined. Any changes in the level of VVP5CS are relatively transient. These results demonstrate that the significant increase in free proline observed late in berry development is not associated with a concurrent increase in steady-state levels of VVP5CS protein. The levels of VVP5CS illustrated in Figures 4.2, 4.3, 4.4 and 4.5 cannot be directly compared due to the nature of the detection method (ECL). Nevertheless, there are no obvious or consistent differences between any of the varieties which might correlate with the level of proline accumulated in their mature berries. Analysis of VVP5CS levels in Cabernet Sauvignon berries over development (Figure 4.2) indicates that there was a drop in levels of both VVP5CS and overall extractable protein levels at 8 weeks postflowering. This sampling date occurred during a heatwave where temperatures exceeded $40^0$C for over a week. It appears, therefore that these high temperatures had a significant effect, in terms of protein turnover, on the Cabernet Sauvignon berries and also on VVP5CS protein levels.

### 4.3.3 Analysis of VVP5CS protein levels in *V.vinifera* tissues

Approximately equal amounts of protein extracted from skin, pulp and seeds of *V.vinifera* cv. Chardonnay berries harvested at 16 weeks postflowering, flower buds (i.e. two weeks preanthesis), flowers (i.e. at anthesis), young leaves and mature leaves and roots were electrophoresed (Figure 4.6A) and subjected to Western analysis using polyclonal antibodies raised against VVP5CS (Figure 4.6B). The VVP5CS protein was detectable in all tissues except seeds and was present at highest levels in berry pulp, flower buds and roots.

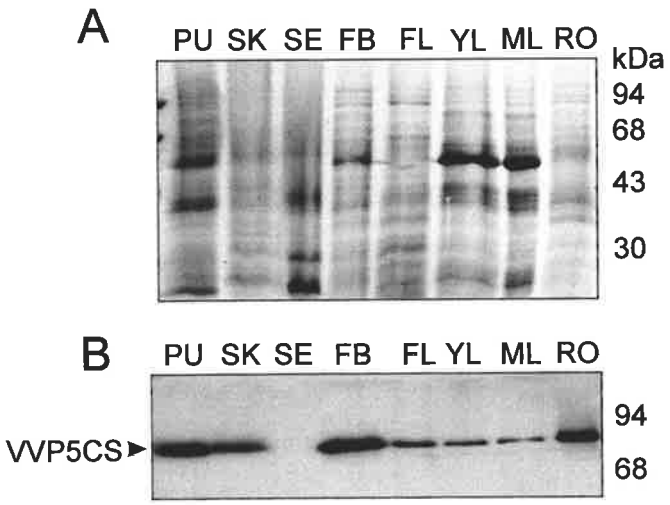



**Figure 4.6** Relative levels of VVP5CS in a range of *V.vinifera* Chardonnay tissues. **A.** Protein was extracted from berry pulp (PU), berry skin (SK), seeds (SE), flower buds (FB), flowers (FL), young leaves (YL), mature leaves (ML) and roots (RO). **B.** A replica gel was transferred to nitrocellulose membrane and subjected to western analysis using anti-VVP5CS antibodies.

## 4.4 Discussion

In order to determine which factors lead to high levels of proline accumulation in developing grape berries, the expression of the key proline biosynthetic enzyme P5CS was examined by measuring the steady-state levels of VVP5CS mRNA and protein. Northern analysis of RNA isolated from the berries of cv. Chardonnay showed that steady-state levels of VVP5CS mRNA remain relatively constant during the sixteen weeks from flowering to fruit maturity, with only transient increases seen at 4 weeks and 12 weeks postflowering. These transient increases could be a result of the peaks in berry ABA concentration observed at flowering and approximately 2-3 weeks postveraison (Coombe and Hale, 1973). Treatment with exogenous ABA has been shown to enhance levels of P5CS mRNA in *A.thaliana* seedlings (Yoshiba *et al.*, 1995; Savouré *et al.*, 1997; Igarashi *et al.*, 1997; Strizhov *et al.*, 1997). In any case, the increased VVP5CS mRNA levels at 4 and 12 weeks postflowering do not appear to be translated into significant changes in the steady-state levels of VVP5CS protein (Figure 4.2). Thus, unlike previous reports on stress-induced proline accumulation in vegetative tissues (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995; Peng *et al.*, 1996; Igarashi *et al.*, 1997), the primary basis for proline accumulation late in berry development does not appear to be induction of VVP5CS mRNA or protein levels.

Our results, which suggest that developmental accumulation of proline occurs independently of changes in P5CS gene expression, are significantly different to those in most of the literature on stress-induced proline accumulation (see Chapter 1 for review). Recent studies, however, on the expression of genes encoding P5CS isoenzymes

isolated from *A.thaliana* (Strizhov *et al.*, 1997) and tomato (Garcia-Rios *et al.*, 1997) suggest that regulation of proline biosynthesis and accumulation in plants may not be as simple as was first thought. Although the first P5CS gene isolated from *A.thaliana* (AtP5CS1) is transcriptionally induced up to 8-fold upon osmotic stress, the second *A.thaliana* P5CS gene (AtP5CS2) showed little transcriptional response to the same treatment, and was described as a 'housekeeping gene' (Strizhov *et al.*, 1997). Our results clearly demonstrate that the VVP5CS gene is not induced during berry ripening, despite steady increases in berry osmotic pressure post veraison (Downton and Loveys, 1978). It is possible, therefore, that the VVP5CS encoding clone isolated from berries may be functionally homologous to the AtP5CS2 gene from *A.thaliana*. Alternatively the 'stress' signals responsible for the induction of P5CS observed in other plants may not be operating in developing berries where changes in osmotic pressure occur relatively gradually. A recent report by Fujita *et al.* (1998), demonstrated that there may be other exceptions to the previously established connections between osmotic stress, transcriptional activation of P5CS and proline accumulation. These researchers showed that whilst tomato plants subjected to osmotic stress accumulated extremely high levels of proline (up to 100 µmol/ g fwt), only a slight change in the level of *tomPRO2* mRNA occurred. Furthermore, the expression of the second, 'dicistronic' P5CS tomato gene (*tomPRO1*) was said to be too low to account for the increase in proline that was observed during the osmotic stress response. The same researchers observed that tomato pollen contained exceedingly high levels of proline (200 µmol/g fwt), but had the lowest level of P5CS mRNA of all tomato tissues examined. These results suggest that the high amounts of free proline present in salt stressed tomato tissue and pollen are not due to substantial transcriptional activation of P5CS genes. Taken together, the results

from tomato and those reported in this thesis suggest that further investigation of other factors which may contribute to proline accumulation is warranted.

Virtually all of the published information regarding P5CS gene expression in plants is restricted to analyses at the mRNA level (Hu *et al.*, 1992; Delauney and Verma, 1993; Kishor *et al.*, 1995; Savouré *et al.*, 1995; Yoshiba *et al.*, 1995; Peng *et al.*, 1996; Strizhov *et al.*, 1997, Roosens *et al.*, 1998). Therefore it is not clear whether the transcriptional activation of P5CS under osmotic stress is also accompanied by an increase in its protein levels. A direct relationship between mRNA and protein is assumed by most researchers, however, post-transcriptional and post-translational regulation mechanisms are an important and sensitive means of controlling cellular metabolism. As mentioned above western blot analyses were used in this study to examine whether P5CS mRNA levels reflected the actual amount of P5CS protein in developing berries. The western blot results for all four *V.vinifera* cultivars examined confirm the level of VVP5CS protein remained relatively throughout development (Figures 4.2-4.5). Further there are no consistent differences observed between cultivars which accumulate different levels of proline. Indeed, in a study carried out in collaboration with the author of this thesis, it was found that the concentrations of VVP5CS protein in berries of Chardonnay (a high proline accumulating cultivar) and Gewurztraminer (a moderate proline accumulating cultivar) were very similar (Møller, unpublished results).

Clearly there are a number of points of regulation other than simple changes in P5CS mRNA and protein levels which could account for the net accumulation of free proline in developing grape berries. Western blot analysis of different grapevine tissues

demonstrated that the skin and pulp of grape berries contain relatively high levels of P5CS protein compared with other tissues such as seeds and leaves. The lack of VVP5CS protein detected in seeds may be a result of compounds present in the protein extract interfering with the analysis. Alternatively, the proline content in the seeds may arise through the import of the amino acid from another source and not synthesis via VVP5CS. The high level of VVP5CS protein in skin and pulp suggests that the capacity for proline synthesis, in terms of enzymic protein, remains relatively high in berry tissue throughout development. The lack of proline accumulation early in development could be explained by a deficiency of glutamate, the VVP5CS substrate. This does not appear to be the case, however, as the concentrations of both glutamine and glutamate are relatively high in the early stages of berry development (see Chapter 2).

Modification of P5CS enzyme activity could be involved in regulating proline accumulation, for example by the presence of an inhibitor of P5CS early in grape berry development. To investigate this aspect of regulation, attempts were made to assay crude protein extracts from berries for P5CS activity, however no activity could be detected. P5CS activity could also not be detected by other workers in extracts of control tobacco or *V.aconitifolia* plants, but was measurable in extracts of transgenic tobacco plants overexpressing the *V.aconitifolia* P5CS cDNA (Zhang *et al.*, 1995; Kishor *et al.*, 1995). This suggests that the level of P5CS activity may be too low to be detected in wildtype plant tissues. The activity of glutamine synthetase also interferes with the P5CS assay as it competes for the substrate glutamate. This problem was overcome by ammonium sulphate fractionation of the proteins extracted from plant tissue (Zhang *et al.*, 1995). However, this fractionation alters the metabolic context of

the enzyme preparation, potentially removing any possible inhibitors and hence may also alter its relative activity. Therefore, due to the aforementioned problems and time considerations, the changes in VVP5CS activity *in situ* during berry development remain to be determined.

The results described in this chapter demonstrate that proline accumulation late in grape berry development is independent of changes in steady-state levels of both P5CS mRNA and protein. This suggests that the mechanisms regulating proline accumulation during normal plant development may be quite different to those operating during the abiotic stress response. In Chapter Five experiments designed to investigate whether the alternative pathway of proline synthesis, namely that catalysed by δ-OAT plays, a role in grape berry proline accumulation, will be described.

# Chapter 5
# *Expression of VVOAT in* V.vinifera

## 5.1 Introduction

Proline can be synthesised in plants by two different pathways. The most studied route is the conversion of glutamate to proline by the enzymes P5CS and P5CR. Alternatively proline can be synthesised via the less well characterised OAT pathway, which is linked with arginine metabolism (Chapter 1). Many studies have demonstrated that the glutamate pathway is the predominant pathway of proline synthesis in osmotically stressed plants (Rhodes and Bressan, 1986; Hu *et al.*, 1992; Delauney *et al.*, 1993, Roosens *et al.*, 1998). In contrast, the significance of OAT to proline synthesis and accumulation in plants is still subject to debate. This is due, in part, to conflicting reports in the literature on its relative contribution to proline accumulation in water stressed plants. The level of δ-OAT mRNA in *V.aconitifolia* plantlets was reduced by salt stress (Delauney *et al.*, 1993). In contrast, the level of δ-OAT mRNA in 12 day old *A.thaliana* plantlets was induced by salt stress, although no δ-OAT mRNA could be detected in 4 week old *A.thaliana* plants under the same conditions (Roosens *et al.*, 1998). Earlier reports demonstrated that production of proline from labelled arginine and ornithine is enhanced by water stress (Boggess and Stewart, 1976; Boggess *et al.*, 1976). OAT enzyme activity was measured in extracts of water and salt stressed radish (*Raphanus sativus*) cotyledons and water stressed Ragi (*Eleusine coracane*) leaves, and was demonstrated to be increased in these plants in conjunction with an increase in proline concentration (Kandpal and Rao, 1982; Hervieu *et al.*, 1994). In the radish cotyledons, inhibition of OAT activity *in vivo* with gabaculine reduced the stress induced increase in proline concentration by up to 64% (Hervieu *et al.*, 1994). Experiments with transcription and translation inhibitors indicated that the stress-

induced increase in OAT activity was due to induction at the level of translation, rather than transcription, indicating the importance of measurements of OAT protein levels and activity in studies of its regulation (Billard *et al.*, 1997).

The level of δ-OAT mRNA was shown to be increased in *V.aconitifolia* plantlets supplied with excess nitrogen (Delauney *et al.*, 1993). It was suggested that the accumulation of arginine or ornithine under high nitrogen input may induce OAT gene expression. OAT gene expression is induced by exogenous arginine in yeast (Brandriss and Magasanik, 1980). Roosens *et al.* (1998) suggested that the prime role of δ-OAT in plants is the recycling of amino acids such as glutamate when levels of nitrogenous compounds are high, for example in young plants. The hypothesis that δ-OAT is important when levels of nitrogenous compounds are high is supported by previous work in which it was shown that in cotyledons and germinating seeds, where large nitrogen reserves in the form of arginine need to be mobilised, the ornithine produced from the hydrolysis of arginine, is converted into P5C by δ-OAT and thence into proline or glutamate (Mazelis and Fowden, 1969; Splittstoesser and Fowden, 1973). Further, all aminotransferases are, in general, involved in nitrogen redistribution in addition to other aspects of metabolism including: amino acid synthesis, photorespiration and synthesis of secondary metabolites (for review see Lea and Ireland, 1999). In this chapter the expression of VVOAT in developing grape berries is examined.

## 5.2 Materials and Methods

### 5.2.1 RNA isolation

Total RNA was extracted from grapevine tissues as described previously in section 3.2.1.

### 5.2.2 Qualitative RT-PCR analysis of VVOAT mRNA levels

First strand cDNAs were generated with RNase H reverse transcriptase (Superscript II, Gibco BRL) according to the manufacturer's instructions using 2 μg total RNA isolated from berries and various other *V.vinifera* tissues (as described in section 4.2.1 above) as template and a $(dT)_{15}$ primer. A primer pair for subsequent PCR was chosen that would include an intron within the amplified region, to enable ready differentiation between the RT-PCR product and any product resulting from amplification of genomic DNA which might contaminate the RNA preparations. The primers were as follows:

OAT1B 5'-GGAGCTTTGGTGCTCTCCCTG-3'

RACE2OAT 5'-CCTTTGGATGACAGTGTCCCTG-3'.

These primers are located on the VVOAT cDNA sequence at +139-159bp and +327-348bp respectively (Figure 3.5). The expected size of the RT-PCR product was 210bp. Based on preliminary DNA sequence determination of a VVOAT genomic clone, a fragment of at least 300bp would be expected if genomic DNA was amplified (data not shown). The PCR mixes were prepared as described in section 3.2.2.1, then denatured at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min 30 sec, followed by 1 min 30 sec of extension at 72°C. The reaction was

completed by a 3 min extension at 72°C. PCR products were resolved in a 0.8% agarose gel buffered in TAE and visualised with ethidium bromide under UV light.

### 5.2.3 Protein extraction from plant tissue

Proteins were extracted from plant tissue as described in section 4.2.3

### 5.2.4 Protein quantification and western analysis

All techniques were performed as described in sections 3.2.7 and 3.2.9

### 5.2.5 Measurement of VVOAT enzymic activity throughout fruit development

OAT assays were carried out as described previously (section 3.2.12). Protein extracts were prepared as described in section 4.2.3 from cv. Chardonnay berries at each stage of development. However, before the assays were conducted, 3 ml of crude berry protein extract was desalted on a Biogel P-6 desalting column (BioRad) and eluted in 4 ml buffer consisting of (200 mM Tris-Cl (pH 8.8), 50 mM NaCl, 10 mM DTT, 20% (v/v) glycerol). The eluants were then concentrated by ultrafiltration using a YM-30 membrane (Diaflo, Centricon).

### 5.2.6 Gel filtration chromatography of *V.vinifera* cv. Gewurztraminer crude berry protein extract

Crude protein extracted from 10 g of cv. Gewurztraminer berries sampled at 12 weeks postflowering ($18^0$Brix) was subjected to gel filtration on a Superdex-200 column (26/60, Hiload, Pharmacia) equilibrated in 25 mM Tris-Cl (pH 8.0) and 50 mM NaCl at $4^0$C. Proteins were chromatographed at a flow rate of 100 ml/h and 10 ml fractions

collected and stored at -70$^0$C until required. Selected fractions were concentrated by ultrafiltration using a YM-30 membrane (Diaflo, Centricon) and assayed for presence of OAT by determination of enzyme activity as described in section 3.2.12, and western blot analysis as described in section 3.2.9.

## 5.3 Results

### 5.3.1 Analysis of VVOAT mRNA levels throughout grape berry development and in other grapevine tissues

Conventional Northern blot analysis of total berry RNA using a 580bp fragment of VVOAT as a probe yielded no detectable hybridisation signal for VVOAT mRNA. However, the isolation of both a RT-PCR product and cDNA clone using berry RNA as the template (Chapter 3) suggested that VVOAT is expressed during berry development, albeit at low levels. The more sensitive qualitative RT-PCR approach was therefore used to confirm the presence or absence of VVOAT transcript during the development of Chardonnay berries. The results in figure 5.1A show that VVOAT transcripts appear to be present throughout berry development. However, as a constitutively expressed control transcript has not yet been defined for use in quantitative RT-PCR of RNA from grapevine tissues, including berries, a quantitative evaluation of the levels of VVOAT mRNA cannot be made at this stage. The use of the qualitative RT-PCR method further demonstrated that VVOAT is expressed in a variety of *V.vinifera* tissues (Figure 5.1B), although again at apparently low levels since the mRNA could not be detected using Northern blot analysis of the same RNA preparations.

### 5.3.2 Analysis of VVOAT protein levels throughout grape berry development and in other grapevine tissues

The production of a polyclonal antibody against VVOAT (section 3.3.6) enabled us to examine the levels of VVOAT protein present in a variety of grapevine tissues.

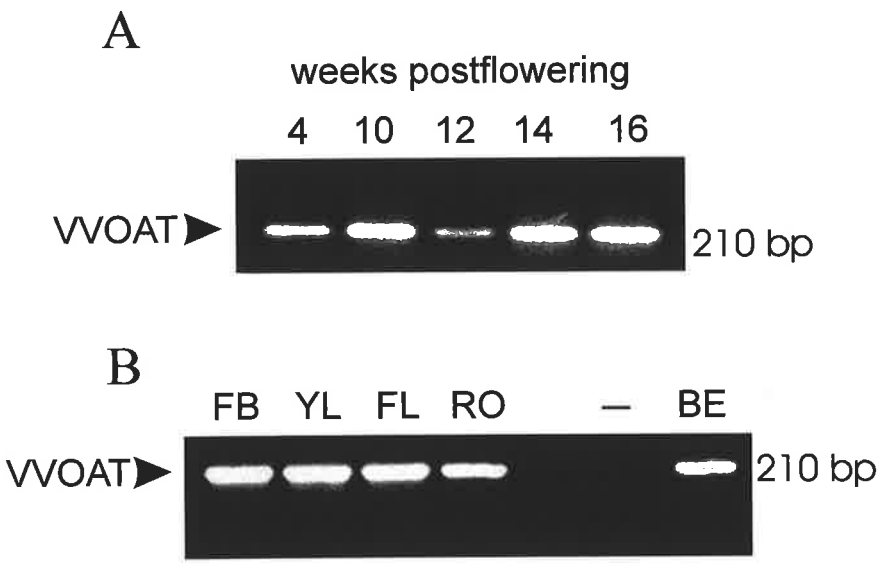



**Figure 5.1 A.** RT-PCR analysis of total RNA extracted from *V.vinifera* cv. Chardonnay at several developmental time points demonstrates the presence of VVOAT transcripts throughout berry development. (A minus template control was run on a separate part of the gel and contained no amplified products, data not shown).**B.** RT-PCR analysis of total RNA extracted from a variety of grapevine tissues indicates that VVOAT transcript is present in all the tissues examined. FB, flower buds; YL, young leaves; FL, flowers; RO, roots; -, minus template control; BE, mature Chardonnay berries.

Although many enzymatic studies have been previously carried out this is the first known report of determination of actual VVOAT protein levels in any plant. The VVOAT protein is present in the developing berries of all four varieties examined (Figures 5.2 A, B, C and D). The protein appears to remain at a relatively constant steady-state level throughout development in berries of Chardonnay and Cabernet Sauvignon and is not significantly induced at the time when berry proline levels increase dramatically. In contrast, there is an increase in VVOAT steady-state levels in berries of Gewurztraminer and Muscat Gordo during development. Interestingly, VVOAT appears to be present as a doublet in these protein extracts although the intensity of the upper band varied from sample to sample.

VVOAT protein was also detected by western analysis in the roots flowers, young leaves and mature leaves of *V.vinifera* cv. Chardonnay (Figure 5.3). Again, two bands were detected with the anti-VVOAT antibody in protein extracts from all tissues. Within the tissues of a mature Chardonnay berry, it appears that the VVOAT protein is present in the pulp, with very little being detected in protein extracts from skin and seed.

### 5.3.3 Measurement of VVOAT enzymic activity in developing *V.vinifera* berries

To determine whether modification of VVOAT enzyme activity could be involved in regulating proline accumulation in developing berries protein extracts of *V.vinifera* Chardonnay berries were desalted and concentrated then assayed for OAT activity. It was determined that VVOAT activity per g/fwt tissue remains relatively constant until 12 weeks postflowering, after which it increases slightly (Figure 5.4B). If OAT activity is expressed on the basis of mg total protein in the extract (Figure 5.4A), there appears

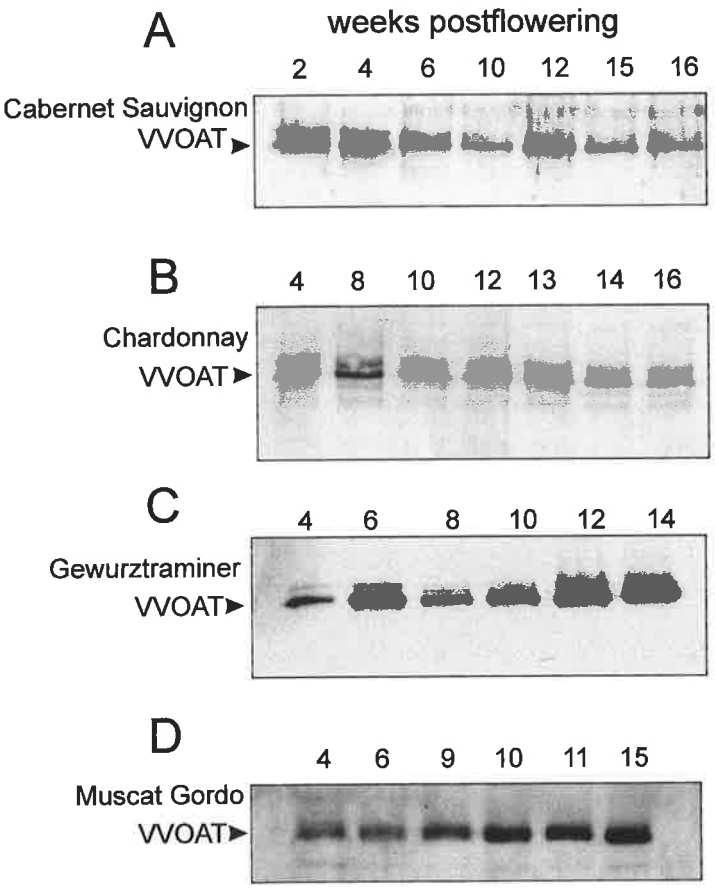



**Figure 5.2** Steady-state levels of VVOAT protein in *V.vinifera* berries throughout development. Proteins extracted from whole berry homogenates were separated by SDS-PAGE, transferred to nitrocellulose membranes and subjected to western analysis using antibodies raised against VVOAT. Berry protein extracts are loaded such that all lanes within each panel contain equivalent amount of total protein. **A.** Cabernet Sauvignon, **B.** Chardonnay, **C.** Gewurztraminer, **D.** Muscat Gordo.

to be two peaks of activity at 4 and 13 weeks postflowering respectively, however in general the activity levels remain relatively constant throughout berry development. The production of P5C by this assay was significantly decreased (<90%) in the absence of α-ketoglutarate, a substrate of OAT, and in the presence of 50 μM gabaculine. This confirmed the specificity of the assay for measurement of OAT activity.

### 5.3.4 Assessment of the specificity of anti-VVOAT antibodies

The specificity of the antibodies raised against VVOAT was investigated because of the presence of the doublet band in the western blot analyses described above. Fractions obtained after gel filtration chromatography of protein extracts of Gewurztraminer berries were subjected to OAT enzyme assays as well as western analysis with anti-VVOAT antibodies. A single peak of OAT activity was detected, primarily in fraction 12, with a smaller amount of activity in fraction 14 (Figure 5.5A). Western blot analysis indicated that fraction 12 also contained the highest concentration of immunoreactive protein (Figure 5.5B). Extended electrophoresis of these protein fractions reveals that the immunoreactive band in both fractions 12 and 14 is present as a doublet. The relative proportion of upper to lower bands is the same in both fractions (data not shown). Although an explanation for the presence of a doublet of immunoreactive bands cannot be given at present, this experiment demonstrates that the reactivity of the antibodies correlates with the presence of proteins able to synthesise P5C from ornithine in grapes. Native VVOAT behaved as a multimer by gel filtration on a Superose-6 high performance liquid chromatography column (data not shown). Time constraints did not allow experiments to determine an accurate size to be carried out.

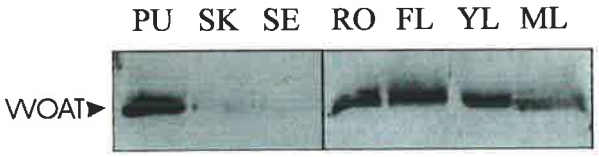



**Figure 5.3** Presence of the VVOAT protein in a range of grapevine tissues. Protein was extracted from berry pulp (PU), berry skin (SK), seeds (SE), roots (RO) flowers (FL), young leaves (YL), and mature leaves (ML) of *V.vinifera* cv. Chardonnay, separated by SDS-PAGE, transferred to nitrocellulose membrane and subjected to western analysis using anti-VVOAT antibodies.

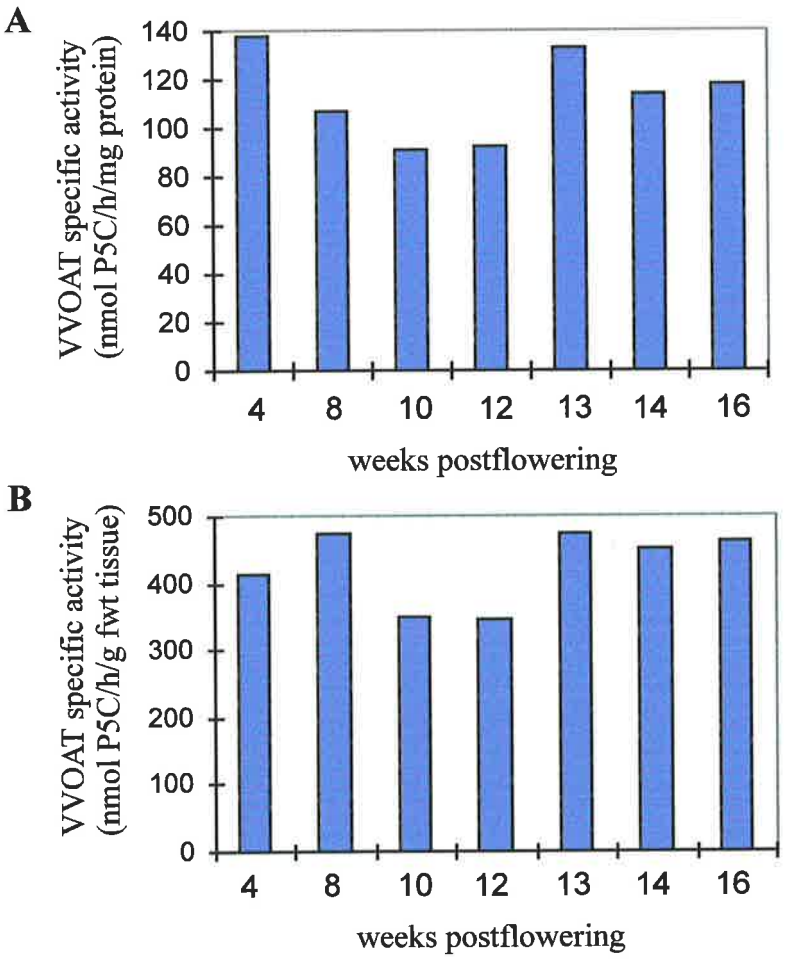



**Figure 5.4** OAT activity throughout berry development. Crude protein extracts from whole berry homogenates of *V.vinifera* cv. Chardonnay were desalted, concentrated and assayed for OAT activity. **A.** The specific activity of OAT was determined as the amount of product (P5C) produced/h/mg berry protein in each extract. **B.** The specific activity of OAT calculated on a per g fwt tissue basis. (values are an average of duplicate measurements)

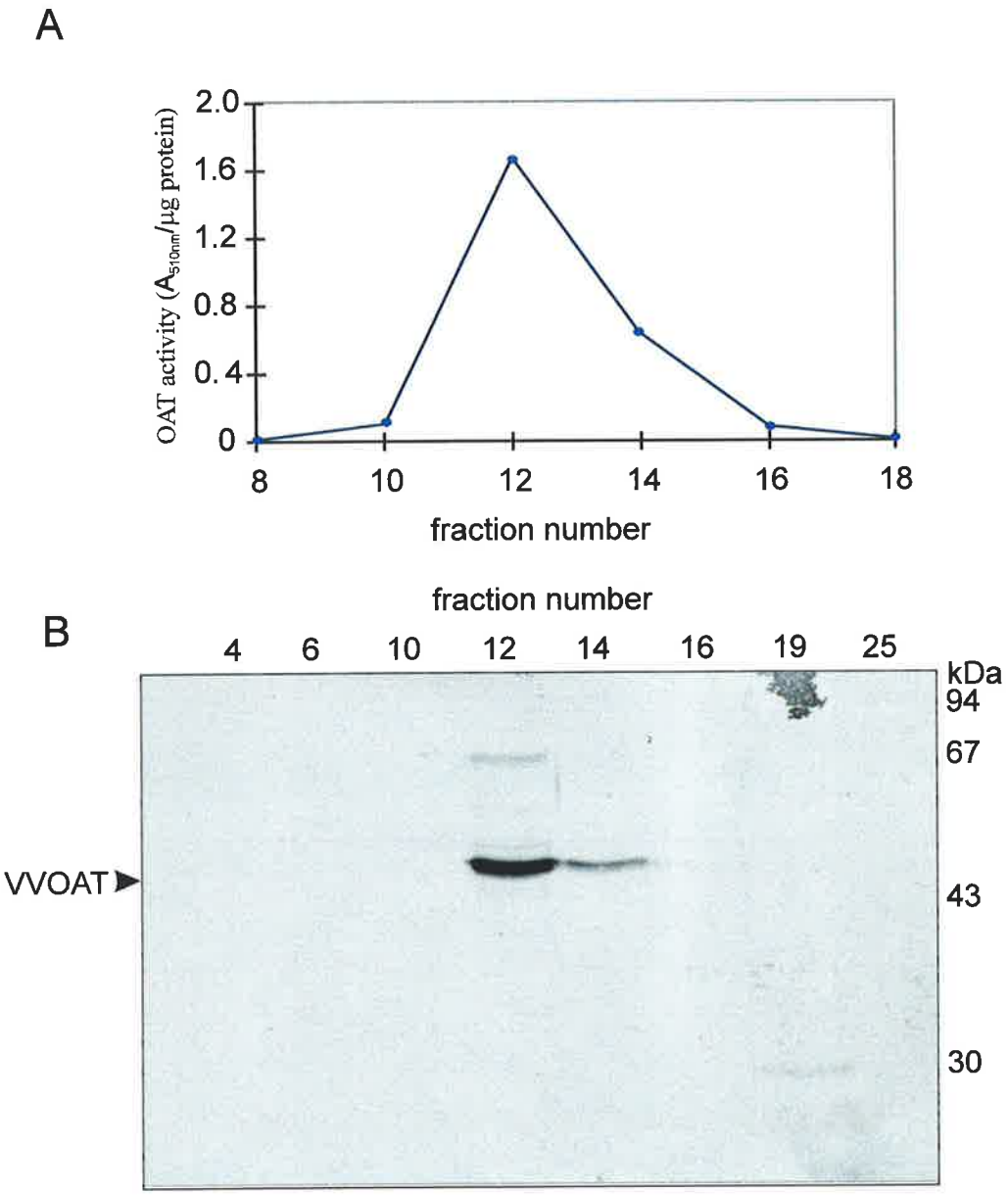



**Figure 5.5** The specificity of the anti-VVOAT antibodies for proteins catalysing the conversion of ornithine to P5C was investigated by carrying out gel filtration chromatography of protein extracts from whole berry homogenates of *V.vinifera* cv. Gewurztraminer followed by measurements of OAT activity and western blot analysis with anti-VVOAT antibodies. **A.** OAT activity of selected chromatography fractions measured as the absorbance of the reaction product at 510 nm/µg protein in the assay. **B.** Eighty µl of the same chromatography fractions were electrophoresed on a SDS-PAGE gel, transferred to a nitrocellulose membrane and subjected to western blot analysis with anti-VVOAT antibodies.

## 5.4 Discussion

The observation that VVOAT mRNA could not be detected using conventional Northern blot analysis suggested that it was expressed at very low levels in *V.vinifera* tissues. The isolation of a cDNA clone for VVOAT from a berry cDNA library indicates that the mRNA is present in berry tissue, although in very low amounts (Chapter 3). Using RT-PCR we were able to detect the presence of VVOAT mRNA in all tissues examined and also throughout berry development. However, a quantitative estimation was not able to be made due to the lack of a constitutively expressed control template. Nevertheless, this result indicates that the mRNA for VVOAT is present in a wide range of tissues and that the ornithine pathway of proline synthesis is probably operating in grape berries. Northern blot analyses used to examine OAT gene expression in previous reports may not have been sensitive enough to determine the presence of the enzyme in all cases (Delauney *et al.*, 1993; Roosens *et al.*, 1998).

In this study, antibodies were raised against VVOAT so that the highly sensitive method of western blot analysis could be used to detect the presence of the protein. This technique revealed that VVOAT was indeed present throughout berry development and also in other plant tissues. It also appears that the majority of VVOAT protein present in the berry is localised in the berry pulp. This tissue specific expression may be contributing to the different patterns of proline and arginine accumulation which occur in berry skin and pulp of cvs such as Chardonnay and Cabernet Sauvignon (results presented in Chapter 2). Interestingly, two immunoreactive protein bands which electrophorese as a doublet on SDS-PAGE are observed in the western blots of VVOAT. The Southern analyses described in chapter 3 strongly suggest that only one

VVOAT gene is present in the *V.vinifera* genome. In order to determine if one or both bands represented VVOAT, gel filtration chromatography of proteins extracted from mature Gewurztraminer berries was carried out and measurements of OAT activity and western blots were performed on a number of the resulting fractions. This gel filtration chromatography demonstrated that a single peak of OAT enzyme co-chromatographed with the fraction containing the most immunoreactive protein, detected with anti-VVOAT antisera. This strongly supports that at least one of the immunoreactive bands detected in the western blots represents a protein with OAT activity. Preliminary gel filtration chromatography experiments indicated that VVOAT is likely to be a multimer in its native confirmation. In *V.aconitifolia*, δ-OAT was reported to be a monomer (Delauney *et al.*, 1993). In *Bacillus* the native enzyme is a homodimer (Takechi *et al.*, 1994) and in humans it has been reported as a homotetramer (Ohura *et al.*, 1982) and also as a hexamer of three dimers (Shen *et al.*, 1998). The functional significance of these differences in native size has not been established. Interestingly the immunoreactive doublet was present in all gel filtration chromatography fractions containing OAT, indicating that they have very similar size and/or shape in the native confirmation. These bands may therefore be a result of slight protein degradation during tissue extraction or they may represent two alternative forms of the enzyme. These different forms could arise through processes such as alternative splicing of a single mRNA, alternative translational start points, or they could represent pre- and post-mitochondrial forms of the one protein. Purification of the native VVOAT, followed by amino acid sequence determination could resolve some of these questions.

To determine whether the level of VVOAT enzyme activity changes during berry development, VVOAT activity was measured in crude, desalted extracts of cv. Chardonnay berries. These measurements indicate that the specific activity of the enzyme measured in terms of total berry protein remains relatively constant when comparing early and late stages of development. However, on a per g fwt basis, OAT specific activity increases moderately towards berry maturity, possibly as a result in increasing concentrations of total protein at this time.

Some studies have suggested that the pathway mediated by δ-OAT may be important for recycling of amino acids in situations where the endogenous concentration of amino acids such as ornithine and arginine are high, for example during seed germination or in the presence of excess nitrogen (Delauney *et al.*, 1993; Hervieu *et al.*, 1994; Roosens *et al.*, 1998). The berry contains relatively high amounts of nitrogenous compounds, and especially proline and arginine. It is not surprising, therefore, that VVOAT gene expression is activated under these conditions. However, the possible function of VVOAT in grape berries needs to be considered in the context of the other major metabolic pathways with which it interconnects, those of proline synthesis and degradation and arginine synthesis and degradation. In chapter 2 it was suggested that the differences in the extent of interconversion of arginine and proline might explain the differences observed in berry proline:arginine ratios between cultivars.

Clearly VVOAT enzyme activity is present throughout berry development and could contribute to the accumulation of free proline observed in the later stages of berry ripening. Interestingly, the levels of VVOAT protein, relative to total berry protein, appear to increase in the later stages of berry development in the cultivars examined

which accumulate only moderate levels of proline, those of Gewurztraminer and Muscat Gordo, but not in the cultivars examined which accumulate high levels of proline, namely Cabernet Sauvignon and Chardonnay. This suggests that whilst VVOAT may be involved in proline synthesis in the berry it is not *per se* a key regulatory factor in the high proline accumulation in the berries of some cultivars, although direct comparisons of the concentrations of VVOAT protein and the levels of VVOAT enzyme activity between all of these cultivars should be made to further support this suggestion.

It is likely that OAT would be involved in the interconversion of arginine and proline, and, therefore, it is possible that it is involved in determining the proline:arginine ratio characteristic for any one cultivar. Gewurztraminer and Muscat Gordo accumulate relatively high levels of arginine in their berries and therefore have a low proline:arginine ratio, whilst Chardonnay and Cabernet Sauvignon accumulate relatively low levels of arginine and have a high proline:arginine ratio (Chapter 2). The increasing levels of VVOAT protein observed late in development of Gewurztraminer and Muscat Gordo could be in response to the accumulation of arginine which may have an effect on VVOAT gene expression (Brandriss and Magasanik, 1980; Delauney *et al.*, 1993). In addition, the increase in arginase activity in berries at veraison, demonstrated for cv. Chenin Blanc (Roubelakis-Angelakis and Kliewer, 1981) would result in an increased supply of ornithine. Again however, it is difficult to reconcile the increasing levels of VVOAT protein in Gewurztraminer and Muscat Gordo, but not Chardonnay and Cabernet Sauvignon, with a key regulatory role for this enzyme in the conversion of arginine to proline. Indeed, although these trends need to be confirmed by direct comparisons of VVOAT protein and enzymatic activity levels between different

cultivars, the observations here raise the possibility that VVOAT may also be involved in the conversion of proline to arginine by catalysing the glutamate dependent transamination of P5C (or GSA) to form ornithine and α-ketoglutarate. This reverse reaction is catalysed by the mammalian OAT (Katunuma *et al.*, 1964). However, it not known whether the plant OAT also catalyses the reverse reaction. The recombinant VVOAT enzyme produced in this study (Chapter 3) provides an excellent tool, with which this possible reverse activity of OAT can be investigated. In any case, neither P5C nor ornithine accumulate to a significant extent in mature berries (Chapter 2), suggesting that any interconversion that may take place between proline and arginine is complete, and therefore all the enzymes in both the proline and arginine biosynthetic pathways must be operating in grape berries throughout the later stages of development. The regulatory role of OAT, if any, in this interconversion remains to be determined.

Clearly both P5CS and OAT are present in *V.vinifera* berries throughout development, suggesting that both the glutamate and ornithine pathways of proline synthesis are in operation. However, the results described in this and the previous chapter suggest that changes in gene expression of VVP5CS and VVOAT are not primarily responsible for regulation of final proline levels in mature berries and that additional factors are likely to be making a significant contribution. Preliminary investigations into some of these additional factors are presented in the following chapter.

# Chapter 6

## *Preliminary Investigation of Other Factors which may be Involved in the Regulation of Proline Accumulation in Developing Grape Berries*

## 6.1 Introduction

The net accumulation of free proline in the grape berry will be a result of the combination of proline synthesis in the berry, import from the phloem, release from peptides, proline degradation and assimilation into other molecules. In addition to the in depth investigation of the key enzymes of proline biosynthesis, discussed in the previous three chapters, a number of preliminary investigations into other factors which could be involved in proline accumulation were also carried out. These factors include the degradation of proline by PDH and the incorporation of free proline into cellular protein.

The first step of proline degradation in plants is mediated by the mitochondrial enzyme PDH which converts proline into P5C (Huang and Cavalieri, 1979). Recent studies examining the proline degradation pathway have shown that it is regulated coordinately with proline synthesis via the glutamate pathway under osmotic stress conditions (Kiyosue *et al.*, 1996; Peng *et al.*, 1996). The steady-state levels of PDH mRNA increase significantly in *A.thaliana* plants recovering from osmotic stress, concomitant with a reduction in P5CS mRNA. The steady-state levels of PDH mRNA are also induced by high proline levels, but this induction is inhibited in salt stressed plants when levels of P5CS mRNA are high. This coordinate, reciprocal regulation of PDH and P5CS mRNA levels indicates that cellular free proline levels are tightly controlled, at least during the osmotic stress response (Kiyosue *et al.*, 1996; Peng *et al.*, 1996; Nakashima *et al.*, 1998). Under 'normal' non-stressed conditions, however, PDH mRNA levels are highest in those tissues of *A.thaliana* which also contain the highest

concentrations of proline i.e. pollen, pistils and seeds (Nakashima *et al.*, 1998). The coexistence of high proline concentrations and high levels of PDH mRNA, in the same tissue, suggests that either the location of proline accumulation and the degradative pathway are separated into distinct subcellular compartments, or that a high rate of proline turnover is occurring in these tissues. Indeed, it was suggested that the presence of high levels of PDH mRNA indicate that proline is being metabolised and used as a source of energy, carbon and nitrogen for pollen and seeds during rapid tissue growth (Nakashima *et al.*, 1998).

The cell's metabolic demand for proline incorporation into peptides also influences the net accumulation of free proline. Hence high levels of free proline may arise as a result of inhibition of protein synthesis or enhanced protein turnover. During berry development, the accumulation of total protein is reported to occur primarily during stage I of development with a second increase in protein content at veraison, after which it declines (Ghisi *et al.*, 1984; Tattersall *et al.*, 1997). Changes in the net accumulation of proteins during berry development will, at least in part, determine the cellular demand for proline for biosynthetic purposes and will, therefore, potentially also have an impact on the levels of free proline which accumulate in the berry.

## 6.2 Materials and Methods

### 6.2.1 Western analysis of PDH levels in developing berries of *V.vinifera* cv. Chardonnay

Berry proteins were extracted and analysed as described in sections 4.2.3 and 3.2.9 using anti-AtPDH (*A.thaliana* PDH) serum (kindly supplied by N.Verbruggen, University of Ghent, Belgium) as the primary antibody.

### 6.2.2 Analysis of protein accumulation patterns in developing berries of *V. vinifera* cv. Cabernet Sauvignon

Proteins were extracted from berries of cv. Cabernet Sauvignon tissues as described in section 4.2.3 and electrophoresed by SDS-PAGE as described in section 3.2.6. Quantification of total berry proteins was carried out according to the method of Ball (1986).

## 6.3 Results

### 6.3.1 Analysis of PDH protein levels in developing berries of *V.vinifera* cv. Chardonnay

Using polyclonal antibodies raised against At-PDH, to probe a western blot of berry proteins from Chardonnay grapes sampled throughout development produced a single band corresponding to a protein of size ~55kDa, which is the size expected for the mitochondrial enzyme PDH (Verbruggen *et al.*, 1996). The level of this cross-reactive protein increased steadily in berries to 13 weeks postflowering, after which it remained relatively constant (Figure 6.1).

### 6.3.2 Analysis of protein accumulation in developing berries of *V.vinifera* cv. Cabernet Sauvignon

The total amount of protein present in extracts from berries from different developmental stages was determined after electrophoresis of the proteins by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and elution and quantification of the Coomassie Brilliant Blue according to the method of Ball (1986) (Figure 6.2). The polypeptide pattern observed upon SDS-PAGE analysis and measurement of protein content clearly established that accumulation of total berry protein had ceased by 12 weeks postflowering.

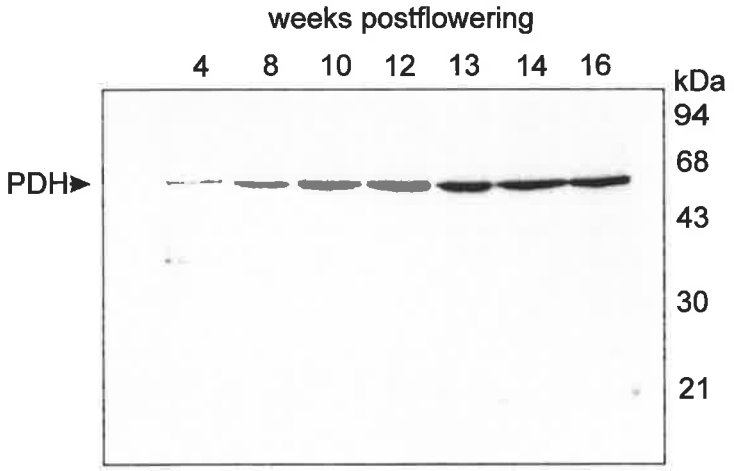



**Figure 6.1** Steady-state levels of PDH protein throughout development of *V.vinifera* cv. Chardonnay berries. Protein extracts from equivalent amounts of whole berry homogenates on a fresh weight basis were separated by SDS-PAGE and transferred to a nitrocellulose membrane and subjected to western analysis using antibodies prepared against AtPDH.

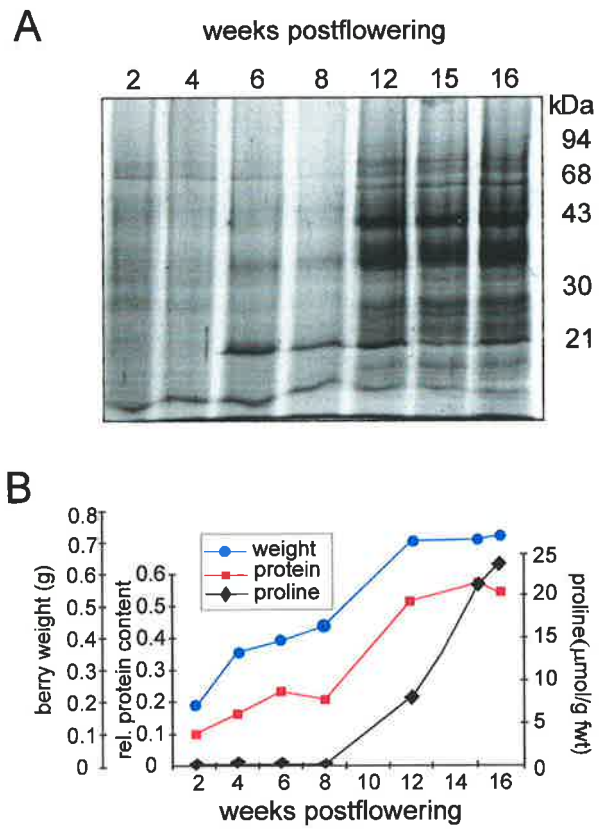



**Figure 6.2** A dramatic increase in free proline accumulation correlates with the cessation of growth and protein synthesis in the grapevine berry. **A**, SDS-PAGE of total protein extracted from *V.vinifera* cv. Cabernet Sauvignon berries throughout development. **B**, The changes in the relative amounts of total protein, measured in each lane of the gel in A, and the concentration of free proline, and berry weight during development.

## 6.4 Discussion

Proline accumulation in ripening berries may result from a developmentally programmed decrease in the degradation of proline catalysed by the mitochondrial enzyme PDH (Elthon and Stewart, 1981). Using antibodies raised against AtPDH, a protein of ~55kDa representing a putative grapevine PDH homologue was detected by western blot analysis. The steady-state levels of PDH protein increased throughout berry development to 13 weeks postflowering, possibly in response to the increases in free proline which occur during this period. PDH mRNA levels in *A.thaliana* increase in the presence of high concentrations of proline, providing 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 rapid proline accumulation. This suggests that proline accumulation late in development is independent of changes in the steady-state levels of PDH protein. It is possible , however that postranslational mechanisms may regulate PDH activity in berries. To assess this possibility, measurements of PDH enzyme activity need to be made. Assays for PDH are based on the coupled reduction of cytochrome C and are therefore subject to much interference by other enzyme activities in crude extracts. In addition, PDH is a mitochondrial enzyme, which relies on the electron transport chain for activity (Elthon and Stewart, 1981). Consequently, isolation or enrichment of berry mitochondria may be required to carry out the PDH assay (N.Verbruggen, University of Ghent, Belgium, pers. comm.). Due to time considerations this was not attempted during the present study.

The growth of the berry and concurrent synthesis of cellular protein is clearly another potential nitrogen sink that might compete with free proline accumulation. Indeed, there appears to be a correlation between the cessation of berry growth and net protein accumulation during the late phases of berry development and rapid proline accumulation in the grapevine cultivar examined. This suggests that the demand for free proline for biosynthetic purposes may decline during the late phases of berry development, whilst production is likely to continue due to the continued presence of VVP5CS and VVOAT. The relative contribution of this decreased demand for proline in protein synthesis to net proline accumulation remains to be determined.

Enhanced import of proline combined with decreased export of proline could also affect intracellular levels of free proline. Recently a gene encoding a transporter which mediates proline transport across the plasma membrane (LeProT1) was isolated from tomato and was shown to be expressed in pollen, a tissue which accumulates high levels of free proline (Schwacke *et al.*, 1999). This suggests that the high level of free proline in pollen may result from the import of free proline rather than its *de novo* synthesis *in situ*. The possible contribution of proline transport to proline accumulation in grape berries was not investigated during the present study as research by Gholami *et al,* (1996) indicated that glutamine was the major amino acid species imported via the phloem into the developing berry. As glutamine is a precursor of proline synthesis, this suggests that the high level of free proline present in mature grape berries is most likely to result from *de novo* synthesis of proline in the berry rather than its import via the phloem.

# Chapter 7
## *Conclusions and Future Directions*

The work described in this thesis was undertaken to document the accumulation of amino acids during grape berry development and to investigate the mechanisms underlying the significant accumulation of proline late in ripening in some cultivars.

In Chapter 2, amino acid analysis of the berries from four different grapevine cultivars (Chardonnay, Cabernet Sauvignon, Gewurztraminer and Muscat Gordo) was described and it was revealed that proline accumulation does not occur uniformly throughout berry development, but only during the last 4-6 weeks of ripening. Measurement of the proline concentration in a range of grapevine tissues demonstrated that the degree of proline accumulation which occurs in the berry skin and pulp is significantly different from that of other tissues.

The research described in Chapter 3 resulted in the isolation of cDNA clones encoding the key proline biosynthetic enzymes VVP5CS and VVOAT from a grapevine cDNA library. This indicated that grape berries are capable of synthesising proline *de novo* from either glutamate or ornithine, via P5C. The cDNA clones enabled the production of recombinant VVP5CS and VVOAT proteins which are useful tools for studying the properties of these enzymes. Furthermore, the recombinant proteins were used to produce polyclonal antibodies, which proved invaluable in the following studies on analysis of gene expression.

In Chapter 4, the steady-state levels of VVP5CS protein were determined in the developing fruit of the four *V.vinifera* cultivars, and were found to be relatively uniform from flowering through to full fruit maturity. This was in contrast to the pattern of proline accumulation, where substantial increases in free proline concentration occurred

late in berry development. This demonstrated that changes in P5CS protein levels are not a major factor in the changes in proline levels and suggests that the mechanisms regulating proline accumulation during normal plant development are quite different to those operating during the abiotic stress response. Chapter 5 described an investigation whether the alternative pathway of proline synthesis, mediated by VVOAT, plays a role in grape berry proline accumulation. VVOAT protein was shown to be present and active throughout berry development. Clearly both pathways of proline synthesis are operating in *V.vinifera* berries throughout development, however, the results described in Chapters 4 and 5 suggest that regulation of VVP5CS and VVOAT is not primarily responsible for the changes observed in proline levels during berry development and that additional factors must be involved. Preliminary investigations into some possible additional factors were presented in Chapter 6. In particular, it was shown that a downregulation of levels of PDH protein and therefore of proline degradation is not likely to contribute to the increases in proline accumulation observed late in fruit ripening.

Based on all of the above, the developmental pattern of proline accumulation in ripening berries does not appear to be attributable to direct regulation of the levels of the key biosynthetic enzymes VVP5CS and VVOAT, but rather may be the result of an inherent high capacity for proline biosynthesis combined with a more indirect mechanism of regulation. An example of such a mechanism of regulation would be the control of metabolic flux through the proline synthesis pathways through availability of the primary substrate glutamate. Glutamate is derived from the deamination of glutamine, which in turn can result from the assimilation of ammonium ions through the activities

of GS, GOGAT and possibly GDH (Kanellis and Roubelakis-Angelakis, 1993). Both GS and GDH activities have been detected in berries of cv. Merlot, suggesting that berries possess the ability to assimilate ammonium ions via these pathways (Ghisi *et al.*, 1984). The synthesis of proline is, therefore, likely to be influenced by the inflow of glutamate, glutamine and ammonium ions into the berry and also by the metabolic conversions that take place between these three substrates within the berry. The total nitrogen content of grape berries increases in an almost linear fashion from just prior to veraison to full maturity (Gholami, 1996; D. Wheatley, pers. comm.). This is presumably due to a steady inflow of nitrogen via the phloem. The supply of glutamate and competing demands for its use as a substrate in the synthesis of glutamine, proline, arginine and other amino acids would affect the rate of proline synthesis. Preliminary data from this study suggests that the levels of both a GS mRNA and GS activity increase during berry development (results not shown). A systematic study of GS, GDH and GOGAT gene expression and levels of enzyme activity in developing berries would therefore add valuable insight into the metabolic environment in which proline synthesis and accumulation is taking place. Due to time constraints, this research was not carried out during this study.

As the berry ripens, it experiences large changes in osmotic pressure, due to the accumulation of sugars in the vacuole (Downton and Loveys, 1978). A high concentration of proline in the cytoplasm may create a counteracting solute effect, enabling osmotic adjustment. This type of developmentally imposed osmotic stress may occur gradually, as opposed to the rapid and severe osmotic stress imposed by external factors such as exposure to salt used in many previous studies on proline accumulation

in plants. It may, therefore, elicit a different response in the plant tissue. The results described in this thesis demonstrate that the expression of VVP5CS and PDH in developing berries is distinct to that observed during the abiotic stress response in the vegetative tissues of a number of plant species. This suggests that the accumulation of proline in developing berries is indeed occurring through signals and mechanisms different to those operating during the abiotic stress response.

It is possible that the accumulation of proline in grape berries may serve a metabolic role during development. The results described in this study suggest that both proline synthesis and degradation may be occurring in the developing berry. The turnover of proline may serve as a possible source of energy for berry cells through the production of NAD(P) and NAD(P)H. These energy equivalents are required in many different metabolic cycles and pathways and the ratio of the two can affect the activity of key enzymes, such as those in the TCA cycle. Further research into the actual rate of proline turnover in the berry may provide more information about this potential role for proline in general berry metabolism. This would require measurement of both P5CS and PDH enzyme activity *in situ* in the berry.

Future study of the genes encoding VVP5CS and VVOAT and their promoter regions would improve our understanding of the factors involved in the developmental regulation of their expression in berries. Creating transgenic grapevines with berry specific down- and/or up-regulation of VVP5CS or VVOAT expression could provide valuable insights into both the relative contribution of the glutamate and ornithine pathways to proline accumulation, and the role of proline in berry function and development. In addition this could provide a basis for manipulation of the levels of

certain amino acids in the berry such that the amino acid composition of the grape juice is optimal for efficient fermentation by yeast, without the associated formation of hydrogen sulfide or ethyl carbamate (Jiranek *et al.*, 1993). To this end, an enhancement in the levels of glutamate and glutamine is desired through a reduction in the conversion of these substrates to proline and arginine. The research described in this thesis has provided the tools and analytical techniques to make this next step possible.

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# ERRATA

In various places in the text of this Thesis, the following corrections should be inserted:

1. Where $^{0}$C is used, it should be replaced by °C
2. Where 3' appears in the text, it should be replaced by 3′
3. Where the term 'variety' is used, it should be replaced by 'cultivar'