

**DIFFERENTIAL GENE EXPRESSION DURING BERRY  
RIPENING IN *VITIS VINIFERA* (CV CHARDONNAY):  
ISOLATION OF SPECIFIC SEQUENCES THROUGH  
SUBTRACTIVE CLONING**

By

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*Declaration*

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

## **ABSTRACT**

Grapevine is worldwide an agronomically important crop. Traditionally selective breeding has been used to improve existing cultivars. In the last ten years, however, the advent of biotechnology has shortened these breeding programmes by producing transgenic grapevine. Because this new technology is aimed at the possible genetic manipulation of the ripening process in grape berries, it is important to elucidate all the mechanisms that may be involved in ripening. The aim of the present study was the identification of genes that play an important role during the ripening process in grape berries. This was achieved by investigation of putative differentially expressed genes in ripening Chardonnay berries isolated through subtractive hybridisation. Two subtraction libraries, representing early and late ripening stages were constructed. Four of the ten genes analysed exhibited expression during berry ripening. One of the four genes was expressed in a tissue and stage specific manner. Further characterisation of eight of the DNA and protein sequences revealed that the putative translation products of these clones had homologues that are involved in amongst others cell wall structure in other species. These included UDP-glucose dehydrogenase, which is involved in the synthesis of hemicellulose precursors. The remaining seven clones encoded putative stress response proteins. These included two heat shock proteins, a vacuolar pyrophosphatase and a protein involved in cell division. It is suggested that specific grape mRNAs accumulate in response to stresses such as the storage of high concentrations of sugars and rapid cell expansion. These processes occur rapidly during the ripening of berries. Accumulation of specific mRNAs can be attributed to part of the normal ripening developmental programme.

## OPSOMMING

Druive is wêreldwyd 'n belangrike landbougewas en kultivars word tradisioneel deur middel van tydsame selektiewe teling verbeter. Die tyd wat hieraan bestee word, kan verkort word deur die implementering van biotegnologie en die produksie van transgeniese duiwe. Omdat hierdie nuwe tegnologie op die moontlike genetiese manipuleringsproses in duiwe gemik is, is dit belangrik dat alle meganismes betrokke by rypwording ondersoek en verstaan word. Die doel van hierdie studie was om gene wat moontlik tydens die rypwordingsproses in duiwe 'n rol kan speel, te identifiseer. Hierdie doel is bereik deurdat differensieel uitgedrukte gene uit die kultivar Chardonnay geïsoleer is met behulp van verrykingsbiblioteke vanuit jong en volwasse duiwekorrels. Vier van die tien gene wat geanaliseer is, word uitgedruk tydens die rypwordingsproses. Verder het een van die vier gene weefsel- en rypwordingstadium- spesifisiteit getoon. Volledige karakterisering van agt van die DNA- en proteïenvolgordes het aangedui dat die proteïenprodukte van hierdie gene homolog is aan volgordes wat onder andere by selwandstruktuur betrokke is. Dit sluit UDP-glukose dehidrogenase in, wat betrokke is by die sintese van hemi-sellulose boustene. Die ander sewe gene kodeer vir moontlike spanningsproteïene. Twee hiteskokproteïene, 'n vakuolêre pirofosfatase en 'n proteïen wat betrokke is by selverdeling is geïdentifiseer. Daar word voorgestel dat duiwe mRNA versamel in reaksie op spanningsituasies soos die berging van hoë konsentrasies suikers en selvergroting. Hierdie prosesse vind baie vinnig plaas tydens rypwording. Versameling van spesifieke mRNAs kan toegeskryf word as 'n normale deel van die rypwordingsproses.

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## **PREFACE**

The experimental work in this thesis was supervised by Prof. F.C. Botha and Dr. S. Groenewald, and conducted in the Institute for Plant Biotechnology, at the Department of Botany, University of Stellenbosch, South Africa. The results presented here are original, and have not been submitted in any form to any other university.

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To the staff and students of the IPB:...if I had do it again I would only choose one team!

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## LIST OF ABBREVIATIONS

ACC-oxidase	1-aminocyclopropane-1-carboxylate-oxidase
ACC-synthase	1-aminocyclopropane-1-carboxylate-synthase
ABA	abscisic acid
Asr-like	abscisic acid-, stress-, ripening-induced-like
$\beta$ -ME	beta-mercaptoethanol
BLAST	basic local alignment search tool
BTOA	benzothiazole-2-oxyacetic acid
cDNA	complimentary DNA
Ci	Curie
cia	chloroform:isoamyl alcohol
CsCl	cesium chloride
cv.	cultivar
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dTTP	deoxythymine triphosphate
dUTP	deoxyuracil triphosphate
EDTA	ethylenediamine tetra-acetic acid
ELIP	early light-induced proteins
Grip	grape ripening induced proteins
HE-buffer	Hepes and EDTA containing buffer
IBA	isobutyric acid
KWV	Koöperatieve Wijnbouwers Vereniging
LEA	late embryogenesis abundant
LiCl	lithium chloride
mRNA	messenger RNA
NaOAc	sodium acetate
NaCl	sodium chloride

NaOH	sodium hydroxide
Na-perchlorate	sodium perchlorate
NCBI	National Center for Biotechnology Information
nsLTP	non-specific lipid transfer proteins
PCR	polymerase chain reaction
PEG	polyethylene glycol
P/HRGP	proline/hydroxyproline-rich glycoproteins
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
rRNA	ribosomal RNA
RuBisCo	ribulose-1,5-bisphosphate carboxylate
Sde	subtractive, differentially expressed
<i>sdi</i>	sunflower drought-induced
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
SSH	suppression subtractive hybridisation
SSPE	standard saline phosphate EDTA
UDP	uridine diphosphate
Udpgdh	UDP-glucose dehydrogenase
UFGT	UDP-glucose flavonoid-3-glucosyl transferase
<i>Vvht1</i>	<i>Vitis vinifera</i> hexose transporter 1
TE buffer	tris and EDTA containing buffer
Tris-HCl	tris(hydroxymethyl)aminomethane-hydrochloric acid

## CHAPTER 1

### INTRODUCTION

Previous advances in the improvement of grapes for viticultural purposes have been through traditional breeding programmes aimed at the production of either new cultivars or the improvement of existing ones (Vivier & Pretorius, 2000). Using existing breeding programmes, desired traits in vines were identified and incorporated in new or improved vines. Frequently, these traits are polygenetically inherited and under the control of groups of genes of minor effect (Mullins *et al.*, 1992), thereby complicating traditional breeding programmes. The demand of the grapevine industries to preserve the desirable characteristics of certain cultivars while generating improved yield, disease and pest resistance, etc. in plant material accentuates the shortcomings of traditional breeding programmes (Robinson *et al.*, 1999).

Almost all plants and animals of agricultural importance today are the result of some sort of genetic manipulation through traditional breeding techniques (Meredith, 1999). The challenge facing the grapevine industry today is how to successfully integrate these existing breeding programmes with the application of biotechnology (Thomas *et al.*, 2000).

In grapevine biotechnology, the main areas are disease management and berry ripening (Vivier & Pretorius, 2000). Concomitant studies on the various processes of pathogenesis, cell wall structure and morphogenesis, plant-pathogen interactions, signal transduction on infection stimuli and sugar-acid metabolism have been aimed at enhancing knowledge in the field of diseases and ripening (Vivier & Pretorius, 2000). A thorough knowledge on these subjects is key to the successes obtained in the manipulation of these processes. Biotechnological applications rely heavily on the availability of fundamental knowledge as well as technologies (new methodology), to identify, isolate and characterise target genes and useful promoter sequences (Robinson *et al.*, 1999).

Current strategies aimed at elucidating the underlying mechanisms involved in grape berry ripening have focused on the isolation and characterisation of genes involved in the ripening process. One direction of research has focused on the isolation of sucrose and hexose transporter-encoding genes exhibiting developmental regulation (Atanassova *et al.*, 2000; Fillion *et al.*, 1999). The aim of these studies was to clarify questions regarding sugar transport and the control of sugar accumulation in ripening grape berries (Atanassova *et al.*, 2000; Fillion *et al.*, 1999).

Coinciding with the accumulation of hexoses in the ripening berry is the increase in the formation of anthocyanins leading to the characteristic skin colour of grape berries (Boss *et al.*, 1996a). Several of the genes involved in anthocyanin biosynthesis in grapevine have been cloned (Sparvoli *et al.*, 1994) and were used to study the control of anthocyanin biosynthesis during berry ripening (Boss *et al.*, 1996a, 1996b).

Another area under investigation is that of stress management. Different developmental stages of plant growth including reproductive growth, seed maturation and senescence are differentially affected in response to stress conditions (Grime, 1989; Khanna-Chopra & Sinha, 1998). As the berry enlarges during ripening, several stress-related proteins seem to be up-regulated. It is speculated that this could be in response to enlargement itself or as a defense mechanism against possible pathogen attack (Saltzman *et al.*, 1998).

The genes and gene products involved in cell wall synthesis, anthocyanin metabolism and stress during the ripening process mentioned above are only three examples of areas under investigation.

The hypothesis of the current study was two-fold. First, by isolating and comparing mRNA transcripts of differentially expressed genes isolated from the first and last stages of berry development, unique sequences involved in the ripening of berries could be identified. Second, by using subtractive hybridisation, not only abundantly expressed genes would be identified, but genes expressed at low abundance as well.

In order to test these hypotheses, this study had three goals. First, the isolation of high quality RNA from recalcitrant grape berry tissue (Chapter 3). Extracting nucleic acids

from grape berries are notoriously difficult due to the high concentration of tannins and phenolic compounds (Franke *et al.*, 1995). This remains the foundation of any subsequent experimentation. Second, the production of two stage specific cDNA libraries: a library representing expressed sequences in the early stage of ripening and one representing expressed sequences in the late stage of ripening (Chapter 3). Third, the isolation of differentially expressed genes that exhibit tissue specificity and developmental regulation in grape berries, using subtractive hybridisation (Chapter 4).

## CHAPTER 2

### LITERATURE REVIEW

This chapter is aimed at presenting recent findings in the field of molecular characterisation and gene expression in grapevine fruit ripening. The importance of distinguishing between climacteric and non-climacteric fruit will be discussed, because of the possible molecular implications as far as developmental stage-specific gene expression is concerned. Since one of the goals of the present study was the isolation of differentially expressed genes, the concept of differential gene expression will be introduced, specifically emphasising the progress that has been made in relation to grape berry ripening. Subtractive hybridisation will be discussed in the context of gene expression in plants and the rationale for the use of this method in the present study will be presented. Finally, the results obtained in this study (Chapters 3 and 4) will be presented together with ideas for future research.

#### 2.1 Fruit ripening

It is not surprising when looking at the complex nature of fruit ripening, that the process is under genetic control. Changes in flavour, texture and colour must be regulated and co-ordinated (Tucker, 1993). Fruit ripening was originally seen as primarily a catabolic process in which cellular organisation and control were breaking down (Blackman & Parija, 1928). However, over the last few decades, it has become apparent that ripening, like other plant senescent processes, is under strict genetic control (Tucker, 1993).

Early evidence for anabolic processes occurring during ripening came from *in vivo* radio-labelling studies to investigate turnover of proteins and nucleic acids during ripening. It was found that in many fruit, such as banana (Brady & O'Connell, 1976) and tomato (DeSwardt *et al.*, 1973), protein synthesis continued during ripening. Similarly, nucleic acid synthesis continues (Richmond & Biale, 1967). It has been shown that cell wall carbohydrate polymers also continue to be synthesised during ripening (Mitcham *et al.*, 1989). It is thus generally held that during ripening the



processes of nucleic acid and protein synthesis continue, albeit at perhaps a reduced rate. Of more significance was the realisation that not only was protein synthesis continuing but that to a certain extent this was also being redirected. Thus analysis of mRNA and protein species during ripening of both avocado (Christoffersen *et al.*, 1982) and tomato (Biggs *et al.*, 1986; Rattanapanone *et al.*, 1978) showed the synthesis of distinct ripening-related proteins.

These findings led to the concept that ripening is controlled, at least partially, at the level of gene expression. The search is underway to identify these 'ripening-related' proteins and some have already been identified. Amongst others, these proteins include polygalacturonase (Grierson *et al.*, 1986), 1-aminocyclopropane-1-carboxylate (ACC) synthase (Van der Straeten *et al.*, 1990) and ACC oxidase (Hamilton *et al.*, 1990).

### **2.1.1 Grape berry ripening**

Berry fruit growth follows a double sigmoidal curve. The exact form of the growth curve can vary considerably between grape cultivars and divisions into two, three, or four phases have been proposed (Coombe, 1992). For the purposes of the present study, berry growth (cv. Chardonnay) was divided into 6 stages of development.

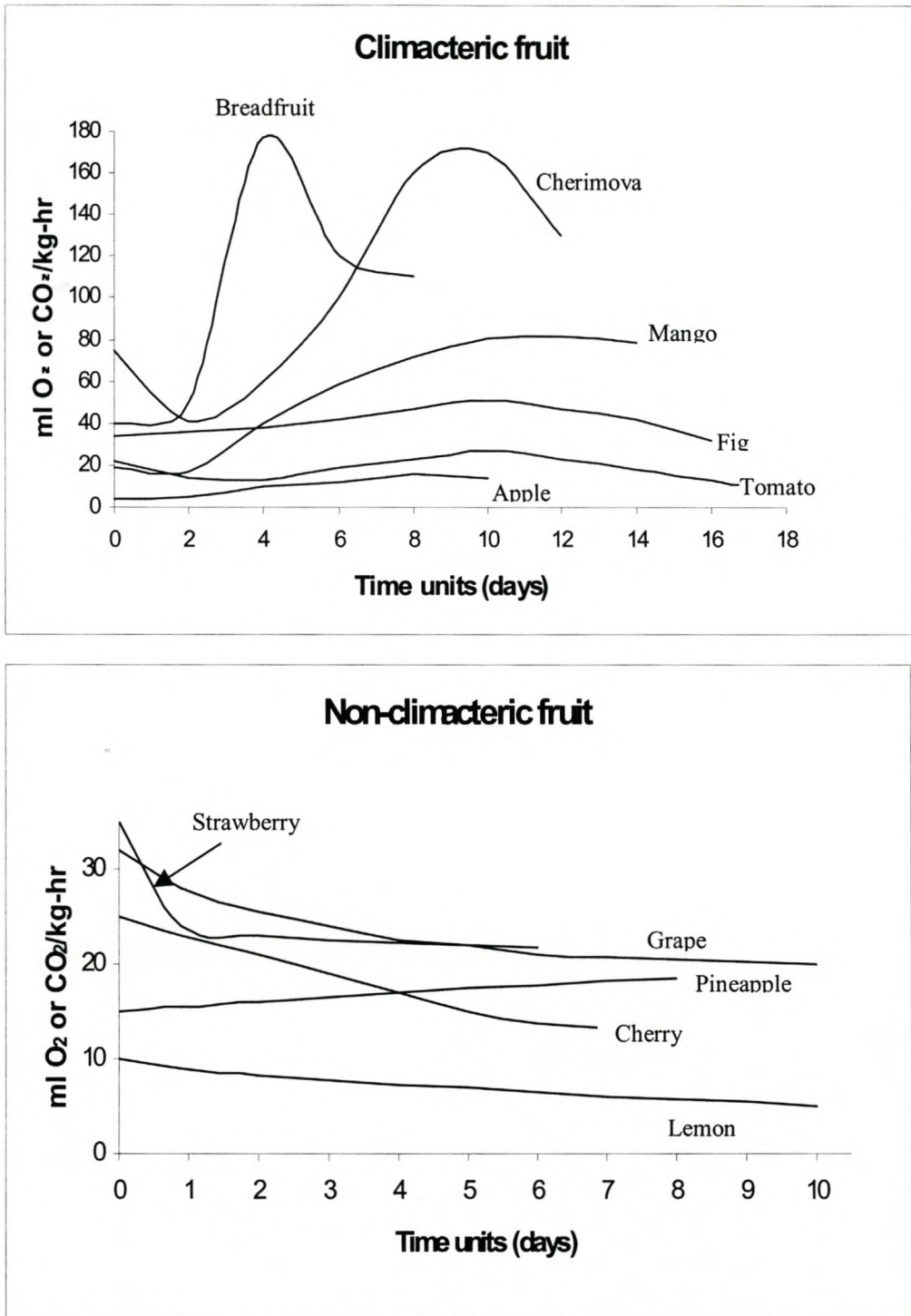
The first two stages, varying from 25 to 60 days after anthesis, are characterised by a period of about 2 to 3 weeks of rapid cell division followed by a marked cell enlargement (Coombe, 1976; Pratt, 1971). The major difference between stage 1 and 2 is the size of the berries. By the end of stage 2, the seeds attain nearly their full size (Pratt, 1971), whereas seed dry weight shows a biphasic pattern during the whole fruit growth. The growth of the embryo shows no relation to the double-sigmoidal growth of the pericarp and final size is reached 70-75 days after anthesis (Staudt *et al.*, 1986). During stages 1 and 2, chlorophyll is the predominant pigment and berries display active metabolism, with high rates of respiration and rapid acid accumulation. Acid content is comparable to that of sugar content ( $20 \text{ g kg}^{-1}$ ) (Peynaud & Ribéreau-Gayon, 1971; Winkler *et al.*, 1974).

Stages 3 to 4 starts 35 to 80 days after anthesis (Winkler *et al.*, 1974) depending on the cultivar. The duration of the slow growth phase or lag phase also depends on the cultivar, timing of flowering and competition between clusters and the vine's environment (Coombe, 1976; Pratt, 1971). Chardonnay is characterised by a shorter lag phase of 3 – 4 weeks (Archer, 1981). During the lag phase, the embryos develop rapidly and generally reach their maximum size towards the end of this period (Kanellis & Roubelakis-Angelakis, 1993). The berries also lose chlorophyll and soften. In addition, acidity reaches its highest level (Kanellis & Roubelakis-Angelakis, 1993).

The inception of stage 5 is called *véraison* and is characterised by a rapid change in appearance and constitution of the berries. The events that take place, as stage 5 begins, are acceleration of growth, softening of the berry and increase in deformability, increase in the content of glucose, fructose, total and free amino acids (especially arginine and proline), total proteins and total nitrogen (Coombe, 1973; Peynaud & Ribéreau-Gayon, 1971). Decreases in the concentration of organic acids (mainly malic acid) and ammonia along with a loss of chlorophyll from the skin and accumulation of anthocyanins (only in red grapes) are found during this stage. A decrease in respiration rates, and finally an increase in activity of some enzymes, including sucrose phosphate synthetase (EC 2.4.1.14), sucrose synthase (EC 2.4.1.13) and hexokinase (EC 2.7.1.1) are also found during stage 5 and 6 (Winkler *et al.*, 1974).

### **2.1.2 Climacteric and non-climacteric ripening**

Ripening, like other plant developmental processes, is probably under the control of plant growth regulators (Bruinsma, 1983; McGlasson *et al.*, 1978). All five major growth regulators affect ripening if applied exogenously. Thus auxin, gibberellin and cytokinin generally act to retard ripening, while ethylene and abscisic acid act to enhance the ripening process (Tucker, 1993). Much work has been carried out in an attempt to monitor endogenous levels of these regulators during ripening. Some correlations can be found, but only ethylene is routinely found to be associated with ripening and hence has led to this plant growth regulator being considered the 'ripening hormone' (Tucker, 1993). Ethylene is only considered to be involved with ripening in a certain type of fruit, namely climacteric fruit. Grapevine, however, is considered a non-climacteric fruit (Alleweldt & Koch, 1977; Coombe & Hale, 1973).



**Figure 2.1.** Respiratory rates of climacteric and non-climacteric fruits (according to Tucker 1993)

The difference between climacteric and non-climacteric ripening lies in the characteristic patterns of respiration observed during ripening. In Fig. 2.1 the respiration (measured as the amount of O<sub>2</sub> or CO<sub>2</sub> produced by each fruit type per hour) pattern of climacteric fruit, like breadfruit and cherimoya, is characterised by a sudden

rise in the production of  $O_2/CO_2$ , whereas fruit like tomato and apple exhibit very low increases. Thus, climacteric fruit are characterised by a burst of ethylene production that occurs during ripening and most often precedes the respiratory climacteric (Chalmers & Rowan, 1971), as can be seen by the respiratory patterns of breadfruit, cherimoya and to a lesser extent, mango (Fig. 2.1). However, in some climacteric fruit, like apple for instance, it is difficult to establish whether the burst of ethylene comes before or coincides with the respiratory climacteric (Bennett *et al.*, 1987). Like the respiratory climacteric, different fruit have different peak levels of ethylene production (Fig. 2.1). Non-climacteric fruit do not exhibit a burst of ethylene production, but simply a decline in ethylene production from the mature green to ripe stage of development, a pattern reminiscent of their respiration during ripening (Brady, 1987).

Climacteric and non-climacteric fruit also appear to differ in the control of ethylene synthesis. The biosynthesis of ethylene in climacteric fruit is said to be autocatalytic (McMurchie *et al.*, 1972). This was best demonstrated by the response of fruit to the ethylene analogue, propylene. It was shown that climacteric fruit exposed to propylene begin to synthesise ethylene in an autocatalytic manner; non-climacteric fruit, however, showed no such response (Phillips & Huttly, 1994).

The literature does not support a role for ethylene in the control of non-climacteric fruit ripening. However, the cloning of two rate limiting enzymes (ACC synthase and ACC oxidase) of the ethylene biosynthetic pathway from pineapple, a non-climacteric fruit, suggests that there may be a putative role for ethylene in the ripening process of non-climacteric fruit (Cazzonelli *et al.*, 1998).

### **2.1.3 Other hormones involved in grapevine fruit ripening**

Other hormones implicated in fruit ripening include auxin and ABA as mentioned before. In grapes, auxin reaches a maximal level just after anthesis, subsequently decreasing to very low levels post-véraison (Cawthon & Morris, 1982). Moreover, exogenous application of a synthetic auxin, benzothiazole-2-oxyacetic acid (BTOA), delays the ripening of grape berries (Davies *et al.*, 1997). In contrast, it seems that ABA

levels increase in ripening berries and that ripening is delayed when ABA-increase is blocked (Hale & Coombe, 1974).

The synthetic auxin BTOA, was used to evaluate the effect on developmentally regulated genes by monitoring the expression of a putative vacuolar invertase, a chalcone synthase, UFGT, a chitinase and a ripening-related gene (Davies *et al.*, 1997). From these results it was evident that the normal expression of these genes was altered by the BTOA. The invertase expression that usually decreases with the onset of ripening, extended beyond véraison, whereas expression of the other genes, which are usually induced at the onset of ripening, were delayed. It was also shown that the normal accumulation of ABA levels during ripening was delayed due to the BTOA treatments (Davies *et al.*, 1997). These results support the case for a hormonal signal controlling the ripening process and it hints at the possible linkage of the control of auxin and ABA levels.

## **2.2 Current research**

Emphasis in research has shifted toward the isolation and characterisation of ripening-related proteins (Vivier & Pretorius, 2000). In previous years research was conducted on the molecular aspects of grape berry ripening covering areas like aroma development (Coombe & McCarthy, 1997), changes in pigment (Boss *et al.*, 1996c) and accumulation of enzymes (Saltzman *et al.*, 1998). Researchers are now focusing more on the genes and promoter elements involved in the ripening process (Vivier & Pretorius, 2000).

### **2.2.1 Sugar regulation in the grape berry**

Glucose and fructose are the major solutes accumulating in grape berry vacuoles and originates from sucrose (Coombe, 1992). The sucrose is translocated in the phloem from source organs (*e.g.* leaves) to the ripening grape berries (sink organs) (Coombe, 1992). In grapes, the pathways of sugar loading/unloading are still poorly understood and it is still not clear whether phloem loading/unloading proceeds symplastically or apoplastically (Fillion *et al.*, 1999). It was postulated that plasmodesmata connecting the flesh cells of storage parenchyma in the berry, and significant plasma membrane

surface area on the phloem/storage parenchyma surface, provide scope for either of the loading mechanisms (Fillion *et al.*, 1999).

Sugar utilisation and/or compartmentalisation is dependent on the flux of sugar transport into the sink organs (Fillion *et al.*, 1999). During the post-véraison hexose accumulation phase it remains unclear where sucrose hydrolysis occurs. It has been shown that the bulk of the invertase activities in berries are soluble (Davies & Robinson, 1996). Two vacuolar invertase-encoding genes have been isolated from grape berries and shown to have increased expression levels well in advance of the rapid hexose accumulation phase (Davies & Robinson, 1996). This result seems to suggest that the synthesis of invertase is not linked to the rapid accumulation of hexoses in the berry vacuole, and that other factors probably play a regulatory role (Davies *et al.*, 1997; Davies & Robinson, 1996). Sucrose synthase activity is also low during the maturation of the berries (Hawker, 1969), again implying a control mechanism of compartmentalisation rather than sugar utilisation or metabolism in the ripening grape berry (Fillion *et al.*, 1999).

In order to investigate the mechanisms involved in sugar transport, research involving sucrose and hexose transporter-encoding genes have tried to shed some light on this enigma (Atanassova *et al.*, 2000; Fillion *et al.*, 1999). A study of one of the hexose transporters from grapevine showed that the cDNA sequence shared strong homology (70-78% identity) with other hexose transporters from herbaceous hosts (Atanassova *et al.*, 2000; Fillion *et al.*, 1999). Studies revealed two peaks of expression in berries, with the first peak at anthesis and the second major peak occurring approximately five weeks after véraison (Fillion *et al.*, 1999). Another population of hexose transporters has been identified that exhibited high expression levels early during ripening (Atanassova *et al.*, 2000). The fact that plasma membrane hexose transporters are expressed during the post-véraison stage seems to suggest that at least some of the sucrose imported into the ripening berry is already hydrolysed before accumulation occurs in the flesh cells (Fillion *et al.*, 1999).

It seems a likely conclusion, when looking at ripening, that sugar loading is up-regulated during véraison. This up-regulation would have to involve the engagement of

the promoter area of the genes involved in sugar uptake. A study that investigated promoters with the abovementioned in mind analysed a putative promoter of the isolated hexose transporter. It revealed several potential *cis* elements such as ethylene- or abscisic acid (ABA)-responsive elements (Atanassova *et al.*, 2000). Promoter deletion constructs, using reporter genes, were evaluated in tobacco and grapevine transformants to analyse the role of these elements. Preliminary results seem to suggest that the hexose transporter from grapevine (encoded by *Vvht1*) is at least under partial control of glucose (Atanassova *et al.*, 2000).

### **2.2.2 Regulation of colour in the grape berry**

Coinciding with the accumulation of hexoses in the ripening berry is the increase in the formation of anthocyanins leading to the characteristic skin colour of red berries. In grapes, anthocyanins are produced by the flavonoid pathway consisting of several successive enzyme reactions which convert colourless precursor molecules into coloured anthocyanin compounds (Boss *et al.*, 1996a). Several of the genes involved in flavonoid biosynthesis in grapevine have been cloned (Sparvoli *et al.*, 1994) and were used to study the control of anthocyanin biosynthesis during berry ripening (Boss *et al.*, 1996a; 1996b).

From these studies and expression analyses comparing white, red and black grape varieties, it was clear that one of the last proteins in the pathway represents a controlling step in the production of anthocyanins in grapevine (Boss *et al.*, 1996a; b; c). This control step was attributed to UDP glucose flavonoid-3-glucosyl transferase (UFGT) which was not expressed in non-pigmented tissues of Shiraz berries, nor in the skins of white cultivars (Boss *et al.*, 1996a).

### **2.2.3 Stress and grape berry ripening**

It seems that the ripening process in grape berries is a stress-associated process (Davies & Robinson, 2000). Ripening involves the influx of high concentrations of sugars as well as the concomitant rapid cell expansion and ripening-induced softening of the fruit (Davies & Robinson, 2000). Previously, ripening-related cDNAs were isolated and two groups of proteins could be identified. The first group consisted of several examples of

proteins putatively involved with cell wall structure and includes members of the diverse P/HRGP family that are thought to be involved in the strengthening of polysaccharide networks in cell walls (Sommer-Knudsen *et al.*, 1998). These proteins might function in stabilising the rapidly expanding and “softening” cell wall during ripening, or alternatively be involved in pathogen elimination, since their expression in other species has been up-regulated by pathogen attack. In grapevine, there also seem to be a developmentally controlled defence response during fruit ripening as evidenced by coordinate accumulation of antifungal proteins and hexoses (Saltzman *et al.*, 1998).

Another group of proteins identified by differential screening comprise stress-related proteins (Davies & Robinson, 2000). As argued by the authors, the adjustment to rapid increases in vacuolar hexose levels during ripening might involve proteins usually employed in stress management. Since ABA levels usually increase during ripening, it might be involved in the regulation of these putative stress-response genes as has been shown in other instances (Bray, 1997).

### **2.3 Differential gene expression and screening**

Many different processes take place during fruit ripening and it has been shown that the genes regulating these biochemical processes are differentially expressed (Nam, *et al.*, 1999). This invariably leads to different mRNA transcript levels (Scutt, 1997). These differences can occur between different tissues, at different developmental stages, in response to various environmental stimuli or between genetically dissimilar individuals. Differential screening is a method of identifying cloned DNA sequences homologous to differentially regulated genes (Scutt, 1997). Early examples of such genes include the small sub-unit of ribulose-1,5-bisphosphate carboxylase (RuBisCo) (Bedbrook *et al.*, 1980) and  $\alpha$ -amylase (Rogers & Milliman, 1983). Several hundred differentially expressed plant genes have been identified (Oh *et al.*, 1995).

There are several differential screening strategies available today. One such method makes use of PCR-based differential display for efficient identification and isolation of genes whose expression patterns are correlated with changes in growth, development, physiology and/or environmental response (Oh *et al.*, 1995). Other methods include



screening of cDNA libraries (Nam *et al.*, 1999) and subtractive cloning (Balzer, *et al.*, 1996).

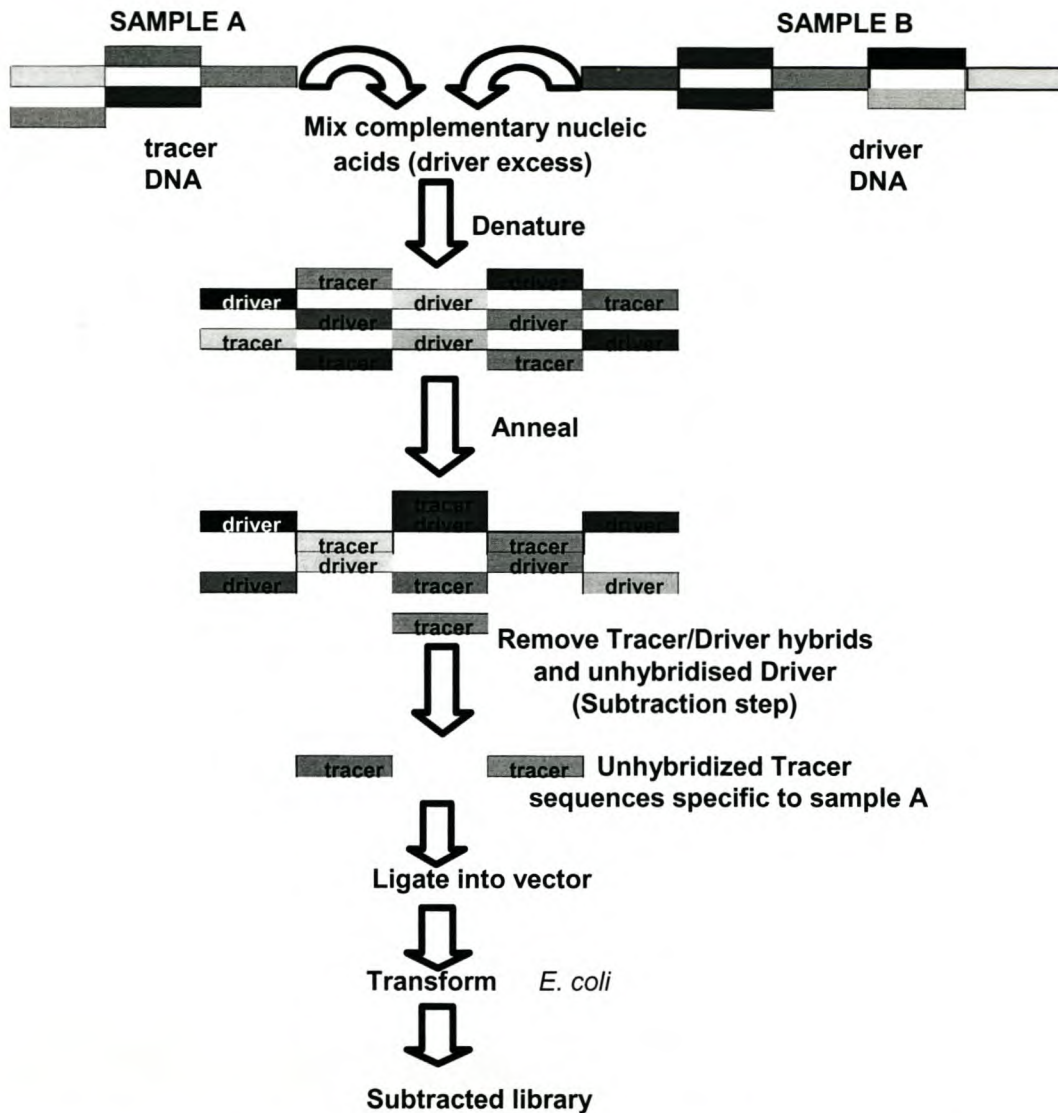
### **Differential gene expression in grapevine**

The isolation of ripening-enhanced cDNAs from strawberry (Nam *et al.*, 1999) and grapevine (Davies & Robinson, 2000) by differential screening has demonstrated that this technique will be useful in the study of ripening in non-climacteric fruit.

Using this technique, researchers have been able to isolate grape ripening-induced (*Grip*) cDNAs from grape berry cDNA libraries (Davies & Robinson, 2000). A number of differentially expressed clones were isolated and their sequences and expression patterns in grape tissues analysed. Seven clones found homology with sequences encoding putative cell wall proteins and ten clones encoding putative stress response proteins were isolated (Davies & Robinson, 2000). The researchers proposed that the putative *Grip* proteins might function to protect tissues that are more at risk of pathogenic infection due to changes in cell wall properties during ripening. Equally important may be the role of some of the putative *Grip* proteins in protecting ripening berry tissues from the consequences of an important part of the ripening process itself, i.e. the storage of large amounts of hexoses (and water) in the cell vacuole (Davies & Robinson, 2000).

### **2.4 Subtractive cloning**

Subtractive cloning is a powerful technique that allows isolation of the differences in the nucleic acid composition of two cell samples (Sagerström *et al.*, 1997). Differences can be at the level of RNA species represented within each sample or within the complement of genomic DNAs. Such differences include genes whose differential expression distinguishes one cell type from another, one growth phase from another, or a normal state from a diseased state (Sagerström *et al.*, 1997).



**Figure 2.2.** A simple schematic representation of the subtraction process. Amplification rounds are built in after each step to supplement tracer and driver populations for further rounds of subtraction.

Subtractive cloning uses a process called driver excess hybridisation. For this reason it is sometimes called subtractive hybridisation. Nucleic acid from which one wants to isolate differentially expressed sequences (the tracer) is hybridised to complementary nucleic acid that is believed to lack sequences of interest (the driver) (Sagerström *et al.*, 1997). Driver nucleic acid is present at a much higher concentration (at least 10-fold) than tracer, and it dictates the speed of the re-annealing reaction. The driver and tracer nucleic acid populations are allowed to hybridise, and only sequences common to the two populations can form hybrids. This is the subtraction step (Sagerström *et al.*, 1997). The tracer that remains behind is enriched for sequences specific to the tracer tissue source and depleted for sequences common to tracer and driver (the hybrids)

(Fig. 2.2). An important parameter controlling the success of the hybridisation is the tracer:driver ratio, which should be at least 1:10 to allow the driver to govern the subtraction (Wang & Brown, 1991). This minimises the risk of tracer:tracer hybrids forming. In the present study a ratio of 1:20 was used as described by Ausubel and co-workers, (1998).

A variety of methods have been used to separate target sequences from the rest of the hybridisation. Most current methods employ biotin labelling of the driver. This allows the use of techniques exploiting the very high affinity of biotin binding to capture hybrids and unhybridised driver, *e.g.* avidin affinity columns (Duguid *et al.*, 1988) and streptavidin-coated paramagnetic beads (Houben *et al.*, 1996). In the current study, streptavidin-phenol:chloroform was used (Ausubel *et al.*, 1998). After subtraction, remaining nucleic acid can be used to prepare a library enriched in tracer-specific clones or to make a probe that can be used to screen a library for tracer-specific clones (Sagerström *et al.*, 1997).

Differentially expressed genes are of importance as they confer identity to a stage or tissue, and often permit specific functions to be carried out. Historically, methods for isolating such genes have been among the most challenging in molecular biology (Birch *et al.*, 2000). The advent of the polymerase chain reaction (PCR) has led to methods that allow profiles of gene expression (after conversion of mRNA to cDNA) to be readily visualised. This would make it possible to begin with very small quantities of RNA and, by performing repeated subtractions, achieve maximal enrichment of differentially expressed genes in both RNA starter populations (Ausubel *et al.*, 1998).

There are several methods for investigating differentially regulated genes in plants (Kuhn, 2001). These include cDNA-AFLP (Bachem *et al.*, 1996), microarrays (Schena *et al.*, 1995) and suppression subtractive hybridisation (SSH) (Diatchenko *et al.*, 1996). In the present study it was decided to use subtractive hybridisation. Subtractive hybridisation has found several practical applications in the world of molecular biology of which some examples will be discussed. Furthermore, interesting findings from these studies will be mentioned. One such research project used this method coupled with differential screening to isolate three cDNA clones that show enhanced expression

during cold, moist dormancy-breakage treatment of Douglas fir seeds (Jarvis *et al.*, 1996).

Sequence analysis revealed a high degree of similarity between the three cDNA clones and Late Embryogenesis Abundant (LEA) protein genes. This was the first isolation of LEA protein genes from gymnosperms (Jarvis *et al.*, 1996).

More detailed knowledge about the DNA and protein composition of plant centromeres is not only an indispensable prerequisite for understanding the mechanisms that regulate chromosome segregation during nuclear divisions and of the evolutionary convergence/divergence of this chromosomal 'organelle' between phyla, but also for future construction of artificial plant chromosomes (Houben *et al.*, 1996). Characterisation of a *Vicia faba* centromere/kinetochore complex was performed using subtractive DNA-DNA hybridisation. After six rounds of subtraction results showed that, **1.** either centromere-specific DNA sequences are not very conserved in higher plants and may occur intermingled with complex dispersed repeats; or **2.** dispersed repeats themselves specify the centromeres by stereophysical parameters (and specific interaction with kinetochore proteins) rather than simply base sequence (Houben *et al.*, 1996).

Plants respond to drought through modifications of their morphological, physiological and metabolic processes. Researchers selected two genotypes of sunflower in the field as drought-tolerant (R1) or drought-sensitive (S1). Using subtractive hybridisation, six cDNA clones were identified corresponding to transcripts accumulated in R1 and S1 leaves during adaptive response. These so-called sunflower drought-induced (*sdi*) genes found sequence similarities with protein homologues like dehydrin, ACC oxidase, nsLTP (an epidermal cell wall protein) and ELIP (chloroplast thylakoid protein) (Ouvrard *et al.*, 1996).

Subtractive hybridisation can also be used to distinguish between specific crop lines. Researchers have used this method to isolate genes specifically expressed at the initiation of plant embryo development in three isogenic wheat lines. It was demonstrated for the first time that a grass pollen allergen-like sequence thought to be specific for pollen tissue is also expressed in wheat ovaries (Balzer *et al.*, 1996).

Buchanan-Wollaston and Ainsworth, (1997) developed a technique in which cDNAs representing genes that showed enhanced expression during leaf senescence in *Brassica napus* were cloned. A number of genes were identified that showed different patterns of expression during leaf development but were all expressed at increased levels during senescence. It is postulated that the genes fall into two broad classes. One class of genes is concerned with degradation and mobilisation of cellular components and the other, more unexpected class is involved with cellular protection to maintain the viability of the senescing cell to optimise the recovery of metabolites (Buchanan-Wollaston & Ainsworth, 1997).

Subtractive hybridisation is a useful tool not only in distinguishing between organ-specific gene expression, but it can be used to differentiate between cell types within a specific plant organ. To understand the functions of the root cap at the molecular level, Matsuyama and co-workers (1999), isolated two unique cDNAs that are specifically localised in the cap by using subtractive hybridisation and differential screening. It was found that these two sequences code for proteins that are possibly involved in the facilitation of cell separation from the root cap proper.

### **Subtractive hybridisation in grapevine**

As far as could be ascertained, no research papers reporting the use of subtractive hybridisation in grapevine were published. Although this method is not novel, the work here remains, to the author's knowledge, the first example of subtractive hybridisation presented in grapevine.

## CHAPTER 3

### RNA ISOLATION FROM RIPENING GRAPE BERRIES: A COMPARATIVE EVALUATION

#### ABSTRACT

The production of high quality cDNA libraries relies on the isolation of large amounts of intact RNA. The choice of a method that delivers large amounts of intact RNA is therefore important. Three methods of RNA extraction using either sodium perchlorate, cesium chloride or guanidine thiocyanate in the extraction buffer, were used to compare yield and quality. The RNA yield was determined fluorometrically, while the quality was evaluated by spectrophotometric analysis, gel electrophoresis, northern blot analysis and the construction of cDNA libraries from young and ripe berry samples. The cDNA libraries contained  $7.5 \times 10^5$  and  $6.5 \times 10^5$  primary recombinants respectively with average insert sizes for both libraries between 1.0 and 1.1 kb. In order to increase the accuracy of RNA quantification, it is suggested that fluorometry be used in addition to spectrophotometric analysis. It was found that the sodium perchlorate method gave the highest yield in the ripening stages leading up to véraison, while the cesium chloride and guanidine thiocyanate methods delivered higher quality RNA, especially in the post-véraison stages.

#### 3.1 INTRODUCTION

In most molecular biology studies, the isolation of high quality RNA is paramount. Thus it is important for any researcher to isolate RNA of sufficient quality to serve as substrate in for example reverse transcription or northern blot analysis.

The presence of large amounts of polyphenols and polysaccharides in grape berry tissue complicates the extraction of high quality RNA (Franke *et al.*, 1995). These compounds can hamper accurate quantification of RNA, especially if it is done spectrophotometrically (Franke *et al.*, 1995). Phenolic compounds can irreversibly

bind to RNA rendering it unusable for such fundamental procedures as reverse transcription and cDNA library construction (Tesniere & Vayda, 1991). It is therefore imperative for any method to include chemicals that will deal with this problem (Franke *et al.*, 1995; Salzman *et al.*, 1999).

There are several methods for the extraction of RNA from grape berries (Franke *et al.*, 1995; Loulakakis *et al.*, 1996; Tattersall *et al.*, 1997). One of these methods draws on classic protocols using a so-called chaotropic salt, like sodium perchlorate to dissociate and denature protein-nucleic acid complexes (Rezaian & Krake, 1987). A second method uses selective precipitations and a cesium chloride cushion to isolate RNA. The cushion provides a density gradient that allows the binding of DNA and other cellular components. Since there is no attainable cesium chloride concentration at which RNA binding will take place, the RNA moves through the cushion to form a retrievable pellet (Glišin *et al.*, 1974; Loulakakis *et al.*, 1996). The third method uses a potent denaturing agent like guanidine thiocyanate to dissociate nucleoproteins from nucleic acids, as protein secondary structure is lost. RNAses can recover activity after many forms of treatment but are inactivated by guanidine thiocyanate and  $\beta$ -mercaptoethanol (Salzman *et al.*, 1999; Sambrook *et al.*, 1989).

There has been a lack of publications addressing comparative RNA isolation using different methods. Loulakakis and co-workers, investigated methods of RNA isolation in grapevine, but in this study RNA isolated from different grapevine tissues using a hot phenol method and three different types of cesium chloride methods were compared (Loulakakis *et al.*, 1996). Therefore, the need for a comparative study that involves three so-called 'classic' methods using one tissue type only, was identified.

In the present study the three methods mentioned above were used for RNA isolation from grape berries and compared as far as quality and yield were concerned. First, it was found that all three methods delivered consistently moderate to high quantities of intact RNA. Second, it was found that fluorometric quantification of RNA yields was more reliable than spectrophotometric readings. Third, it was found that RNA isolated with the Na-perchlorate method could be used in the successful extraction of mRNA as well as the construction of two stage-specific cDNA libraries.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Plant material

Grape berries (*Vitis vinifera* L. cv.. Chardonnay and Merlot) were harvested from the Grondves experimental vineyard of the Koöperatiewe Wijnbouwers Vereniging (KWV), Stellenbosch, South Africa. After harvest, berries were rinsed with water, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . Leaves and roots from aeroponically grown grapevine cuttings were used in this study. Cuttings were harvested after winter and roots were induced with a growth medium containing  $0.035\text{ g.L}^{-1}$  isobutyric acid (IBA) and  $1\text{ g.L}^{-1}$  of an inorganic fertiliser containing 6.5% (m/v) nitrogen, 2.7% (m/v) phosphor and 13% (m/v) potassium.

### 3.2.2 RNA extraction procedures

Frozen berries were ground to a fine powder using an electric mill at maximum speed for 2-4 min (Ika, A10, Janke & Kunkel). The mill was pre-chilled using chips of dry ice that were also included with the berries prior to homogenisation. The homogenised material was either used directly for RNA isolation or stored at  $-80^{\circ}\text{C}$  for future use. Leaf and root material were ground to a fine powder in liquid nitrogen using a mortar and pestle.

All glassware and laboratory equipment used were cleaned with RNase AWAY<sup>®</sup> (Molecular BioProducts) and rinsed with DEPC-treated (diethylpyrocarbonate-treated) water. Solutions were prepared from stocks that had been treated with 0.1% (v/v) DEPC.

***Sodium perchlorate method.*** The extraction buffer contained 5 M Na-perchlorate, 0.3 M Tris-HCl (pH 8.3) (tris(hydroxymethyl) aminomethane), 1 % (w/v) SDS (sodium dodecyl sulfate), 10% (w/v) PVPP (polyvinylpolypyrrolidone), 2% (v/v) PEG-6000 (polyethylene glycol) and 1% (v/v)  $\beta$ -ME ( $\beta$ -mercaptoethanol). The solution containing the SDS, PEG, Tris-HCl and Na-perchlorate was heated at  $65\text{ }^{\circ}\text{C}$  for at least 15 min before addition of PVPP and  $\beta$ -ME. Four grams of pulverised frozen tissue



were added to 20 ml extraction buffer. To properly mix the buffer with the tissue, the samples were placed on ice for 30 min and vortexed vigorously every 10 min. The resulting slurry was filtered through a 1.5 cm length of glasswool, tightly packed in an empty 50 ml syringe tube, by centrifugation at 2000 g. After addition of 2.5 volumes of 100% (v/v) ethanol, the filtrate was placed at  $-20^{\circ}\text{C}$  for 2 h. The solutions were centrifuged at 10000 g for 20 min. After the pellet was washed with 70% (v/v) ethanol, the supernatant was discarded and the resulting pellet was dried for approximately 15 min. The pellet was resuspended in 2 ml TE buffer containing 0.2% (v/v)  $\beta$ -ME. Two to three phenol: chloroform: isoamylalcohol (25:24:1) extractions were performed until the interphase appeared clear. After adding 2.5 volumes 100% ethanol and 0.1 volume 3 M NaOAc (sodium acetate)(pH 5.2) to the supernatant, the solution was placed at  $-20^{\circ}\text{C}$  for 2 hr. The solution was then centrifuged at 12000 g for 20 min at  $4^{\circ}\text{C}$  after which the supernatant was discarded and the pellet washed with 70% (v/v) ethanol. The pellet was dried in a SpeedVac (Savant) and resuspended in 200  $\mu\text{l}$  DEPC-treated water. A 0.3 volume 8 M LiCl (lithium chloride) was added and the solution was incubated at  $4^{\circ}\text{C}$  for at least 12 h. The solution was centrifuged at 12000 g for 30 min. After a 70% (v/v) ethanol wash, the pellet was resuspended in 100  $\mu\text{l}$  DEPC-treated water. When contaminants were still present, the suspension was centrifuged for 5 min at 4000 g and the supernatant recovered. The samples were then ready for RNA quantification using both spectrophotometry and fluorometry.

***Cesium chloride method.*** A modified version of a method by Tesniere and Vayda (1991) was used. The extraction buffer was modified in the following way: 300 mM Tris-HCl (pH 8.3) was used and 8.5% (w/v) PVPP was added (Jones *et al.*, 1997). The solution for the cesium cushion remained the same as the one published (Tesniere & Vayda, 1991). Five gram of tissue was added to 35 ml of the extraction buffer after which the homogenate was vortexed vigorously for at least 15 min. After centrifugation at 12000 g at  $4^{\circ}\text{C}$  for 30 min, the supernatant was carefully removed and filtered through silanised glasswool. The latter was tightly packed to a height of 1.5 cm in an empty 50 ml syringe tube. The filtration was done by centrifugation at 2000 g for 10 min at  $4^{\circ}\text{C}$ . Cesium chloride was added to the filtrate and layered onto a cesium chloride cushion (Tesniere & Vayda, 1991). Ultra-centrifugation was done in a Beckman 30 rotor at 28 000 rpm for 20 h at  $20^{\circ}\text{C}$ . The supernatant was removed by

aspiration with a pasteur pipette and discarded. The pellet was washed with 5 ml chilled 70% (v/v) ethanol. The pellet was air dried in a fumehood at room temperature for approximately 15 min after which it was resuspended in 1 ml of DEPC-treated water. After the addition of a final concentration of 0.5 M LiCl and 2.5 ml 100% ethanol, the suspension was incubated at  $-20^{\circ}\text{C}$  overnight. The suspension was centrifuged at 12000 g for 30 min at  $4^{\circ}\text{C}$  and washed with 70% (v/v) ethanol. The pellet was resuspended in 100  $\mu\text{l}$  of DEPC-treated water after which the remaining contaminating viscous components were removed by slow addition of 95% (v/v) ethanol to a final concentration of 30% (v/v). After the solution was centrifuged at 12000 g for 5 min, the supernatant was collected and quantified by spectrophotometry and fluorometry.

***Guanidine thiocyanate method.*** The method of Salzman and co-workers (1999) was followed exactly as described. The extraction buffer contained 4 M guanidine thiocyanate, 100 mM Tris-HCl (pH 8.0), 25 mM sodium citrate (pH 8.0) and 0.5% (v/v) N-lauryl sarcosine. Prior to addition of the sample material, 0.1 g PVP and 200  $\mu\text{l}$   $\beta$ -ME were added to the extraction buffer. Two grams of pulverised, frozen tissue were added to 10 ml extraction buffer. After shaking vigorously for 1 min, an equal volume of chloroform:isoamyl alcohol (24:1) was added. After shaking vigorously for 10-20 min, the solution was centrifuged at 16 000g for 10 min to separate the two phases. The upper phase was carefully transferred to another centrifuge tube. If the interphase remained unclear after one addition of chloroform:isoamyl alcohol (cia), the cia wash was repeated until the interphase cleared. After adding 2 times the volumes of 100% (v/v) ethanol and 0.1 times volume 5 M NaCl, the homogenate was placed at  $-20^{\circ}\text{C}$  for at least 3 h to allow for precipitation. The solution was centrifuged at 16 000g for 10 min at  $4^{\circ}\text{C}$  and the pellet was resuspended in 10 ml extraction buffer. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. After shaking the suspension for 5-10 min at room temperature, it was centrifuged at 13 000g for 10 min at room temperature to separate the two phases. The phenol:chloroform:isoamyl alcohol washes were repeated until the interphase appeared clear of visible material. A two times volume of 100% (v/v) ethanol together with a 0.1 times volume of 5 M NaCl was added and the previously mentioned precipitation step was repeated at  $-20^{\circ}\text{C}$  for at least 3 h. After centrifuging the suspension at 16 000g for 15 min at  $4^{\circ}\text{C}$ , the pellet

was resuspended in 1 ml DEPC-treated water. After addition of 333 $\mu$ l of 8 M LiCl, the suspension was placed at 4 °C for at least 3 h to allow for RNA precipitation. The solution was centrifuged at 12 000g for 20 min at 4 °C and the recovered pellet was washed with a 70% (v/v) ethanol solution. After the pellet was dried, it was resuspended in 100 $\mu$ l DEPC-treated water and quantified using spectrophotometry and fluorometry.

**Other methods.** A phenol extraction method (Bugos *et al.*, 1995), Qiagen's RNeasy kit (Qiagen, Valencia, USA) and Promega's SV-Total RNA (Promega, Madison, USA) kit were used.

### 3.2.3 Analysis of RNA

RNA concentrations were determined fluorometrically using Ribogreen<sup>®</sup> dye (Molecular Probes) and an FL-600 fluorometer (Bio-Tek Instruments) according to the manufacturer's specifications (Molecular Probes, Eugene, USA). To determine RNA quality and assess degradation, 1  $\mu$ g RNA samples were subjected to gel electrophoresis along with RNA molecular weight markers (Gibco) in a 1.2% (w/v) agarose gel containing 0.5  $\mu$ g.mL<sup>-1</sup> ethidium bromide. Ribosomal bands of 18S and 28S RNA were visualised and photographed under UV light. To gauge possible contamination by polysaccharides and polyphenols, RNA was quantified spectrophotometrically (PowerWaveX, Bio-Tek Instruments). Absorbance was measured at wavelengths of 230, 260, 280 and 320 nm respectively. To calculate purity of RNA samples, the  $A_{260}/A_{280}$  ratio was used, while the  $A_{230}/A_{260}$  ratio gave an indication of the polysaccharide/polyphenol contamination. Pure RNA was indicated by an  $A_{260}/A_{280}$  ratio of 2.0. The 320 nm reading was taken as an indication of background absorbance and was used in the calculation of RNA yields. RNA was visualised in a 1% (w/v) agarose gel containing 10  $\mu$ g RNA/lane. Northern blot membranes were prepared using total RNA isolated from berry ripening stages 1-6 (see 2.1.1), leaves and roots. Verification of the presence of tubulin mRNA was obtained from northern blots probed with a grape  $\beta$ -tubulin probe (obtained from Sarah Picaud, Laboratoire de Physiologie et Biochimie Végétales, Université de Poitiers, France). RNA was transferred to positively charged nylon membranes (Boehringer Mannheim) by upward

capillary blotting using 10x SSC (standard saline citrate) (Sambrook *et al.*, 1989). The grape  $\beta$ -tubulin probe was radioactively labelled using 25  $\mu$ Ci [ $\alpha$ - $^{32}$ P] dCTP and the random primer labelling kit, Prime-It<sup>®</sup> II (Stratagene). Hybridisation occurred overnight using ULTRAhyb<sup>™</sup> hybridisation buffer (Ambion). All washing procedures were carried out as described by the manufacturer (Ambion, Austin, USA). Hybridisation signals were visualised using the Cyclone<sup>®</sup> Storage Phosphor System (Packard Instruments).

### 3.2.4 cDNA library construction

cDNA libraries from ripening stages 1 and 5 were prepared for this study. Total RNA from stages 1 and 5 of berry ripening were subjected to poly A<sup>+</sup> selection using a PolyATtract<sup>®</sup> kit (Promega, Madison, USA). The mRNA was quantified fluorometrically to assess percentage yield. First- and second-strand cDNA was synthesised from the purified mRNA using a Universal Riboclone<sup>®</sup> cDNA Synthesis System (Promega, Madison, USA). The cDNA was treated with T4 DNA Polymerase (Promega) to create blunt-ends. A modified version of a method by Jepson and co-workers (1991) were used to construct the libraries. Two oligonucleotides were synthesised according to the abovementioned publication: cDNA 1 (5'-ATGCTTAGGA ATTCCGATTTAGCCTCATA-3') and cDNA 2 (5'-TATGAGGCTAAA-3'). After annealing of the two primers to create adapters (linkers), the adapters were ligated to the blunt-ended cDNA.

To remove smaller fragments (less than 300bp) and unligated linkers, the cDNA was size fractionated using Sephacryl<sup>®</sup> S-400 columns. Ligated fragments were amplified for 35 cycles using Taq Polymerase (Promega) in the presence of 1.28  $\mu$ M primer (cDNA 1) and 0.1 mM dNTPs under the following conditions: denaturation at 94°C for 48 s, annealing at 68°C for 66 s, and elongation at 72°C for 3 min. Preceding the 35 cycles, 1 cycle at 73°C for 2 min is needed to melt off the short primer (cDNA2). PCR products were subjected to restriction digestion with *EcoRI* (Promega) overnight. The libraries were constructed using a Lambda Zap<sup>®</sup> II vector cloning kit and packaged using Gigapack Gold<sup>®</sup> III packaging extracts according to the manufacturer's

instructions (Stratagene, La Jolla, USA). Subsequent library analysis, titering and amplification were performed as described by Stratagene.

### 3.3 RESULTS AND DISCUSSION

Extraction of RNA from fruit tissue is complicated by several factors including the presence of interfering polysaccharides, phenolic compounds and RNases (Franke *et al.*, 1995). Today there are a plethora of extraction methods proclaiming validity using various criteria to boost yield and quality of RNA (Loulakakis *et al.*, 1996; Franke *et al.*, 1995; Salzman *et al.*, 1999; Bugos *et al.*, 1995). An examination of these extraction protocols revealed the foundation set in either one of three methods using Na-perchlorate, CsCl or guanidine thiocyanate respectively. The objective in the comparison of the three methods was to provide a basis for researchers to make an informed decision as to which method would be more suitable for their purposes.

Standard methods for RNA extraction from plant tissues employing phenol/SDS failed due to the binding of polyphenolic compounds to nucleic acids under phenol extraction (Salzman *et al.*, 1999). This makes the addition of PVPP (Na-perchlorate & CsCl methods) or PVP (guanidine thiocyanate method) to the extraction buffer crucial, because it binds phenolics and allows for subsequent phenol extractions to selectively separate RNA from the remaining cellular components (Guena *et al.*, 1998).

Early RNA extraction methods (Rezaian & Krake, 1987; Chomczynski & Sacchi, 1987 and Glišin *et al.*, 1974) did not use a LiCl precipitation step, however the selective precipitation of RNA and RNA only is ensured if this step is included (Bugos *et al.*, 1995). Several methods for RNA isolation including Qiagen's RNeasy kit, Promega's SV-Total RNA kit and a phenol extraction method of Bugos and co-workers, (1995) were examined. No RNA could be isolated when these methods were used.

The data in Table 3.1 show the yield (fluorometrically determined) and spectral quality of RNA isolated from six berry ripening stages. For the early stages of ripening (1 and 2), the cesium chloride extractions delivered the lowest yields at an average of  $17\mu\text{g.gfw}^{-1}$ , whereas the sodium perchlorate method gave an average of  $75\mu\text{g.gfw}^{-1}$  and

the guanidine thiocyanate protocol yielded  $\sim 50 \mu\text{g.gfw}^{-1}$ . In stages 2 and 3 the average yield obtained using the Na-perchlorate method decreased to  $50 \mu\text{g.gfw}^{-1}$ , while the other two methods exhibited slight decreases in RNA yields.

**Table 3.1** Purity and yield of RNA isolated from grape berries using three methods at different developmental stages. Values are means from three to five replicates.

Stage	Method	Spectral quality		Yield ( $\mu\text{g.gfw}^{-1}$ ) <sup>d</sup>
		$A_{260}/A_{280}$ <sup>b</sup>	$A_{260}/A_{230}$ <sup>c</sup>	
1 (young) <sup>a</sup>	Na-perchlorate	1.9	2.2	100
	CsCl	1.8	2.5	18
	Guanidine	2	2.1	61
2 (young) <sup>a</sup>	Na-perchlorate	2	2.8	50
	CsCl	1.8	2.6	16
	Guanidine	2	2	38
3 (lag) <sup>a</sup>	Na-perchlorate	1.9	2	50
	CsCl	1.7	1.5	14
	Guanidine	2	1.8	34
4 (véraison) <sup>a</sup>	Na-perchlorate	1.9	1.5	24
	CsCl	1.7	1.4	10
	Guanidine	2	1.8	28
5 (ripe) <sup>a</sup>	Na-perchlorate	1.6	1	17
	CsCl	1.8	1.1	7
	Guanidine	2	1.5	23
6 (overripe) <sup>a</sup>	Na-perchlorate	1.6	0.9	15
	CsCl	1.9	2.2	7
	Guanidine	1.8	1.3	17

<sup>a</sup>Stages are representative of two weekly intervals taken from a week after anthesis. Stage 4 marks the onset of ripening.

<sup>b</sup>The ratio of absorbances at 260 and 280 nm.

<sup>c</sup>The ratio of absorbances at 260 and 230 nm.

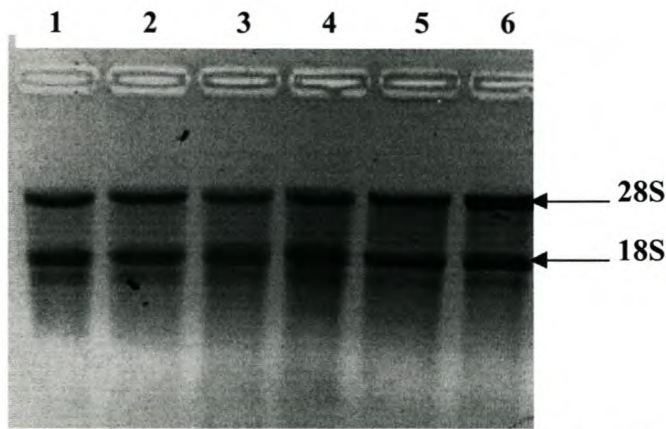
<sup>d</sup>Yield was determined fluorometrically and is presented as micrograms RNA per gram fresh weight.

The results obtained with the Na-perchlorate method showed a sharp decline in the spectral quality with an accompanying decline in the fluorometrically determined RNA concentrations obtained from berries shortly after stage 4. The other two methods showed a continued gradual decrease in RNA yields with a concomitant decline in purity. RNA of highest purity was isolated from stages 1 to 5 using the guanidine thiocyanate method. The  $A_{260}/A_{280}$  ratio here was 2, indicating a pure RNA sample (Loulakakis *et al.*, 1996). The Na-perchlorate method showed a consistent  $A_{260}/A_{280}$  ratio of 1.9 for the stages leading up to and including véraison (stage 4). In the last two stages, the purity fell to 1.6, whereas the CsCl method delivered slightly purer samples in stages 5 with  $A_{260}/A_{280}$  ratios in stage 6 rising to 1.9.

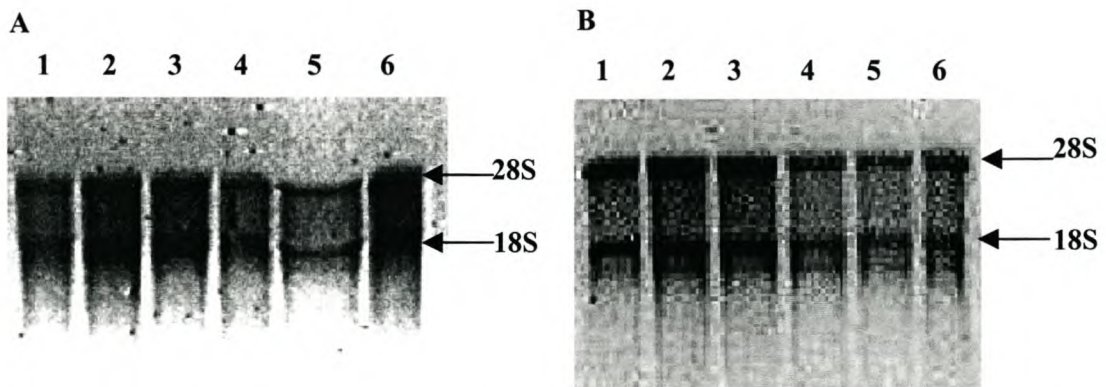
The CsCl method gave RNA of relatively good quality but with low actual yield per extracted sample as reported earlier (Loulakakis *et al.*, 1996). The guanidine thiocyanate method delivered a higher yield, with the purest samples isolated from late stage berries. These tissues are traditionally more difficult to extract from than the early stages due to the accumulation of interfering polysaccharides (Franke *et al.*, 1995). Both of the previous methods are apparently not only ideal for post-véraison berries, but can be applied successfully to leaf tissue as well. The Na-perchlorate method described here gave high yields for the stages leading up to véraison and was subsequently used to isolate RNA from leaf and root tissues.

Characteristically, yields fell with interference of polysaccharides as the berries matured (Franke *et al.*, 1995). This is reflected in the ratio of absorbance at wavelengths of 260 to 230 nm ( $A_{260}/A_{230}$ ). It consistently fell from a 2.8 high to a low 0.9 during ripening. The high number (2.2-2.8) is indicative of low polysaccharide/polyphenol contamination (Loulakakis *et al.*, 1996). As the berries matured, the ratio dropped concomitantly suggesting accumulation of these substances. The ratio  $A_{260}/A_{280}$ , an indicator of purity, showed a 1.9 – 2.0 ratio during the stages leading up to véraison, with pure RNA having an  $A_{260}/A_{280}$  of 2.0 (Loulakakis *et al.*, 1996).

The Na-perchlorate method described here was very effective for the isolation of high amounts of intact RNA from berry ripening stages 1 to 4. RNA yields from stages 5 and 6 were not only lower than the previous stages but also less pure than RNA



**Figure 3.1.** Gel electrophoresis of RNA extracted with the Na-perchlorate method. Lanes 1-6: RNA isolated from stages 1-6 of berry ripening. Ribosomal RNA fragments are indicated with arrows.



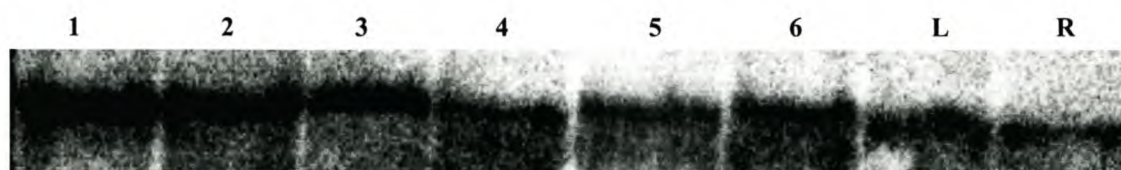
**Figure 3.2.** Gel electrophoresis of RNA extracted using the guanidium thiocyanate method (A) and the cesium chloride method (B). Lanes 1-6: RNA isolated from stages 1-6 of berry ripening.

samples extracted with the other two methods. Examination of the gel electrophoresis of RNA extracted using the CsCl and guanidine thiocyanate methods showed possible salt contamination (carried over from extractions) in samples extracted from the later stages (5 and 6). This can be seen in the distortion of lanes in both figures. Analysis of the spectrophotometric readings, however, showed that both these methods delivered, in general, higher purity ratios ( $A_{230}/A_{260}$ ) than what is evident after gel electrophoresis. A possible explanation for this is that the data in Table 1 are means of 3 to 5 RNA samples whereas the RNA visualised on the gel electrophoresis are single samples. Establishing an average reading of three to five RNA extractions can help conceal contamination in individual samples.



In Figs. 3.1 and 3.2 total RNA extracted with the three methods investigated in this study is visualised. The presence of contaminants (possibly salt from extractions) in both CsCl and guanidine thiocyanate methods can be seen in Fig. 3.2. This is indicated by distortions in lane 5 (Fig. 3.2A) and in lane 6 (Fig. 3.2B).

Tesniere and Vayda (1991) reported that the 28S ribosomal RNA was under-represented in the purified RNA preparations that were isolated using the protocol described. The authors suggested that this could be a characteristic of the maturity stage of the berry. Using a CsCl-based protocol, Loulakakis and co-workers (1996), however, reported that a normal banding profile of total RNA was evident in all the maturity stages tested. Although smearing was evident with gel electrophoresis, the 28S rRNA bands in the present study was equal to or stronger in intensity than the 18S rRNA bands, indicating that little RNA degradation occurred during the extraction (Salzman *et al.*, 1999). It was also noted that the banding profiles, as reported above, did not seem to vary significantly between the various ripening stages.



**Figure 3.3.** Northern hybridisation analysis of RNA isolated from grape berries using the Na-perchlorate method. Lanes 1-6: total RNA from ripening stages 1-6 of grape berry. Lane 7: leaf RNA and lane 8: root RNA. The membrane was probed with  $^{32}\text{P}$ -labelled grape  $\beta$ -tubulin.

In order to further verify the intactness of the isolated RNA, northern blot analysis was performed using grape  $\beta$ -tubulin as a probe (Fig. 3.3). This constitutively expressed gene (Fillion *et al.*, 1999) exhibited expression in the six berry ripening stages as well as the leaf and root RNA as a clearly defined single band. This result confirms the presence of intact mRNA in the isolated total RNA samples.

In Table 3.2 the comparative spectrophotometric and fluorometric quantification of RNA isolated with the Na-perchlorate method is summarised. The comparison was done to illustrate the need for both spectrophotometric and fluorometric quantification. The values showed a difference between the yields calculated from fluorometrical and spectrophotometrical readings. The fluorometric readings were up to 3 times higher

than the spectrophotometric readings. The spectrophotometric yields were calculated using the Beer-Lambert law,  $A = \epsilon \cdot c \cdot l$  where  $A$  was the absorbance at a particular wavelength,  $c$  the concentration of RNA,  $l$  the pathlength of the spectrophotometer cuvette and  $\epsilon$  the extinction coefficient. In our case the  $A$  was calculated by subtracting the absorbance readings at 320nm from the readings taken at 260nm. At a concentration of 1 M, the  $\epsilon$  for RNA will be equal to  $0.025 \mu\text{g}\cdot\text{ml}^{-1}$  if the pathlength,  $l$ , is 1 cm. However, the pathlength used in the present study changed from 1 cm to 0.288 cm, because RNA quantitation was done in a 96-well microtiterplate (Held, 2000). Thus, based on the Beer-Lambert law, the formula used was:

$$\frac{A_{260} - A_{320}}{0.0072} \times \text{dilution factor.}$$

**Table 3.2** Spectrophotometric and fluorometric readings of RNA isolated using the Na-perchlorate method.

Tissue and ripening stage	RNA concentration ( $\mu\text{g}\cdot\text{gfw}^{-1}$ )	
	$A_{260}$	Fluorometry
Leaf	2.65	7.5
Root	6.56	13
4	4.75	15
5	1.83	4.3
6	1.15	2.88

Fluorometric yields were calculated by comparison with a RNA standard curve. The RNA standard was provided in the RiboGreen kit (Molecular Probes). The standard curve was set up according to the specifications set out in the assay protocol (Molecular Probes).

The most commonly used technique for measuring nucleic acid concentration is the determination of absorbance at 260 nm. The major disadvantages of the absorbance-based method are the large relative contribution of proteins and free nucleotides to the signal, the inability to distinguish between DNA and RNA, the interference caused by contaminants commonly found in nucleic acid preparations (*e.g.* phenol) and the relative insensitivity of the assay (Jones *et al.*, 1998). Therefore, in the present study it was

decided to present the comparison between the two quantification methods using tissues and berry ripening stages that are known to contain higher amounts of polysaccharides and compounds that may interfere in the extraction process. It was found that the berry yields (stages 4-6), as well as RNA yields from root and leaf tissue, calculated from fluorometric analysis was 2 to 3 times higher than the same readings obtained through spectrophotometric analysis.

A reason for the discrepancy between fluorometric and spectrophotometric readings may be the fact that the dye used (RiboGreen<sup>®</sup>) in the fluorometric assay binds directly to each RNA molecule, thereby increasing the accuracy of the reading and therefore the yield obtained (Jones *et al.*, 1998). The RiboGreen assay allows detection of as little as 1.0 ng.ml<sup>-1</sup> RNA, surpassing the sensitivity achieved by more traditional methods (Jones *et al.*, 1998).

Perhaps the best indication that the Na-perchlorate method we describe yields good quality RNA is its suitability for cDNA library construction. Two libraries were constructed representing expressed sequences in stage 1 and stage 5 of berry ripening. Not only was it used as a means of RNA quality control, but it can be used subsequently as a basis for further molecular studies in grape berry ripening (*e.g.* library screening). All mRNA extractions delivered between 0.6% and 0.9% (w/w) yield of total RNA used. The stage 1 cDNA library contained  $7.5 \times 10^5$  primary recombinants per ml with an average insert size of 1 kb. The stage 5 cDNA library contained  $6.5 \times 10^5$  primary recombinants per ml with an average insert size of 1.1 kb.

### 3.4 CONCLUSIONS

In conclusion it was found that, depending on the ripening stage, all three methods gave moderate to high intact RNA yields, with lower yields in stages 5 and 6 of ripening. RNA obtained with the Na-perchlorate method delivered sufficient amounts of intact RNA for mRNA isolation and the subsequent production of high quality cDNA libraries. However, it is suggested for future extractions that in order to increase purity and possibly yield when extracting from stages 5 and 6, that a combination of two of the methods evaluated here be investigated. Combining the effectiveness of a strong chaotropic agent, like Na-perchlorate, in the extraction buffer followed by placement on

a CsCl gradient should ensure the selective isolation of RNA. Combining two methods have been suggested before, where guanidine thiocyanate is used to disrupt the cells, and the resulting homogenate is then layered on a cushion of CsCl (Sambrook *et al.*, 1989). Furthermore, it is advisable, based on comparative data analysis between fluorometric and spectrophotometric assays in this study that RNA should be quantified both fluorometrically and spectrophotometrically to ensure RNA quality and accuracy in yield estimations.

## CHAPTER 4

### CLONING AND CHARACTERISATION OF DIFFERENTIALLY EXPRESSED GENES DURING RIPENING IN GRAPE BERRIES THROUGH SUBTRACTIVE HYBRIDISATION

#### ABSTRACT

Subtractive hybridisation and differential screening were used to isolate putative differentially expressed sequences from maturing Chardonnay grape (*Vitis vinifera* L.) berries. Using a PCR-based subtraction cloning method, two subtraction libraries were established representing differentially expressed cDNAs in early and late berry ripening respectively. A rapid increase in the mRNA expression profiles of cDNAs not present in unripe fruit occurred in grape berries at the onset of ripening. The putative translation products of some of these clones had homologues in other species that are involved in cell wall structure. These included, UDP-glucose dehydrogenase, which is involved in the synthesis of hemicellulose precursors. The remainder of the clones encoded putative stress response proteins. These included two heat shock proteins, an abscisic stress ripening protein, a ribulose biphosphate carboxylase, a vacuolar pyrophosphatase and a protein involved in cell division. Many of the homologues of the grape cDNAs thought to be involved in cell wall structure or stress-related responses also accumulate in a developmental manner in other plants. This may indicate that the grape mRNAs accumulate in response to stresses such as the storage of high concentrations of sugars and rapid cell expansion, or they may accumulate as part of the ripening developmental programme.

#### 4.1 INTRODUCTION

It seems that the ripening process in grape berries is a stress-associated process (Davies & Robinson, 2000). Ripening involves the influx of high concentrations of sugars as well as the concomitant rapid cell expansion and ripening-induced softening of the fruit (Coombe, 1992). Part of the adjustment to the rapid increase in vacuolar

sugar levels may be the synthesis of proteins involved in stress management (Davies & Robinson, 2000).

From a differential screening analysis, two groups of proteins involved in the abovementioned process have been isolated (Davies & Robinson, 2000). The first group consisted of several examples of proteins putatively involved in cell wall structure and includes members of the diverse P/HRGP family that are thought to be involved in the strengthening of polysaccharide networks in cell walls (Sommer-Knudsen *et al.*, 1998). These proteins might function in stabilising the rapidly expanding and softening cell wall during ripening, or alternatively be involved in pathogen elimination, since their expression in other species has been up-regulated through pathogen attack (Sommer-Knudsen *et al.*, 1998). In grapevine, there also seem to be a developmentally controlled defence response during fruit ripening as evidenced by coordinate accumulation of anti-fungal proteins and hexoses (Saltzman *et al.*, 1998).

The second group of proteins identified by the differential screening comprised stress-related proteins and it is argued that the adjustment to rapid increases in vacuolar hexose levels during ripening might involve proteins usually employed in stress management (Davies & Robinson, 2000). Since ABA levels usually increase during ripening, it might be involved in the regulation of these putative stress-response genes as has been shown in other instances (Bray, 1997).

Similar differential screening techniques have also been used to investigate the molecular biology of ripening in non-climacteric fruit such as strawberry (Medina-Escobar *et al.*, 1997) and pepper (Proust *et al.*, 1996). The isolation of ripening-enhanced cDNAs from strawberry (Manning, 1998) and black currant (Woodhead *et al.*, 1998) by differential screening has demonstrated that this technique will be useful in the study of ripening in non-climacteric fruit.

Many genes are differentially expressed and may be amenable to identification by differential screening, as was shown above. Subtractive hybridisation is a powerful method of detecting and isolating gene sequences that are differentially expressed (Harper, 1997). Subtractive methods have successfully been used to identify

differentially expressed genes in plants, and these include genes induced by environmental stimuli (Jarvis *et al.*, 1996; Ouvrard *et al.*, 1996) and those involved in development (Buchanan-Wollaston & Ainsworth, 1997; Matsuyama *et al.*, 1999). It should however be noted, that subtractive hybridisation, although useful in the isolation of less abundant expressors, can not be used to detect sequences with an abundance lower than approximately 0.1% of the original population (Hodge *et al.*, 1992). In the present study, subtractive hybridisation was used to clone several Sde (subtractive, differentially expressed) cDNAs from early and late grape berry ripening stages. Two subtraction libraries were established representing early and late berry ripening respectively. Initial screening of the expression arrays identified ten subtracted sequences. Four of these sequences were shown to be differentially expressed and were subsequently characterised further via sequence analysis.

It was found that many of the homologues of the grape cDNAs isolated in the present study are thought to be involved in cell wall structure or stress-related responses and also accumulate in a developmental manner in other plants. This may indicate that the grape mRNAs accumulate in response to stresses such as the storage of high concentrations of sugars and rapid cell expansion, or they may accumulate as part of the ripening developmental programme.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 Plant material**

Grape berries, leaves and roots were harvested and frozen as described earlier (see 3.3.1). Flowers were harvested from the same vineyard as the other material (see 3.3.1), frozen immediately in liquid nitrogen and stored at -80°C.

### **4.2.2 mRNA isolation and cDNA synthesis**

Total RNA was extracted from grape berry, leaf and root material with a modified sodium perchlorate method (Rezaian & Krake, 1987) (see 3.3.2). Total RNA from grape flowers was extracted using a modified cesium chloride method (see 3.3.2).

Poly(A)<sup>+</sup> RNA was extracted using a PolyATtract<sup>®</sup> mRNA isolation kit (Promega, Madison, USA).

First- and second-strand cDNA was synthesised from the purified mRNA from both target tissue and driver tissue using a Universal Riboclone<sup>®</sup> cDNA Synthesis System (Promega, Madison, USA). In the present study both forward (target tissue: stage 5; driver tissue: stage 1) and reverse subtractions (target tissue: stage 1; driver tissue: stage 5) were performed. The ends of the cDNAs were made blunt using T4 DNA polymerase.

### 4.2.3 Construction of subtracted libraries

The method developed was based on that published by Ausubel and co-workers (1998), which involved alternating rounds of short subtractive hybridisation, long subtractive hybridisation and PCR amplification. The procedure used a biotinylated primer in the amplification reaction to produce the biotinylated driver DNA, and the use of streptavidin to remove the driver and hybridising target DNA fragments. In addition, ligation of a specific primer to the target cDNA fragments allows amplification and cloning of fragments derived from the target cDNA. The cDNA for library construction was synthesised using mRNA isolated from five pooled samples of total RNA. The starting quantity of mRNA was 500 ng. The average yield was ~2.5 µg of poly(A)<sup>+</sup> RNA from 10 g of tissue.

Both sets of target cDNA were digested to obtain small fragments (200 to 600bp) using the restriction enzymes *RsaI* and *AluI*. Two oligonucleotides were synthesised: A1 (5'-TAGTCCGAATTCAAGCAAGAGCAC-3') and A2 (5'-CTCTTGCTTGAATTCGG ACTA-3'). Annealing of these two oligonucleotides results in an *EcoRI* restriction site within the linker (underlined). A2 was phosphorylated using T4 polynucleotide kinase (Promega) and the two linkers were annealed together. The double stranded linker (A1/A2) was then ligated to the target cDNA fragments (in this case cDNA from stage 1). The cDNA fragments from the driver tissue were ligated to a different double stranded linker consisting of two manufactured oligonucleotides: B1 (5'-ATGCTGGAATATCTTGGTACTCTTC-3') and B2 (5'-GAGTAC



CAAGATATCCA GCAT-3'). The underlined areas denote the position of an *EcoRV* restriction site. Ligated fragments were amplified for 30 cycles using Taq DNA polymerase (Promega) in the presence of 0.4  $\mu$ M primer and 0.2 mM dNTPs under the following conditions: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 2 min with 25 s of auto-extension per cycle. Target cDNA fragments were amplified using the A2 or B2 primers depending on which subtraction was performed, forward or reverse. B2 was used in a forward subtraction, whereas A2 was used in the reverse subtraction. The same primers were used to amplify driver cDNA. The only difference here was that the driver dNTP mix contained a 0.5 mM biotin-11-dUTP/1.0 mM dTTP ratio.

A twentyfold excess of biotinylated driver fragments (20  $\mu$ g) was mixed with 1  $\mu$ g of amplified target fragments. The DNA was precipitated with ethanol and the pellet was resuspended in 5-8  $\mu$ l of HE buffer (100 mM Hepes pH 7.3, 1 mM EDTA). Five  $\mu$ l of preheated (68°C) 2x hybridisation buffer (1.5 M NaCl, 50 mM Hepes pH 7.3, 10 mM EDTA, 0.2% (w/v) SDS) was added. The solution was incubated at 95°C for 10 min and then allowed to cool slowly over 1 hr to 68°C. The hybridisation was continued at 68°C for 2 h. Subsequent hybridisations alternated between long (30 – 40h) hybridisations and short (2h) hybridisations. Thus, each round of subtraction consisted of one short hybridisation and one long hybridisation. After each hybridisation, 50 mM NaCl in 140  $\mu$ l HE buffer (preheated to 68°C) was added to dilute the reaction. After cooling the reaction mixture to room temperature, 15  $\mu$ l of streptavidin solution (2  $\mu$ g. $\mu$ l<sup>-1</sup> streptavidin, 0.15 M NaCl, HE buffer) was added. This solution was vortexed and then incubated for 5 min at room temperature. After extraction with an equal volume of phenol:chloroform (25:24), the aqueous phase was transferred to new tubes. To ensure that all biotinylated DNA was removed with the former addition, 10  $\mu$ l of streptavidin solution was added. This was vortexed again and then incubated for 5 min at room temperature. Two phenol:chloroform extractions were performed, after which the aqueous phases were retained. This was considered to be round 1 of subtraction. A small amount of the subtracted DNA was then amplified by PCR using A2 or B2 primers. This produced the tracer (target) populations for the next round of subtraction. Driver cDNA for the short hybridisations were used from the original driver populations, synthesised at the start of the subtraction protocol (A<sub>0</sub> and B<sub>0</sub>). All

subsequent driver cDNA for short hybridisations were taken from these two populations. Driver cDNA for the long hybridisations were amplified from the previous round of subtraction.

After three and six rounds of subtractive enrichment and amplification, the subtracted cDNA was cloned into pGEM-T Easy (Promega) and transformed into *Escherichia coli* strain MC106. Resultant colonies were picked and the inserts in the plasmids were amplified by PCR directly from the colonies (colony PCR), using T7 and M13 primers as described in 4.3.5. The sizes of the inserts were estimated by gel electrophoresis.

#### **4.2.4 Expression array analysis**

Subtracted library clones were cultured in 96-well microplates and stored as glycerol stocks at  $-80^{\circ}\text{C}$ . Hybond N<sup>+</sup> membranes (Amersham) were spotted with 1  $\mu\text{l}$  cultures (grown overnight from glycerol stocks) using the Multiprint<sup>®</sup> replicator dot-blot apparatus (VP-Scientific). After the membranes were allowed to dry, the culture cells were lysed with a solution containing 0.5 M NaOH and 1.5 M NaCl. The membranes were then placed in a neutralisation solution consisting of 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0). The membranes were allowed to air dry after which the fixed DNA was denatured with a 0.4 M NaOH solution. The effect of the last solution was neutralised with a 5x standard saline phosphate EDTA (SSPE) solution (Sambrook *et al.*, 1989). After the membranes were air dried, they were ready for hybridisation. The two duplicate membranes contained subtracted fragments from both round three and round six of subtraction. Total cDNA from stage 1 and stage 5 of berry ripening was radio-labelled over 35 cycles with 25  $\mu\text{Ci}$  [ $\alpha$ -<sup>33</sup>P] dCTP using *Taq* DNA polymerase (Promega) in the presence of 1  $\mu\text{M}$  primer and 20  $\mu\text{M}$  dNTPs using the following conditions: denaturation at  $94^{\circ}\text{C}$  for 48 s, annealing at  $68^{\circ}\text{C}$  for 66 s and elongation at  $73^{\circ}\text{C}$  for 3 min. The labelled stage 1 and stage 5 cDNA was hybridised separately to the membranes overnight. Hybridisation results were visualised using the Packard Cyclone<sup>®</sup> Storage Phosphor System (Packard Instruments).

#### 4.2.5 DNA sequencing

After the identification of putatively differentially expressed fragments on the expression arrays, 5 clones from each ripening stage (1 and 5) were picked from the glycerol stocks, cultured and the inserts amplified using the bacteriophage T7 (forward) and M13 (reverse) primers under the following conditions: 10 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 45 s, elongating at 72°C for 45 s followed by 25 cycles of denaturing at 94°C for 20 s, annealing at 45°C for 30 s and elongating at 72°C for 30 s. DNA was sequenced using the Applied Biosystems PRISM<sup>®</sup> Ready Reaction kit (Perkin Elmer) according to the manufacturers instructions. Samples were analysed in an ABI PRISM<sup>®</sup> automated sequencer (Model #377). Homology searches were carried out using the BLAST server developed by the NCBI at the National Library of Medicine, USA (Altschul *et al.*, 1990).

#### 4.2.6 Northern hybridisation analysis

Equivalent amounts of RNA (10µg/lane) were denatured in formamide/formaldehyde and separated electrophoretically in 1.2% (w/v) agarose gels in the presence of ethidium bromide (0.5 µg.mL<sup>-1</sup>). The RNA was transferred to positively charged nylon membranes (Boehringer) using a standard upward capillary blotting method (Sambrook *et al.*, 1989) and 10x SSC. Fragments identified by expression array analysis (4.3.4) were labelled with <sup>32</sup>P according to a protocol by Habu and co-workers (1997) and used as probes. Pre-hybridisation and hybridisation were performed using Ultrahyb<sup>™</sup> hybridisation solution according to the manufacturer's specifications (Ambion). Membrane washes were performed according to Sambrook and co-workers (1989) using SSC/SDS gradient washes. In order to verify that equal amounts of RNA was loaded on gels for membrane transfer and subsequent northern blot analysis, membranes were probed with a grape β-tubulin probe (see Fig. 3.3). Results were visualised using the Packard Cyclone<sup>®</sup> Storage Phosphor System (Packard). The intensity of the hybridisation signals was determined by densitometry. Densitometry involved measuring and subsequently quantifying the hybridisation intensity of each signal on the northern blots by precise delineation of each signal. A computer software programme calculated the amount of pixels per delineation and produced a

representative quantity. Using the same technique, a sample of the un-hybridised background on each northern blot was subjected to densitometry analysis and this quantity was subtracted from quantities obtained for the hybridisations. All visualisations and quantifications were performed using the AlphaImager™ 2000 documentation and analysis system (Alpha Innotec).

## **4.3 RESULTS AND DISCUSSION**

### **4.3.1 Construction and screening of subtracted cDNA libraries**

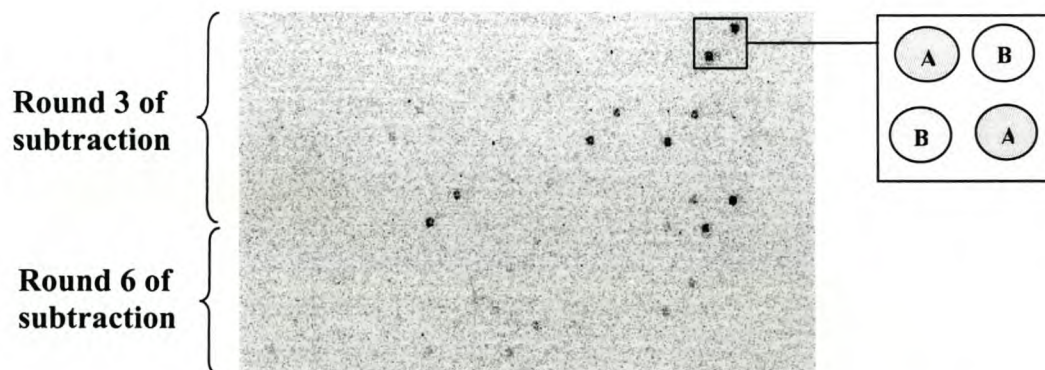
The purpose of subtractive hybridisation is to enrich the target (tracer) cDNA for fragments that represent genes that are specifically expressed in the tissue of interest. This is achieved by removal of DNA sequences representing genes that are also expressed in the driver tissue. In this case the target tissue of interest was grape berry ripening stage 5 for the forward subtraction and ripening stage 1 for the reverse subtraction.

The majority of the clones analysed had inserts between 200 and 400 bp, the small insert size being due to the initial digestion of the target cDNA with the four base frequent cutting restriction enzymes *RsaI* and *AluI*. Initial screening of the subtracted libraries involved the hybridisation of total cDNA from stage 1 and stage 5 of berry ripening to membrane-fixed subtracted fragments (see 4.3.4). The reason behind this strategy was to eliminate abundant cDNAs that "survived" the subtraction process. It was assumed that genes that are expressed abundantly during a specific ripening stage would be present in larger quantities within an mRNA population than the gene products that are the target of subtraction. Thus, by synthesising total cDNA from a specific ripening stage and screening the subtracted fragments from that stage with total labelled cDNA, abundant expressors that were not removed by subtractive hybridisation would hybridise to the labelled cDNA.

Preliminary screening of subtracted clones showed that this assumption might be correct. Six hybridisation signals could be discerned amongst the round 3 clones, whereas only two to three could be discerned (very faintly) amongst the round 6 clones.

This could indicate that the subtraction process was successful in removing most (round 3) to all (round 6) of the abundant gene products. As expected, none of the clones from the early berry ripening stage (A in Fig. 4.1) showed hybridisation. This result was repeated with a duplicate membrane and the same results were obtained but varying hybridisation intensities were observed.

Six rounds of subtraction were considered to be sufficient (Balzer *et al.*, 1996; Buchanan-Wollaston & Ainsworth, 1997; Houben *et al.*, 1996). The tracer populations from both stages 1 and 5 were cloned after round three and round six of subtraction. This was done to monitor the efficiency of the subtraction process. Theoretically, the number of subtraction rounds should be negatively correlated with the variety of sequences present in the cDNA pool. More rounds of subtraction should also allow the detection of low-abundance, differentially expressed cDNAs (Birch *et al.*, 2000).

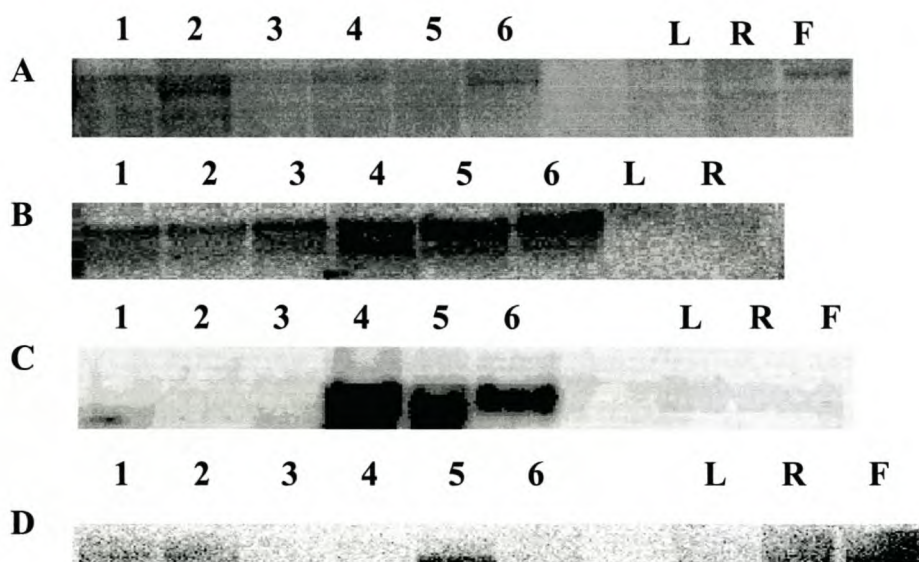


**Figure 4.1.** Expression array probed with total cDNA from the late berry ripening stage. The array contained 96 clones from the early (A) and late (B) ripening stage. Fifty clones from round 3 and 46 from round 6 of subtraction were blotted. The enlarged area represents one clone from each ripening stage together with an identical copy (diagonally across from) to ensure the validity of the first hybridisation signal.

#### 4.3.2 Northern hybridisation analysis and densitometry

The expression patterns of 10 of the isolated clones were investigated in different tissues. Two of the clones exhibited differential expression as well as berry specificity (SdeVI 19 and SdeIII 13). Low expression levels of Sde VI 2, isolated during early berry ripening, were observed in berry development stages 2 and 6 and in flowers (Fig.

4.2A). The round 3 subtraction clone, SdeIII 13, exhibited a gradual increase in expression from stage 1 to the inception of véraison during stage 4. Expression in stage 4, 5 and 6 was the highest with no detectable expression in the leaves or roots (Fig. 4.2B). SdeVI 19, a clone from the late berry ripening stage, was isolated from round 6 of subtraction. Strong hybridisation signals in lanes 4, 5 and 6 suggest that the gene was up-regulated in stage 4 (Fig. 4.2C) and that expression remains high in stages 5 and 6. The fourth clone, which was isolated from the late berry ripening stage of round 6 of subtraction, exhibited very low levels of expression (Fig. 4.2D). Faint hybridisation signals in stage 5 of the berry ripening stage, roots and flowers could be discerned. Very faint hybridisation signals seem to be present in stage 1 and 2 as well.



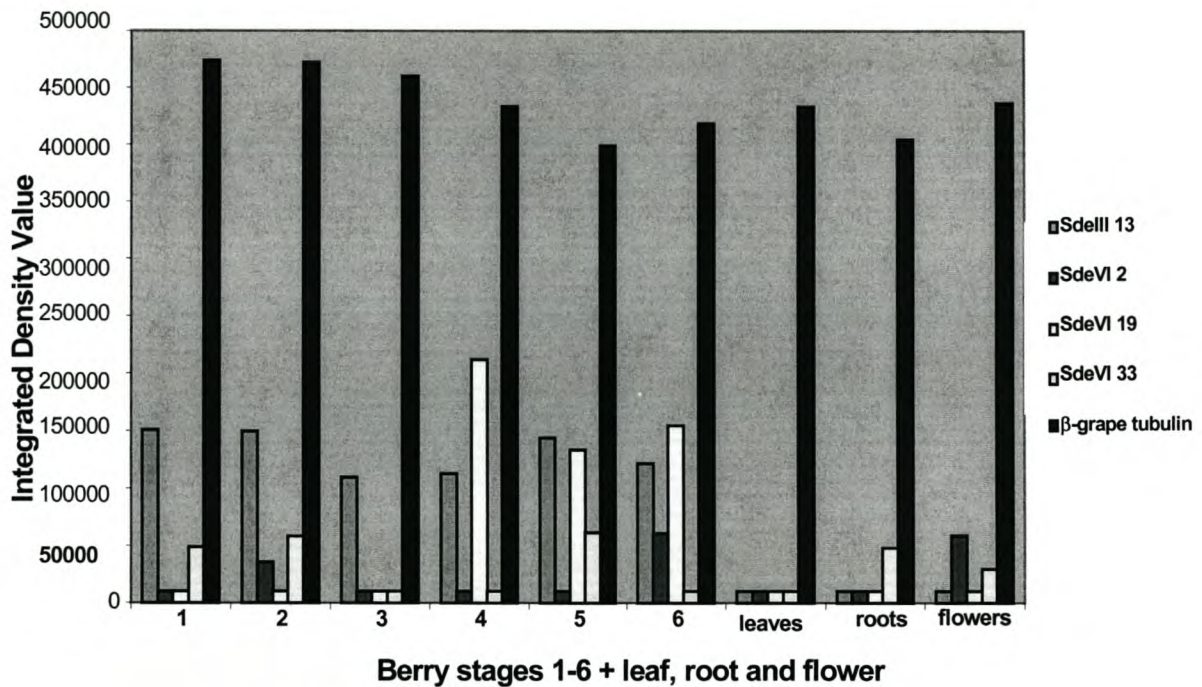
**Figure 4.2.** Northern hybridisation analysis of cDNA fragments isolated from subtracted libraries in grapevine tissues. Probes: SdeVI 2 (A), SdeIII 13 (B), SdeVI 19 (C) and SdeVI 33 (D). Lanes 1 to 6 contain 10  $\mu$ g of total RNA isolated from the six stages of berry fruit ripening (from early to ripe). L: leaf, R: root and F: flower RNA.

**Table 4.1.** Ripening-induced clones encoding enzymes and putative stress response proteins in cv. Chardonnay grape.

Subtraction Clone	Insert Size (bp)	Ripening Stage	Nearest Genbank Match (Accession No.)	Score <sup>c</sup>	Amino Acid Identity (%) <sup>d</sup>	Proposed Protein
<sup>a</sup> SdeIII 13	293	late	<i>Prunus armeniaca</i> (U93164)	74	85	Abscisic stress ripening protein
<sup>b</sup> SdeVI 2	133	early	<i>Arabidopsis thaliana</i> (AB015479)	36	86	Sodium proton exchanger
Sde VI 4	132	early	<i>Zea mays</i> (Z11973)	135	92	Ribulose biphosphate carboxylase (large subunit)
Sde VI 6	306	late	<i>Arabidopsis thaliana</i> (Y07613)	176	91	Heat shock protein
Sde VI 19	268	late	<i>Oryza sativa</i> (AAK16194)	196	100	UDP-glucose dehydrogenase
Sde VI 32	271	late	<i>Oryza sativa</i> (AAK16194)	236	-	UDP-glucose dehydrogenase
Sde VI 33	316	late	<i>Pharbitis nil</i> (M99431)	113	63	Heat shock protein
Sde VI 41	209	late	<i>Daucus carota</i> (AB0296635)	62	95	S-phase specific protein
Sde VI 46	189	early	<i>Beta vulgaris</i> (L32792)	58	-	Vacuolar pyrophosphatase
Sde VI 10	238	early	<i>Sorghum bicolor</i> (AW922752)	56	-	unknown

<sup>a</sup>Subtracted, differentially expressed sequence from round 3 of subtraction.<sup>c</sup>As calculated by the NCBI.. A score of 80 or above denotes significance.<sup>b</sup>Subtracted, differentially expressed sequence from round 6 of subtraction.<sup>d</sup>% amino acid residues of subtracted sequences that showed homology.

To quantify the level of expression observed in the northern hybridisations (Fig. 4.2), the expression level of  $\beta$ -tubulin was used as a reference (Fig. 4.3). It is evident that the expression of  $\beta$ -tubulin is higher than that of any of the subtraction fragments. SdeVI 2 had the lowest expression and SdeVI 19 exhibited the highest expression of the isolated clones.



**Figure 4.3.** Relative abundance of the isolated genes and  $\beta$ -tubulin during ripening. Expression levels are based on desitometry scanning of the signal intensities on northern hybridisations.

### 4.3.3 Analysis of subtracted clones

To characterise the cDNA fragments that appeared to be ripening related, the inserts of 10 of the clones (4 from the early and 6 from the late berry ripening stage) were sequenced from both ends using the T7 and M13 primers. The sequences obtained, and deduced protein sequences that could be translated from them, were compared to DNA and protein sequences in the EMBL/GenBank databases using the BLAST database search analysis. A summary of the sequence similarity BLASTX (protein) searches of the putative ripening-related clones is presented in Table 4.1.



The putative Sde proteins described in this thesis were divided into two general groups based on their proposed functions in the berry. One group consists of proteins that may be involved in a stress response. This is suggested by the proposed functions of their homologues in other plants and even animals. The presence of stress response at a molecular level in grape berries is expected and has been linked to the presence of several different proteins (Davies & Robinson, 2000; Sommer-Knudsen *et al.*, 1998). The presence of heat shock proteins (HSPs) has been reported in grapevine (Morrell *et al.*, 1997a; Morell *et al.*, 1997b), but not in grape berries as such.

The second group consisted of enzymes involved in normal cell functions, such as photosynthesis. This group contained all the clones isolated from the early ripening stage, whereas the putative stress-induced proteins were all isolated from ripe berries. This may further support findings that suggest that there is a definite stress induction mechanism that is initiated in the grape berry upon inception of véraison (Davies & Robinson, 2000).

### *SdeIII13*

**SdeIII 13 D Y R K G E K H H K H L E H 14**  
 Apricot **D Y K K E E K H H K H L E H 14**  
 Pummelo **D Y K K E E K H H K H L E H 14**  
 Rice R Y K K E E K Q H K H K Q H 14

**Figure 4.4** Amino acid sequence alignments of target clone, SdeIII 13, with three abscisic stress ripening proteins. The highest homology was with the two dicotelydons: apricot (protein acc. no. AAB97140.1) and pummelo (protein acc. no. AAA82741.1). The monocotelydon, rice (protein acc. no. AAB96681.1) found only between 57% (SdeIII 13) and 70% (apricot and pummelo) homology with the other sequences. The area of homology is in boldprint.

The highest percentage (70%) of homology SdeIII 13 showed was with an abscisic acid stress ripening protein (acc. no. U93164) isolated from a ripe apricot fruit library. It was suggested that the ABA-, Stress-, Ripening-induced (ASR) protein sequence from apricot fruit is involved in stress-related ripening (Mbeguie-A-Mbenguie *et al.*, 1997). It seems that there is a conserved area of homology between different plant ASRs in the region from residue 113 to 200 (Mbeguie-A-Mbenguie *et al.*, 1997). The area of sequence homology that SdeIII 13 shares with the other plant sequences (Fig. 4.4) starts

at residue 113. Another typical aspect of ASR proteins appears to be the poly-histidine and poly-alanine domains (Mbenguie-A-Mbenguie *et al.*, 1997). Although histidine areas could be discerned in SdeIII 13, the area of sequence homology was not sufficient to allocate poly-histidine and poly-alanine domains. It was found that the sequence of rice is characterised by a higher glutamic acid content, with no tryptophan or cysteine residues (Vaidyanathan *et al.*, 1999). However, due to the length of sequence homology between the rice sequence and SdeIII 13, a comparison was not possible.

Stress due to changes in osmotic potential may occur during pathogen infection, drought, salinity and due to the storage of large amounts of osmotically active substances in storage tissues such as fruit (Davies & Robinson, 2000). During berry ripening, cells expand rapidly as they accumulate large amounts of the hexose, glucose, fructose and water. This would result in considerable changes in osmotic pressure and water potential (Davies & Robinson, 2000). Fruit such as grapes, which store particularly high levels of sugars (approximately 20% [w/w] hexoses when fully ripe (Lot & Barrett, 1967)), may have to adjust to the rapid increase in vacuolar sugar levels by using proteins involved in stress management.

It has been shown that ABA-regulated gene products might be involved in stress tolerance (Chandler & Robertson, 1994). However, it was concluded in the apricot study that wounded tissues did not present higher levels of ASRs than normal, unwounded tissues (Mbenguie-A-Mbenguie *et al.*, 1997). In grape berries, a gradual increase in expression of Sde III 13 (Fig. 4.2B) in the berries during ripening supports an osmotic stress response scenario. The levels of ASRs in grape berries should also be investigated to establish whether abiotic stress such as wounding can cause up-regulation of these genes.

*SdeVI6 and SdeVI33*

```

SdeVI 33  FENLCK I IKDI LGDKVEKVV V SDRIV DSP
SdeVI 6   FEALCKTIKDILGDKVEKVVVSDGIVDSP
A. thaliana FENLCKTIKEILGDKVEKVVVSDRIVDSP
Tomato    FESLCKV IKDILGDKVEKVVVSDRIVDSP
Chicken   FETLCKLMKEILDKKVEKVT I SNRLVSSP

CC LVTGEYGW TANMERIMKA QALRDSSMS SYM
CCA V TGEYGW TANMERIMKA QALRDSSMGAYM
CCLVTGEYGW TANMERIMKA QALRDSSMSGYM
CCLVTGEYGW TANMERIMKA QALKDNSMSSYM
CCI VTST YGWTANMERIMKA QALRDNSTMGYM

S SK KTMEINPDNGI ME 77
SS K KTMEINPDNGIME 77
SS K KTMEINPENGIME 77
SS E KT MEINPDNGIVE 77
MAKKH LE INPDHPIVE 77

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**Figure 4.5** Amino acid sequence alignments of target clones, SdeVI 6 and SdeVI 33, with three heat shock proteins. Homology between *Arabidopsis thaliana* (protein acc. no. CAA68885), the tomato (protein acc. no. AAD30456) and SdeVI protein is approximately 90%, whereas the chicken (protein acc. no. CAA49704) similarity is approximately 68% at amino acid level. The area of homology is in boldprint.

Analyses of both SdeVI 6 and SdeVI 33 sequences revealed the highest percentage of homology, at amino acid level, with a heat shock protein in *Arabidopsis thaliana* (acc. no. Y07613). Other heat shock proteins from different organisms that have high percentage homology included one from *Pharbitis nil* (acc. no. M99431, 85% at nucleotide level), tomato (acc. no. AF123259, 83% at nucleotide level) and a heat shock protein from chicken (acc. no. X70101, 89% at nucleotide level) (Fig. 4.5). The only discernable motif in the 77 amino acid sequence of both Sde VI 33 and Sde VI 6 seems to be a VVV motif. This motif is repeated in both tomato and *Arabidopsis*, but is absent in the chicken sequence. An interesting feature present in all five compared sequences is a YGWTANMERIMKAQAL area.

Heat shock proteins (HSPs) that were identified as putative Sde proteins appear to be involved in stress response. The transfer of plants to an elevated temperature produces

stress (Paull & Chen, 2000). These proteins are produced within 30 min after exposure to temperatures in the range 34-42°C (Kanabus *et al.*, 1984). Large HSPs produced in response to high temperature are believed to prevent irreversible protein denaturation that would be detrimental to the cell (Parsell & Linqvist, 1993) and this activity may be enhanced in plants by smaller HSPs (Lee and Vierling, 2000). Because grapes are grown in areas with high midday temperatures in the summer, heat shock protein synthesis may be an important response of grapevines under field conditions (Morrell *et al.*, 1997b). HSPs and associated thermotolerance have been reported in other fruit like papaya (Paull & Chen, 1990) and apples (Ferguson *et al.*, 1998) as well. Xiong and co-workers (1999) reported that there is a synergistic interaction between HSPs and ABA in *Arabidopsis*. In fruit ripening, ABA production is known to be associated with the later stages of the senescing organ. The presence of HSPs in stage 5 of berry ripening in this study could support Xiong and co-workers' finding. Since the present study is, as far as it is known, the first report of the presence of this type of protein in grape berries, further investigation into the precise function of HSPs during grape berry ripening is needed.

### *SdeVI19*

SdeVI 19	<b>A F K K D T G D T R E T P A I</b> 15
Rice	<b>A F K K D T G D T R E T P A I</b> 15
Soya	<b>A F K K D T G D T R E T P A I</b> 15
Human	<b>A F K K D T G D T R E T S S I</b> 15

**Figure 4.6** Amino acid sequence alignments of target clone, SdeVI 19, with two UDP glucose dehydrogenase proteins. A 100% homology was seen at the amino acid level between the clone, rice (protein acc. no. AAK16194) and soya (protein acc. no. AAB58398) protein. The area of homology is in boldprint.

This fragment showed a high percentage homology to UDP-glucose dehydrogenase isolated in rice (acc. no. AAK16194). The area of 15 amino acids from SdeVI 19 showed a 100% homology with not only the aforementioned rice protein, but also a UDP-glucose dehydrogenase from soya (acc. no. U53418). The human UDP-glucose dehydrogenase (Woodhead *et al.*, 1998) also showed a surprisingly high percentage of homology with only three residues that were different (Fig. 4.6). The UDP-glucose

dehydrogenase protein shows large conserved areas between species, however, the only comparable area present in Sde VI 19 are potential catalytic lysine residues, KK.

Hemicellulose is a major component of primary plant cell walls. Many of the glycosyl residues found in hemicellulose are derived from the sugar precursor UDP-glucuronic acid (Tenhaken & Thulke, 1996). The latter can be converted into arabinose, galactose and galacturonic acid, which form part of the pectin group of cell wall polysaccharides (Robertson *et al.*, 1995). Udpgh is the enzyme that controls the biosynthesis of UDP-glucuronic acid and therefore may play an important role during cell enlargement in fruit ripening. Due to the large influx of fructose and glucose at the start of véraison (Davies & Robinson, 1996), the mechanisms of cell enlargement in the berry may be activated or even up-regulated. The expression pattern of the putative Udpgh subtracted sequence, Sde VI 19, exhibited activation at stage 4 of berry ripening suggesting that the Udpgh gene might be up-regulated upon inception of véraison.

Previously, the presence of Udpgh has been shown in other rapidly expanding tissues, like young roots, epicotyls and young leaves (Tenhaken & Thulke, 1996; Robertson *et al.*, 1995). It is therefore expected in the rapidly expanding fruit of grapevine. However, it is expected to be present in the rapidly expanding cells of the growing, early ripening stages of berries. The strong expression of this gene during the late stages of ripening contradicts this expectation and should, in order to confirm functional analysis, be investigated in other fast growing grapevine tissues like young leaves and roots, as well as tendrils.

### ***SdeVI 41 and SdeV I2***

SdeV 41 showed homology with an S phase specific gene that encodes for a cyc07 protein, involved in mitosis (Ito *et al.*, 1991). The precise function of this protein is unknown, but it is probably the same as in all plant cells that are subject to mitotic division.

Sde VI 2 showed homology to an *Arabidopsis thaliana* sequence (acc. no. AB015479) that encodes a sodium proton exchanger. The area that found homology with the *Arabidopsis* sequence was a 22bp sequence with 95% identity similarity.

The protein involved in intracellular transport is a sodium proton exchanger ( $\text{Na}^+/\text{H}^+$  exchanger) that showed homology to a similar protein from *Arabidopsis thaliana* (Nakamura *et al.*, 1998). In plants, the  $\text{Na}^+/\text{H}^+$  exchanger gene family, is up-regulated when it is needed to tolerate high levels of salt (Wyn-Jones, 1981). Plant cells are structurally well suited for the sequestration of ions because of the presence of large, membrane-bound vacuoles (Apse *et al.*, 1999). It has been proposed that in salt-tolerant plants, the compartmentation of  $\text{Na}^+$  into vacuoles, through the operation of a vacuolar  $\text{Na}^+/\text{H}^+$  anti-port, provides an efficient mechanism to avert the deleterious effects of  $\text{Na}^+$  in the cytosol and maintains osmotic balance by using  $\text{Na}^+$  (and chloride) accumulated in the vacuole to drive water into the cells (Glenn *et al.*, 1999).

If Sde VI 12 represents a  $\text{Na}^+/\text{H}^+$  exchanger in grape berries during ripening it would suggest a possible protection mechanism against accumulation of  $\text{Na}^+$  in the fruit itself. It is also observed in the flower tissues, but not in the roots or leaves. This observation does not compare well with findings in *Arabidopsis*, where transcripts of the AtNHX1 gene were found in root, shoot, leaf, and flower tissues (Apse *et al.*, 1999). A possible explanation for this could be that Chardonnay does not have an especially high salt tolerance, or that the salinity levels in the soil were not high enough to induce a strong stress response in all the tissues. The low comparative expression levels of this gene in berry and flower tissue suggest a housekeeping function linked to the more rapid growing organs of the grapevine, like the flowers and the fruit. Further investigation is needed to elucidate the possible mechanisms involved during salt-induced stress in the berry, as well as the presence of a sodium proton exchanger in young leaves.

#### *SdeVI 4 and SdeVI46*

SdeVI 4 showed homology with the large-chain sub-unit of the ribulose biphosphate carboxylase protein (RuBisCo). RuBisCo is one of the more abundant enzymes in plants and it is involved as part of the Calvin cycle in  $\text{CO}_2$  fixation during photosynthesis (Mathews & Van Holde, 1991). The large-chain sub-unit of RuBisCo is expressed in chloroplasts and in this study it was present in the early stages of berry ripening, alluding to the fact that the young berry is still photosynthesising during the early stages of development. Relative abundance of RuBisCo during the early stages of berry development is in concurrence with previously published findings. These findings

stated that a number of enzymes involved in photosynthetic carbohydrate metabolism, such as RuBisCo, declined in fruit during development (Famiani *et al.*, 2000).

SdeVI 46 exhibited homology with a vacuolar H<sup>+</sup>-pyrophosphatase (V-PPase). The H<sup>+</sup>-pyrophosphatase of plant vacuolar membranes catalyses the electrogenic translocation of H<sup>+</sup> from the cytosol to vacuole lumen and establishes the H<sup>+</sup>-electrochemical potential difference responsible for energising the H<sup>+</sup>-coupled transport of solutes into the vacuole (Kim *et al.*, 1994). Young fruit has a high relative growth rate where metabolic activities including the synthesis of RNA, DNA, proteins and cell walls are high. This results in production of a large amount of pyrophosphate as by-product (Suzuki *et al.*, 1999). However, it was found that the level of mRNA in pears was very low in young fruit and increased with maturation, whereas the protein amount and activity of V-PPase was the highest in the early stages and decreased with maturation (Suzuki *et al.*, 1999).

In the present study this cDNA was isolated from young berries. The mRNA levels, the amount of protein and the activity levels of V-PPase during grape berry development need to be investigated in the future to establish if it correlates with the pear study. It has been postulated that the existence of H<sup>+</sup>-PPase in plant cells may be related to the huge size of the plant vacuole and that it is regulated under stress conditions (Nakanishi & Maeshima, 1998). Linked to this is the investigation into physiological questions such as the presence of two distinct proton pumps in the vacuolar membrane in plant cells (Nakanishi & Maeshima, 1998). In rapidly expanding cells, like those of the young grape berry, it might be postulated that both proton pumps are needed to cope with export of protons from the cytosol. This postulation should be investigated in the grape berry and other fruit. Furthermore, sink organs, such as fruit, are said to be good models to elucidate the roles of proton pumps in the storage of metabolites in vacuoles (Suzuki *et al.*, 1999).

## CHAPTER 5

### SUMMARY AND FUTURE STUDIES

The first goal set in this study was the isolation of adequate amounts of high quality RNA from grape berries for applications such as the construction of cDNA libraries. It was necessary to use a method that was not only robust, but that could also deliver reproducibility. It was found that the best results were obtained by using the sodium perchlorate method described here (Chapter 3).

The second goal set was the synthesis of two stage specific cDNA libraries representing early and late berry ripening respectively. The cDNA library representing the early stage in berry ripening contained  $7.5 \times 10^5$  and the late stage cDNA library contained  $6.5 \times 10^5$  primary recombinants with average insert sizes for both libraries between 1.0 and 1.1 kb (Chapter 3).

Subtractive hybridisation has been used successfully to identify differentially expressed genes from various plants (Ouvrard et al., 1996; Balzer et al., 1996; Matsuyama et al., 1999). In the present study, where this method was used for the first time in grapevine, four putative differentially expressed (of which two were also grape berry specific) genes were identified. All four sequences isolated are expressed at a level significantly lower than that of grape  $\beta$ -tubulin. Furthermore, one of the sequences (Sde VI 19) analysed via northern hybridisation analysis, exhibited tissue and stage specificity. Thus, the third goal of this project, namely the isolation of differentially expressed genes that exhibited not only stage, but tissue specificity as well, was achieved.

In grapevine, the role of abiotic stress factors cannot be underestimated. Extensive studies have been done on the stress responses that the grape berry employs to combat abiotic stresses (Vivier & Pretorius, 2000). This field of research, however, still offers a large scope of improvement in the identification of genes that are involved in the process of stress management within the grape berry.



In the present study differential expression of genes was used to investigate berry ripening. As was seen in the study, ripening can be associated with stress-related metabolism. Two stress-induced proteins involved with fruit ripening in grapevine were identified. Previous investigations of heat shock proteins in grapevine focused on the presence of this family of stress-induced proteins in leaves and flowers (Morrell *et al.*, 1997a; Morell *et al.*, 1997b). A possible role for heat shock proteins in grapevine was postulated to be the involvement of the protein in the protection of cells against excessive temperatures (Morell *et al.*, 1997a; Morell *et al.*, 1997b). The current study indicated that at least one member of this protein family is present in ripening grape berries. It is suggested that further research should focus on the isolation and characterisation of all the members of the heat shock family in grape berries.

The second stress-induced protein that was isolated was an abscisic acid-, stress-, ripening-induced-like protein (ASR-like). In an apricot (a climacteric fruit) study it was found that contrary to expectations, the ASR-like protein was not ethylene-regulated since its expression occurred before the expression of the ACC oxidase gene (Vaidyanathan *et al.*, 1999). Since grapes are non-climacteric fruit, a possible connection of ASR-like proteins with ACC oxidase can be discarded. Thus, in the current study it was suggested that the gradual increase in expression during berry ripening of this gene, supports an osmotic stress response scenario. There could, however be a connection between climacteric and non-climacteric ripening. It was found that promoters from two ripening-induced genes (capsanthin/capsorubin synthase and fibrillin) from non-climacteric pepper were induced in transgenic tomato fruit in parallel with ripening (Kuntz *et al.*, 1998). Expression of both genes was enhanced by application of ethylene, suggesting that climacteric and non-climacteric ripening may share common molecular underpinnings. A similar study using ASR-like promoters from the grape berry in a transgenic climacteric fruit could be invaluable.

UDP-glucose dehydrogenase, which was also identified in this study, is an enzyme involved in the production of precursor molecules used in cell wall synthesis. In contrast to the expectation it was found that Udpghd was up-regulated during berry ripening. Normally this enzyme is associated with actively growing and dividing cells (Tenhaken & Thulke, 1996). The enzyme is involved in pentan (*e.g.* xylan) synthesis. The products of this synthesis are used as building blocks in primary cell walls.

Primary cell walls, however, are expected to degrade during softening of the grape berry. In fact, significant modification of specific polysaccharide components was observed during softening of grape berries alluding to the presence of a degradation process (Nunan et al., 1998). At this stage it is not evident why pectin synthesis would be important in ripening tissue, especially the late ripening stages.

The fourth gene identified through subtractive hybridisation was a sodium proton exchanger. This gene seems to be up-regulated in plants that are under salt stress (Wyn-Jones, 1981). Although the presence of this gene product in the early stage of ripening and flower tissue suggests some type of salt stress response mechanism in grape berries, the low levels can also mean that it was simply detected due to the sensitivity of subtractive hybridisation. Further investigation into a possible stress response mechanism in grape berries is needed to answer these and other questions involving salt tolerance in grapevine.

The isolation of fruit ripening-related genes has resulted not only in tools for studying the direct effects of specific gene products on ripening but also in opportunities to isolate and study gene regulatory elements that may illuminate regulatory mechanisms (Giovannoni, 2001). The present study may be used as a foundation to further elucidate the role of ripening-related genes in grape berries. Full-length cDNA clones of the identified stress-response genes should be isolated. Characterisation and sequencing of these full-length clones, should lead to the promoter elements of these genes. In turn, the promoter elements could be isolated for possible manipulation in a transgenic system (*e.g.* strawberry). The study of promoter elements isolated from grape berries could answer many questions with regard to mechanisms of fruit ripening. Future research should also focus on further elucidation of the mechanisms used by the grape berry to cope with abiotic stresses. Isolating and characterising more stress-induced genes from ripening grape berries could achieve this.

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