The isolation and characterisation of a developmentally-regulated gene from *Vitis vinifera* L. berries.

by

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Declaration

I, Anita Burger, hereby declare that the work presented in this dissertation is my own original work, except for the contributions by J.P. Zwiegelaar and L. Watts, as stated in the Preface. I furthermore declare that I have not previously submitted any part of this work to any University for a degree.

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Anita Louize Burger   **Date**

Summary

Despite increased focus on ripening-related gene transcription in grapevine, and the large number of ripening-related cDNAs identified from grapes in recent years, the molecular basis of processes involved in grape berry ripening is still poorly understood. Moreover, little is known about the mechanisms involved in the ripening-related regulation of fruit-specific genes, since the isolation and characterisation of no ripening-related, fruit-specific promoter elements has been reported to date. This study was aimed at the isolation and characterisation of a fruit-specific, ripening-regulated gene from *Vitis vinifera* L.

In the first phase of the work, gene transcription in ripening berries of Cabernet Sauvignon (a good quality wine cultivar) and Clairette blanche (a poor quality wine cultivar) were studied by Amplified Fragment Length Polymorphism analysis of complementary DNA (cDNA-AFLP analysis). Total RNA from immature (14-weeks post flowering, wpf) and mature (18-wpf) berries was used for the analysis. A total of 1 276 cDNA fragments were visualised, of which 175 appeared to be ripening related. Average pairwise difference of the fragments amplified from immature and mature Clairette and Cabernet berries, suggested that ripening-related gene transcription in these two phenotypically different cultivars is remarkably similar. Nevertheless, it was shown that seventy percent of the 175 ripening-related cDNA fragments were cultivar-specific. It was suggested that these differences should be targeted to identify genes related to the phenotypical differences between the two cultivars, but also to identify genes possibly involved berry quality. Moreover, the analysis illustrated the usefulness of cDNA-AFLPs for the analysis of ripening-related gene transcription during grape berry ripening.

In the second phase of the work, one of the ripening-related cDNAs identified by the cDNA-AFLP analysis, was selected for further characterisation. This work highlighted the limitation placed on the isolation of a single specific sequence from a cDNA-AFLP gel, indicating the presence of multiple ripening-related genes in a single band excised from a cDNA-AFLP gel. Steps to overcome this limitation of cDNA-AFLP analysis to identify and clone a specific ripening-related gene, were implemented. In short, the band corresponding to the particular ripening-related cDNA was band was excised from the cDNA-AFLP polyacrylamide gel and re-amplified. Northern blot analysis using the re-amplified, uncloned product confirmed the ripening-related transcription demonstrated by cDNA-AFLP analysis. The re-amplified, uncloned product was then cloned. Sequence analysis of two randomly selected candidate clones revealed two distinctly different sequences, of which neither hybridised to messenger RNA from ripening grape berries. Further

analysis revealed an additional five cDNAs with terminal sequences corresponding to the selective nucleotides of the primers used for selective amplification, in the re-amplified, uncloned product. Of these, only two were abundantly expressed in ripening grape berries, accounting for the ripening-related transcription visualised by cDNA-AFLP analysis. All seven cDNAs identified from the particular excised band were shown to be ripening-regulated during berry development, although most were characterised by low levels of transcription during berry ripening. One of the clones, based on the relative high levels of the transcript and the initiation of gene transcription at the onset of *véraison* (10- to 12-wpf), was identified for isolation and characterisation of the full length coding sequence.

In the third phase of the work, it was shown that this cloned sequence corresponded to a gene encoding a proline-rich protein (PRP) associated with ripening in Merlot and Chardonnay (*mrip*1, Merlot ripening-induced protein 1). It was shown that the gene is specifically transcribed in the fruit tissue, seed and bunchstems of grapes, from 10-wpf (*véraison*) to the final stages of berry ripening. The results showed that *mrip*1 encodes a distinct member of the plant PRP family. Most obvious is the central region of mrip1, which is comprised of eight consecutive repeats of 19 amino acid residues each. In comparison with other grapevine PRPs, mrip1 revealed single amino acid differences and deletion of one of the 19 amino acid residues repeats, all in the central region of mrip1. *In situ* hybridisation studies showed that accumulation of the *mrip*1 transcript in the ripening berry is limited to the mesocarp and exocarp cells of the ripening grape berry. No transcript with high sequences similarity to *mrip*1 could be detected in ripening strawberry or tomato fruit. Based on the properties and proposed function of PRPs, and the results obtained in this study, potential applications for the use of this gene in the control of cell wall architecture in fruits, were proposed. Furthermore, as manipulation of fruit properties in grape berries would be most important in the later stages of ripening, *mrip*1 was proposed an ideal candidate gene for the isolation of a fruit- and late-ripening-specific promoter to achieve transgene transcription in genetically modified grapevine.

The final phase of the work was dedicated to the isolation and characterisation of the *mrip*1 promoter element. A 5.5 kb sequence corresponding to the *mrip*1 5' untranslated (UTR) flanking region was isolated and characterised by sequence analysis. In the 2.8 kb sequence directly upstream of the *mrip*1 transcription initiation site, several putative *cis*-acting regulatory elements were identified. These include a spectrum of hormone-, light-, phytochrome-, sugar-and stress-responsive elements, as well as elements implicated in tissue-specific transcription. Analysis of the sequence further upstream (3.6 – 5.5 kb) of the *mrip*1 transcription initiation site (TIS), revealed the presence of another proline-rich protein directly upstream of *mrip*1. Sequence identity of this sequence (*mprp*2) to the *mrip*1 coding sequence was 88%. This information provided the first

insight into the chromosomal organisation of grapevine PRPs. For functional analysis of the *mrip*1 promoter element, the 2.2 kb sequence directly upstream of the *mrip*1 TIS, was translationally fused to the *sgfp*S65T reporter gene. Functionality of the *mrip*1:*sgfp*S65T fusion was verified by transient expression in green pepper pericarp tissue, before introduction into tobacco by *Agrobacterium*-mediated transformation. In transgenic tobacco, transcription of the *mrip*1:*sgfp*S65T fusion was developmentally-regulated and specific to the ovary and nectary-tissue of the developing flower. Whilst low in immature flowers, the green fluorescent protein (GFP) rapidly accumulated to the high level of expression visualised in the flower in full-bloom, followed by a decrease in the final stages of ovary development. These observations suggested that the 2.2 kb *mrip*1 promoter is functional and that this promoter region harbours *cis*-elements necessary for tissue- and developmental-specific regulation of GFP accumulation. It furthermore suggested that the transcriptional activation of *mrip*1 is mediated by developmental signals present in both grapevine berries and tobacco flowers. Results presented, suggest that the use of tobacco as heterologous system for the analysis of ripening-related promoters, can be more generally applied. Evidently, characterisation of the *mrip*1 promoter region contributes towards a better understanding of the regulatory mechanisms involved in non-climacteric fruit ripening, and forms a basis for future experiments defining the *cis*-acting elements necessary for tissue- and cell-specific gene regulation in fruit, more specifically in grapevine. Moreover, the *mrip*1 promoter is an ideal candidate for the ripening-related, tissue-specific regulation of transgene transcription in genetically modified grapevine.
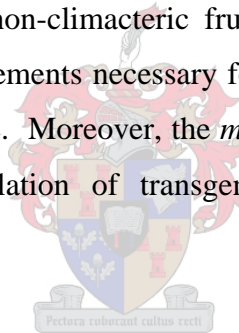
Opsomming

Ten spyte van toenemende fokus op rypwordings-verwante geentranskripsie in druiwe, en die groot aantal rypwordings-verwante komplimentere DNA (cDNA) fragmente wat gedurende die laaste paar jaar in druiwe geïdentifiseer is, word die molekulêre basis van prosesse betrokke by die rypwording van die druif, steeds swak begryp. Nog te meer, is baie min bekend oor die meganismes betrokke in the rypwordings-verwante regulering van vrugspesifieke gene, aangesien die isolering en karakterisering van nie een rypwordings-verwante, vrugspesifieke promoter tot dusver gerapporteer is nie. Die doel van hierdie studie was die isolering en karakterisering van 'n vrugspesifieke, rypwordings-verwante geen uit druiwe (*Vitis vinifera* L).

In die eerste fase van die werk, is geentranskripsie in rypwordende druiwekorrels van Cabernet Sauvignon ('n goeie kwaliteit wyn kultivar) en Clairette blanche ('n swak kwaliteit wyn kultivar) bestudeer deur middel van cDNA-AFLP vingerafdrukke. Totale RNA van onvolwasse (14-weke na blom vorming) en volwasse (18-weke na blom vorming) druiwekorrels was gebruik vir die analise. 'n Totaal van 1 276 cDNA fragmente is gevisualiseer, waarvan 175 as rypwordings-verwant voorgekom het. Gemiddelde paarsgewyse verskille van die fragmente wat vanaf onvolwasse en volwasse Clairette en Cabernet druiwekorrels geamplifiseer is, het aangedui dat rypwording-verwante geentranskripsie in die twee kultivars, wat fenotipies baie van mekaar verskil, merkwaardig soortgelyk is. Nieteenstaande, is daar gewys dat sewentig persent van die 175 rypwordings-verwante cDNA fragmente, kultivar-spesifiek is. Daar is voorgestel dat hierdie spesifieke cDNAs verder geanaliseer word om gene betrokke by die fenotipiese verskille tussen die twee kultivars te identifiseer; maar ook om gene te identifiseer wat moontlik by die kwaliteit van die druiwekorrel betrokke is. Voorts, het die analise die bruikbaarheid van die cDNA-AFLP tegniek vir die karakterisering van rypwordings-verwante geentranskripsie in rypwordende druiwekorrels, geïllustreer.

In die tweede fase van die werk, is een van die rypwordings-verwante cDNAs wat met die cDNA-AFLP analise geïdentifiseer is, geselekteer vir verdere karakterisering. 'n Aantal rypwordings-verwante cDNAs is in die enkele band wat uit die cDNA-AFLP gel gesny is, geïdentfiseer. Dit het die beperking wat geplaas word op die isolering van 'n enkel, spesifieke cDNA uit die cDNA-AFLP gel, beklemtoon. Stappe om hierdie beperking te oorkom, en 'n spesifieke rypwordings-verwante cDNA te identfiseer en te kloneer, is beskryf. In kort, die band oorstemmend met die spesifieke rypwordings-verwante cDNA, is uit die cDNA-AFLP poli-akrielamied gel gesny en ge-reamplifiseer. Noordelike klad analise waarin die ge-reamplifiseerde, ongekloneerde produk as

peiler gebruik is, het die rypwordings-verwante transkripsie soos deur cDNA-AFLP analise aangedui, bevestig. Die ge-reamplifiseerde, ongekloneerde produk is daarna gekloneer. Nukleotied volgorde bepaling van twee ewekansig geselekteerde kandidaat klone, het twee duidelik verskillende cDNAs aangetoon, waarvan nie een enige hibridisering met boodskapper RNA van rypwordende druiwekorrels getoon het nie. Verder analise het die teenwoordigheid van 'n verder vyf cDNAs met terminale nukleotied volgordes ooreenstemmend met die selektiewe nukleotiede van die voorlopers wat gebruik is vir selektiewe amplifisering, aangetoon. Van hierdie, het slegs twee hoë vlakke van geentranskripsie in rypwordende druiwekorrels getoon; heel moontlik verteenwoordigend van die rypwordings-verwante geentranskripsie wat met die cDNA-AFLP analise gevisualiseer is. Die studie het gewys dat al sewe cDNAs rypwordings-verwant is, alhoewel die meeste van hierdie cDNAs baie lae vlakke van geentranskripsie tydens duiwekorrel rypwording getoon het. Gebaseer op relatief hoë vlakke van die transkrip, en die inisiering van geen transkripsie met die aanvang van vrugrypwording (*véraison*, 10- tot 12-weke na blomvorming), is een van die cDNAs geselekteer vir isolering en karakterisering van die vollengte koderings volgorde.

In die derde fase van die werk, is dit aangetoon dat hierdie cDNA ooreenstem met 'n geen wat vir 'n proline-ryke proteïen (PRP), geassosieer met vrugrypwording in Merlot en Chardonnay, kodeer. Hierdie geen is genoem Merlot rypwording-geïnduseerde proteïen 1 (*mrip*1). Die studie het verder aangetoon dat hierdie geen spesifiek in die weefsel van druiwekorrels, saad and stammetjies van die druiwetros getranskribeer word, vanaf 10-weke na blomvorming (*véraison*) tot 16-weke na blomvorming. Resultate het aangetoon dat *mrip*1 vir 'n unieke lid van die plant PRP familie kodeer. Mees opvallend, is die sentrale gedeelte van mrip1, wat uit agt opeenvolgende herhalings van negentien aminosure elk bestaan. In vergelyking met ander druif PRPs, toon mrip1 enkel aminosuur verskille en 'n delesie van een van die negentien aminosuur herhalings, alles in die sentrale gedeelte van mrip1. *In situ* hibridisering het getoon dat akkumulering van die *mrip*1 transkrip net in selle van die mesocarp en eksokarp van die rypwordende druif plaasvind. Geen transkip met hoë nukleotied gelyksoortigheid aan *mrip*1 kon in rypwordende aarbeie of tamatie vrugte aangetoon word nie. Gebaseer op die eienskappe en funksie van PRPs soos voorgestel in die literatuur, en die bevindinge van hierdie studie, is potensiële toepassings vir die gebruik van die geen in die beheer van selwand argitektuur in vrugte, voorgestel. Verder, aangesien die manipulering van vrugkwaliteit in die druif veral belangrik is vanaf die aanvang van vrugrypwording (*véraison*), is daar voorgestel dat *mrip*1 'n ideale kandidaat is vir die isolering van 'n vrugspesifieke en rypwording-verwante promoter vir gebruik in geneties gemodifiseerde druiwe.

Die laaste fase van die studie was gewy aan die isolering en karakterisering van die *mrip*1 promotor element. 'n 5.5 kb fragment ooreenstemmend met die *mrip*1 5' ongetransleerde area is geïsoleer en

gekarakteriseer deur middel van nukleotied volgorde bepaling. In die 2.8 kb area direk stroomop van die *mrip*1 transkripsie inisiasie punt (TIS), is verskeie moontlike *cis*-beherende regulatoriese elemente geïdentifiseer. Hierdie sluit in 'n spektrum van hormoon-, lig-, fitochroom-, suiker- en stress-reagerende elemente, asook elemente geïmpliseer in weefselspesifieke geentranskripsie. Analise van die area verder stroomop (3.6 – 5.5 kb) van die *mrip*1 TIS, het die teenwoordigheid van 'n ander PRP direk stroomop van *mrip*1 getoon. Nukleotied gelyksoortigheid van hierdie geen (*MPRP*2) aan die *mrip*1 koderingsgebied was slegs 88%. Hierdie inligting verskaf die eerste insig in die chromosomale organisasie van druif PRPs. Vir funksionele analise van die *mrip*1 promotor element, is die 2.2 kb area direk stroomop van die *mrip*1 TIS transkripsioneel verenig met die *sgfp*S65T merker geen. Funksionaliteit van die *mrip*1: *sgfp*S65T fusie is bevestig deur middel van kortstondige (transient) geenuitdrukking in die perikarp van groenrissie, voordat dit ingevoer is in tabak met *Agrobacterium*-bemiddelde genetiese transformasie. In transgeniese tabak was transkripsie van die mri*p*1:*sgfp*S65T fusie ontwikkelingsstadium-gereguleerd, en spesifiek in die ovarium en heuningsakkie (nektarium) van die ontwikkelende blomme. Terwyl die vlak van geenuitdrukking laag was in die jong blomme, het GFP baie vinnig akkumuleer tot die hoë vlakke wat in die blomme in volle-blom gevisualiseer is. Daarna het dit weer vinnig afgeneem tydens die finale stadiums van ovarium ontwikkeling. Hierdie waarnemings dui daarop dat die 2.2 kb *mrip*1 promotor element funksioneel is en dit al die nodige *cis*-beherende regulatoriese element bevat wat nodig is vir weefsel- en ontwikkelingsstadium-spesifieke regulering van GFP akkumulering. Dit dui verder daarop dat transkripsionele aktivering van *mrip*1 beheer word deur ontwikkelingsstadium seine teenwoordig in beide die druif en tabakblomme. Hierdie resultate stel voor dat tabak meer algemeen gebruik kan word as heteroloë sisteem vir die analise van rypwording-verwante promotors. Duidelik dra die karakterisering van die *mrip*1 promoter element by tot 'n beter begrip van die regulatoriese meganismes betrokke by die rypwordingsproses van nie-klimateriese vrugte, en vorm die basis vir toekomstige eksperimente waarin die *cis*-beherende regulatoriese elemente vir vrug- en sel-spesifieke geen regulering, meer spesifiek die druif, bepaal sal word. Meer nog, is die *mrip*1 promotor 'n ideale kandidaat vir weefsel-spefieke en rypwording-verwante regulering van transkripsie van die transgeen in geneties gemodifiseerde druiwe.

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Now to Him

who by the power at work within us

is able to do far more abundantly

than all that we ask or think,

to Him be the glory

Ephesians 3: 20 - 21

Acknowledgements

Preface

This dissertation is presented as a compilation of seven chapters. Experimental work is presented in Chapters 3, 4, 5 & 6. Each of these chapters is written according to the style of the journal to which the manuscript was submitted for publication.

Chapter 1   **General Introduction and Project Aim.**

Chapter 2   **Literature Review.**

Chapter 3   **Ripening-related gene transcription during fruit ripening in Cabernet Sauvignon and Clairette blanche.**

Chapter 4   **Cloning of a specific ripening-related gene from the multiple of ripening-related genes identified from a single band excised from a cDNA-AFLP gel.**

Chapter 5   **Characterisation of the gene encoding the Merlot ripening-induced protein 1 (mrip1): evidence that this putative protein is a distinct member of the plant proline-rich protein family.**

Chapter 6   **Grapevine promoter directs gene expression in the nectaries of transgenic tobacco.**

Chapter 7   **Concluding Remarks and Future Prospects.**

I hereby declare that I was the primary contributor with respect to the experimental data presented in the multi-author manuscripts presented in Chapters 3, 4, 5 and 6. *In-situ* hybridisation experiments (Chapter 5) and genetic transformation of tobacco (Chapter 6) was performed by co-authors J.P. Zwiegelaar and L. Watts, respectively. My supervisor, Prof. F.C. Botha, was involved in the conceptual development, and continuous critical evaluation of this study.

Abbreviations

ACC synthase	1-aminocyclopropane-1-carboxylate synthase, EC 4.4.1.14
ACC oxidase	1-aminocyclopropane-1-carboxylate oxidase, EC 1.14.17.4
BAP	6-benzylaminopurine
bp	base pair
cDNA	complementary DNA
cm	centi metre
°C	degrees Celsius
Ci	curie
cpm	counts per minute
cv (s)	cultivar(s)
h	hour
HPLC	high pressure liquid chromatography
iPCR	inverse polymerase chain reaction
kb	kilobase
kPa	kilopascal
M	molar
min	minute
mJ	milli joule
ml	millilitre
mM	millimolar
msec	milli second
NAA	1 naphthalene acetic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
PCR	polymerase chain reaction
PG (endo-polygalacturonase)	EC 3.2.1.15
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SSC	Sodium chloride, sodium citrate solution
Tris	Tris (hydroxymethyl) amino methane
U	units
μ	micro
μm	micro metre
μM	micro molar
5' UTR	5' untranslated region
V	volt
v/v	volume per volume
x g	force of gravity
wpf	weeks post flowering
w/v	weight per volume

Contents

Chapter 5 – **Characterisation of the gene encoding the Merlot ripening-induced protein 1 (mrip1): evidence that this putative protein is a distinct member of the plant proline-rich protein family.**

Chapter 6 – **Grapevine promoter directs gene expression in the nectaries of transgenic tobacco**

Chapter 7 – Concluding Remarks and Future Prospects

CHAPTER 1

General Introduction and Project Aim.

General Introduction and Project Aim

1.1 GENERAL INTRODUCTION

The molecular era opened new avenues to improve our understanding of the physiological and biochemical processes involved in plant development, but moreover, paved the way for the development of genetic engineering as a powerful tool for plant improvement. The capacity to introduce and express foreign genes in plants was first described for tobacco in 1984 (De Block *et al.*, 1984), and has since been extended to over 120 species in at least 35 families. Contributing to this overwhelming agricultural development are 1) the prospects of new cultivars with improved quality and reduced economic and environmental costs; and 2) trends in worldwide population, food production, arable and irrigated land (Bazzaz and Sombroek, 1996; Global Crop Production Review, 2003).

Despite consumer resistance (Cheng, 2003; Huffman, 2003; Lobe, 2004) and the ongoing public debate about the future of agricultural biotechnology, the global area of transgenic crops increased 40-fold, from 1.7 million hectares in 1996 to 67.7 million hectares in 2003 (James, 2003). In 2003, the global area of transgenic crops continued to grow for the seventh consecutive year at a sustained double-digit growth rate of 15 % in 2003, compared to the 12 % in 2002 (James, 2003). The global market value of genetically modified (GM) crops was an estimated $4.5 to $4.75 billion in 2003, having increased from $4.0 billion in 2002 when it represented 15 % of the global crop production market and 13 % of the global commercial seed market. Commercialised transgenic crops currently include soybean, corn, cotton, canola and potato; whereas research to genetically modify plants with a high economic value such as cereals, fruits, vegetables, floricultural and horticultural species, is underway. Indications are that the global hectarage of genetically modified crops will increase to approximately 100 million hectares during the next five years, with up to 10 million farmers growing GM crops in 25, or more, countries (James, 2003).

Despite this large international interest in plant improvement through genetic manipulation, major obstacles prevent successful application of the technology. One of the obstacles is a lack of understanding of plant gene regulation and mechanisms to regulate plant gene transcription; in particular developmentally-regulated- and/or tissue- or cell-type-specific gene transcription. To address this issue, genetic elements (promoter elements) which regulate gene transcription in this manner, need to be isolated and characterised.

Being one of the most widely cultivated plant woody species, and considered the most important fruit crop, grapevine is one of the commercially significant crops which, according to predictions, will soon be included in the range of GM plants worldwide. In grapevine, traits of primary interest are reproductive traits determining yield, pathogen and abiotic stress resistance and quality traits for fruit and wine grapes.

Focusing on the quality traits of wine grapes and the genetic manipulation of fruit metabolism, one of the major obstacles is the availability of promoter elements to regulate transgene transcription specifically in the fruit tissue of the ripening grape berry. As the most dramatic changes in the characteristics that determine the quality of the final product (wine), occurs as the fruit enters into the ripening phase, such a promoter element should ideally be activated at the onset of berry ripening (*véraison*). With even in tomato, the model system for molecular and genetic analysis of fleshy fruit ripening, only a limited number of such promoter elements identified, it was decided to embark on the isolation of such a promoter element from grapevine. Characterisation of such a promoter element would contribute to the current understanding of the regulatory mechanisms involved in grape berry ripening. Unlike tomato, grapevine is a non-climacteric fruit and to date, fruit ripening in non-climacteric fruit is poorly understood. In addition, future studies on this promoter element and other fruit-specific and ripening-related promoters can possibly contribute to the identification of consensus regions for tissue specific and ripening-related transcriptional regulation, and to support or contradict suggestions that climacteric and non-climacteric fruits share common regulatory mechanisms (Kuntz *et al*., 1998). The aim of this study therefore, was to isolate a promoter element from grapevine which can be used to drive transgene transcription specifically in the fruit tissue of the ripening grape berry, particularly from *véraison* and during the post-*véraison* stages of fruit ripening.

Thus, the first objective of the study was to identify a gene which is transcriptionally activated at the onset of fruit ripening (*véraison*), specifically in the fruit tissue of the ripening berry. However, the molecular aspects of grape berry ripening are still poorly characterised, and only a limited number of genes which are fruit- and post-*véraison* specifically transcribed have been identified to date. This prompted our approach to study ripening-related gene transcription during grape berry ripening, from which an appropriate ripening-related gene could be identified and cloned. The second objective of the study was the isolation, characterisation and functional analysis of the promoter element of this gene. Final verification of the functionality and specificity of the promoter element can however only be achieved in plants in which the sequence has been stably integrated. Due to the low genetic transformation efficiency, slow regeneration (about 18 months) and long

reproductive life cycle of grapevine, an alternative system for the evaluation of the promoter element had to be identified.

Hence, the successful isolation of a fruit-specific, ripening-related promoter element from grapevine depends on a reliable and relative rapid, high throughput system for the identification and isolation of an appropriate ripening-related gene and a heterologous system for the functional analysis of the promoter element.

1.2 PROJECT AIM

The isolation and characterisation of a fruit-specific, developmentally-regulated gene from *Vitis vinifera* L. berries and the cloning, characterisation and functional analysis of the promoter element of this gene. Due to our interest in fruit-ripening, the focus will be on the identification of a ripening-related gene.

1.3 PROJECT OBJECTIVES

i) To study ripening-related gene transcription during fruit ripening in Cabernet Sauvignon and Clairette blanche by using the mRNA fingerprinting technique, cDNA-AFLP analysis;

ii) To clone a specific ripening-related gene identified by cDNA-AFLP analysis;

iii) To characterise this ripening-related gene, thereby contributing to the current understanding of the molecular aspects of the processes involved in grape berry ripening;

iv) To clone, characterise and study the functionality of the promoter element of this ripening-related gene.

In this dissertation, studies to address each of these objectives are presented in four manuscripts:

i) "Ripening-related gene transcription during fruit ripening in Cabernet Sauvignon and Clairette blanche". Burger, A.L. and Botha, F.C. Published in Vitis 43(2): 59-64 (2004);

ii) "Cloning of a specific ripening-related gene from the multiple of ripening-related genes identified from a single band excised from a cDNA-AFLP gel". Burger, A.L. and Botha, F.C. Published in Plant Molecular Biology Reporter 22(3): 1-12 (2004);

iii) "Characterisation of the gene encoding the Merlot ripening-induced protein 1 (*mrip*1): evidence that this putative protein is a distinct member of the plant proline-rich protein family". Burger, A.L., Zwiegelaar, J.P. and Botha, F.C. Published in Plant Science 167(5): 1075-1089 (2004);

iv) "Grapevine promoter directs gene expression in the nectaries of transgenic tobacco". Burger, A.L., Watts, L. and Botha, F.C. For submission to Plant Molecular Biology (2004).

The work presented in this dissertation comprises the first report of the isolation of a tissue- and developmental-specific promoter element from grape berries. The isolation and characterisation of this promoter element has laid the foundation for further studies on tissue-specific and ripening-related transcriptional regulation, as well as on the regulatory genes, signalling and coordination mechanisms involved in the onset of grape berry ripening. As the manipulation of fruit properties in grape berries is most important in the later stages of ripening, this promoter element is an ideal candidate for the tissue-specific, post-*véraison* regulation of transgene transcription in genetically modified grape berries.

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CHAPTER 2
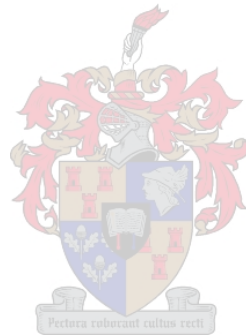
Literature Review.

Literature Review

FRUIT RIPENING – The Increasing Focus on the Manipulation of Quality and Ripening

Fresh or processed fruits form an important/essential part of the human diet providing vital vitamins, minerals, fibre and other health promoting compounds. Moreover, fresh fruits are often attractive to the consumer because of aesthetic qualities of flavour, colour and texture. Fruit therefore constitute a commercially significant food commodity, with an ever-increasing demand, at least in the western society, for both improved quality and extended variety of the fruit available.

Remarkably, the world's most important fruit crop is a berry with an average weight of 1 to 2 grams; the majority of fruit shaped spherical to short oval, and coloured yellow or dark blue (Robinson, 1994). Not an important or essential part of the human diet, or popular because of its aesthetic qualities such as colour or texture, but most valued for its wine-making properties. It is the most widely grown fruit crop in the world with nine million hectares of vineyards producing about 60 million tonnes of fruit in the early 1990's. Its uses include wine-making, distilled liquors, fresh consumption (table grapes), dried fruit (raisins), juice and concentrate, rectified must, and limited industrial products. Wine-making is, however, the most important use accounting for some 80 % of the world's production; totalling between 250 and 300 million hectolitre of wine produced in the world annually (Kanellis and Roubelakis, 1993; Robinson, 1994).

This fruit crop – the grapevine – is a woody perennial angiosperm that reaches reproductive maturity in 4 to 5 years. It belongs to the genus *Vitis*, which was defined in 1700 and was one of the first genera studied by Linnaeus in 1735. *Vitis* are shrubs of the northern hemisphere. It consists of about 60 species, of which the majority originated in the Americas or Asia (Winkler *et al*., 1974). The European species, *vinifera*, has the largest and sweetest berries and is the species most suitable for wine-making. It is to this species that all the most familiar vine varieties belong. It is believed that *Vitis vinifera* originated south of the Black Sea of Transcaucasia, now the disputed territories of Georgia and Armenia, and been spread through the Mediterranean and Europe by the Phoenicians and Greeks and later by the Romans. From there it was distributed through the New World, initially in South America, and subsequently into western North America, the southern tip of Africa (Republic of South Africa), Australia, and then New Zealand. Today, *Vitis vinifera* is grown on all continents except Antartica. Requiring warm summers for fruit maturation, the vine is usually grown approximately between 10 and 20°C isotherm in both hemispheres, or about between latitudes 30 and 50 degrees north, and 30 and 40 degrees south (Fig. 2.1). Hence, most of the world's vineyards

WORLD DISTRIBUTION OF VINEYARDS*
IN 1000S OF HECTARES (1992 FIGURES)
(*including those producing table and drying grapes)

EUROPE	ha				
Spain	1,360	Bulgaria	149	Czechoslovakia (ex)	3
Italy	1,008	Greece	138	Great Britain	1
France	943	Hungary	135	Luxembourg	1
USSR (ex)	813	Germany	107	Malta	1
Portugal	370	Austria	58		
Romania	252	Albania	17		
Yugoslavia (ex)	201	Switzerland	15		

NORTH AMERICA	ha
USA	301
Canada	7

FAR EAST	ha
China	160
Japan	26

LATIN AMERICA	ha
Argentina	209
Chile	121
Brazil	60
Mexico	47
Uruguay	21
Peru	9
Bolivia	4

AFRICA	ha
South Africa	116
Algeria	100
Morocco	50
Egypt	38
Tunisia	33

ASIA/ MIDDLE EAST	ha
Turkey	580
Iran	232
Syria	109
Iraq	58
Libya	30
India	28
Cyprus	23
Jordan	6
Israel	5

AUSTRALIA	ha
Australia	61
New Zealand	6

The hectare is the equivalent of 2.47 acres — Wine growing regions

Isotherm 10° C, 20° C, Equator, 20° C, 10° C

Atlantic Ocean, Pacific Ocean

Figure 2.1. Global distribution of vineyards. Wine growing areas are indicated by the shaded areas. Map on page 1076-7 from "Oxford Companion to Wine 2/e", edited by Jancis Robinson (1994), Free Permission. Reprinted by permission of Oxford University Press (URL www.oup.com).

are in Europe, with three countries, Italy, Spain and France, each having about 1 million hectares of vineyards, and Italy and France each producing almost one-quarter of the world's wine.

With just over one percent of the world's vineyards, the Republic of South Africa (RSA) ranks about 16th in area under vines (115 000 hectares). Its annual output, at some 8 million hectoliter, makes it the world's 8th largest wine producer. Apart from being an important primary source of economical growth and development (specifically in the Western Cape region where most of the vineyards are located), it has been shown that the South African wine industry has an exceptional ability to create prosperity. Nevertheless, considering recent trends in consumer preference and consumption, steep increases in production costs, minimal increases in the final product prices, and the volume and value of wine exports (38 % of the drinking wine exported in 2002), it is evident that the South African wine industry is under pressure to stay economically viable and competitive. Especially in recent years, the value and competitive nature of the grape and wine industry has led to an increasing emphasis on producing quality fruit for the production of quality wines.

Analysis of grapes and must is chiefly concerned with just three components: sugars, organic acids and pH. Ideally, grapes should contain sugars capable of producing wine with a final alcoholic strength of between about 10 and 13 percent by volume, which means that the grapes should have between 18 and 22 percent of fermentable sugar per gram fresh weight. In warm wine regions the accumulation of sugars poses a problem in that resulting wines may have excessive alcohol and insufficient acid. The acidity of the original grape juice has an important influence on wine quality because of its direct influence on colour of the wine, its effect on the growth of yeasts and bacteria, and its inherent effects on flavour qualities. Too little acidity, the consequence of picking too late, or such heat during ripening that the natural plant acids are largely decomposed, results in wines that are flat, uninteresting, and described typically by wine tasters as "flabby". Ideally, the acidity of the grapes or must should be such that the total acidity is in the general range of 7 to 10 gram per litre as tartaric acid. It has been recently established that the chemistry of ageing is strongly influenced by pH, and that there is a close relationship between pH and total acidity. Wines with a pH between 3.2 and 3.5 not only tend to taste refreshingly - rather than piercingly acid, but are also more resistant to harmful bacteria, age better, and have a clearer, brighter colour. On the contrary, wine with pH values higher than this suffer from tasting flat, looking dull, and also from being more susceptible from bacterial attack. In general, hot regions produce wines with high pH, compared to wines with low pH produced in cool regions.

For the South African wine industry – which is already faced with the challenging task to counteract low total acidity, high pH and excessive accumulation of sugars generally associated with hot grape

producing regions - strategies to improve fruit and wine quality and to development novel products, are most challenging. In light of the advent of molecular biology techniques, which provided a powerful tool to probe the biochemistry of fruit ripening, allowing the possible manipulation of aspects of thereof, the South African wine industry embarked on the genetic manipulation of grape berry metabolism. A major obstacle to achieving this aim, however, remains a lack of fundamental knowledge of the biochemistry and molecular biology of the fruit ripening process (Botha, 1999; Boss and Davies, 2001). Successful genetic manipulation of fruit metabolism is further obstructed by 1) the availability of suitable genetic constructs with promoter elements to regulate transgene transcription specifically in the fruit at a particular stage of ripening, and 2) the availability of effective tissue culture systems for genetic transformation and regeneration of transgenic material (Botha, 1999). This study focuses on the identification and characterisation of a ripening-related gene specifically expressed in the grape berry during the post-*véraison* stages of berry ripening, and the characterisation of a promoter element from this gene which can be used to regulate transgene transcription specifically in the fruit-tissue of post-*véraison* berries. In the following sections the reader will be briefed with a short introduction to the fruit ripening process, followed by a discussion of the literature relevant to 1) fruit ripening, in particular ripening-related gene transcription during fruit ripening, highlighting genes specifically expressed in the late stages of fruit development; 2) the isolation and characterisation of ripening-related genes, as well as 3) the analysis of promoter regions identified in ripening-related genes and their characterisation in heterologous systems.

2.1 THE FRUIT RIPENING PROCESS

2.1.1 Introduction

Anatomically fruits are swollen ovaries that may also contain associated flower parts. Their development follows fertilisation, and occurs simultaneously with seed maturation. Fruits enlarge initially through cell division, and then by increasing cell volume. The embryo matures and the seed accumulates storage products, acquires desiccation tolerance, and loses water. The fruit then ripens; a process accompanied by changes in flavour, texture, colour, and aroma. In general, fruit can be classified as either climacteric or non-climacteric on the basis of their respiration pattern during ripening (Tucker, 1993). Climacteric fruit display a characteristic peak of respiratory activity during ripening, termed the respiratory climacteric. In contrast, non-climacteric fruit simply exhibit a gradual decline in their respiration during ripening. The role of the respiratory climacteric, if any, in ripening is unclear. Despite different respiratory and ethylene production behaviour it is suggested that climacteric and non-climacteric fruits share common regulatory mechanisms (Kuntz *et al.*, 1998; Vrebalov *et al.*, 2002).

2.1.2 Fruit Ripening and Ripening-Related Gene Expression

Ripening can be considered as a specialised stage of plant senescence. As such, it was originally deemed that ripening was primarily a catabolic process in which cellular organisation and control were breaking down (Blackman and Parija, 1928). However, over the last few decades, and in particular over the last few years, it has become apparent that ripening, like other plant senescent processes, is under strict genetic control (Richmond and Biale, 1967; Brady and O'Connell, 1976; Mitcham *et al*., 1989). The analysis of mRNA and protein species during ripening of both avocado (Christoffersen *et al*., 1982) and tomato (Rattanapanone *et al*., 1978; Biggs *et al*., 1986) showed the synthesis of distinct ripening-related proteins which led to the concept of ripening as being controlled, at least partially, at the level of gene transcription. Although several of these ripening-specific proteins have been identified, such as Endo-polygalacturonase (PG) (Grierson *et al*., 1986), ACC synthase (Van der Straeten *et al*., 1990) and ACC oxidase (Hamilton *et al*., 1990), the function of most of the ripening-related cDNAs isolated in recent years remains to be elucidated (Moore *et al*., 2002).

With tomato being the model system for studying the biology of fleshy fruit, the molecular biology and genetics of tomato fruit ripening are relatively well characterised (Moore *et al*., 2002; Seymour *et al*., 2002; White, 2002). However, little is known about the genes and the nature of the genetic control which act to initiate and regulate this complex developmental process. Ripening is considered as a set of coordinated but otherwise loosely connected biochemical pathways, and it has been speculated that these pathways are most likely under hormonal control both for their initiation and coordination (Tucker, 1993). Since most of the work of gene expression so far reported has been carried out in avocado and tomato, it is not known whether similar regulatory genes are active in all fruiting species.

Unlike climacteric fruit (avocado, banana and tomato), isolation of ripening-related genes from non-climacteric flesh fruits, received much less attention and the ripening of non-climacteric fruit at molecular level is still poorly understood. Studies on ripening-related gene transcription in these fruits focused mainly on strawberry and grapes and many changes in mRNA populations during the ripening process have been reported (Nam *et al*., 1999; Manning, 1998; Medina Escobar *et al*., 1997; Ablett *et al*., 2000; Davies and Robinson, 2000; Terrier *et al*., 2001a). Other studies include those on pepper and blackcurrant (Proust *et al*., 1996; Woodhead *et al*, 1998; Kim *et al*., 2002).

2.1.3 Grape Berry Ripening, with specific reference to Ripening-Related Gene Expression during Berry Development

The grape berry is classified as a non-climacteric fruit, like strawberry, citrus and pepper, since it does not exhibit a significant increase in respiration or ethylene synthesis at the onset of ripening (Kanellis and Roubelakis-Angelakis, 1993). The growth pattern of the berries follows a double sigmoidal curve (Coombe, 1976) based on the parameters of accumulative berry diameter, length, and volume or weight of the seeded berries. The initial phase (Phase I) is characterised by cell division and cell expansion which results in rapid growth of the berry. This is followed by a lag phase (Phase II) of no or slow growth; and then finally a second growth period (Phase III) in which cell expansion - rather than cell division - is chiefly responsible for the continued growth in berry size and weight (Considine and Knox, 1979). Besides the growth characteristics, each of the three phases is characterised by specific changes in biochemical and physical features of the berry (Fig. 2.2). The most dramatic change in berry development occurs as the fruit enters into the final phase of berry development and ripening. The inception of this phase is referred to as véraison, a French word which has been adopted by viticulturists as a useful term to describe the rapid change in berry skin colour, constitution and texture of the berries. Most obvious changes are the accumulation of hexoses, anthocyanins, metabolism of organic acids, cell wall modifications and the development of compounds involved in flavor and taste (Coombe, 1976; Hawker 1969a and b; Ruffner, 1982a and b; Kanellis and Roubelakis-Angelakis, 1993). It is the development of some of these characteristics that determine the quality of the final product.
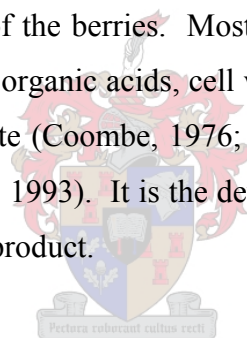
Much of the basic physiology and biochemistry of grape berry ripening has been described (Kanellis and Roubelakis-Angelakis, 1993; Famiani *et al.*, 2000; Roubelakis-Angelakis, 2001), and the knowledge applied in the manipulation of grape berry quality and ripening by means of viticultural practices and hormone treatments (Winkler *et al.*, 1974; Davies *et al.*, 1997). However, far less is known of the molecular events involved. As for other non-climacteric fruit, work only recently focused on the identification and characterisation of ripening-related genes from grapes. In addition, grapevine is often considered "a difficult-to-work-with tissue" as grapevine tissues are rich in phenolic compounds. The high phenolic content, in particular the tannin content, makes the extraction of nucleic acids somewhat difficult. The extraction of adequate amounts of good quality RNA from grape berry tissue is particularly challenging as the yield in general, is low and RNA is easily degraded (Geuna *et al.*, 1998; Salzman *et al.*, 1999).

Véraison

Phase 1
- Period of very rapid cell division rate, followed by a marked cell enlargement,
- Berries display active metabolism, high rates of respiration and rapid acid accumulation.

Phase 2
- Lag phase - Slow or no growth,
- Rapid embryo development,
- Berries starts to loose chlorophyll, and soften,
- Rapid accumulation of sugars and amino acids,Acidity reaches its highest level.

Phase 3
- Accelaration of growth (cell enlargment), softening of the berry and increases in deformability,
- Increase in the content of glucose, fructose, total and free amino acids, total proteins and total nitrogen,
- Decrease in the concentration of organic acids (mainly malic acid) and ammonia,
- Loss of chlorophyll from the skin and accumulation of anthocyanins.

Phase 1   Phase 2   Phase 3

2   4   6   8   10   12   14   16

Weeks post flowering (wpf)

Figure 2.2. Schematic drawing of grape berry ripening. Indicated is the three stages into which the growth pattern of the berries is usually divided, and the structural and biochemical changes associated with each of these stages.

The relationship between fruit ripening and changes in mRNA levels during grape berry ripening was first demonstrated by Boss and co-workers (1996a). Since then, several ripening-related cDNA clones have been isolated from grape. These include genes involved in flavonoid and stilbene biosynthesis (Sparvoli *et al.*, 1994), sugar transport (Davies *et al.*, 1999; Fillion *et al.*, 1999; Ageorges *et al.*, 2000), sugar metabolism (Davies and Robinson, 1996; Takayanagi and Yokotsuka, 1997), anthocynanin biosynthesis (Boss *et al.*, 1996b; Gollop *et al.*, 2002), pathogen-related proteins (Robinson *et al.*, 1997; Tattersall *et al.*, 1997; Salzman *et al.*, 1998); malate accumulation (Or *et al.*, 2000c), fermentative metabolism (Or *et al.*, 2000b; Tesnière and Verriès, 2000), cell wall modification (Nunan *et al.*, 1998; Nunan *et al.*, 2001; Terrier *et al.*, 2001a), proton- (Terrier *et al.*, 2001b) and water transport (Picaud *et al.*, 2003). In recent years, differential screening, RNA fingerprinting and high throughput expressed sequence tags (EST) sequencing projects proved to be highly effective in the identification of differentially expressed genes (Ablett *et al.*, 2000; Davies and Robinson, 2000; Terrier *et al.*, 2001a; Venter *et al.*, 2001). Several putative cell wall and stress response proteins were identified by Davies and Robinson (2000) and Venter *et al.*, (2001), while a large panel of ripening-related genes were identified from ripening berry cDNAs libraries (Terrier *et al.*, 2001a). In their study Terrier *et al.* (2001a) showed that 93 % of independent clones were specific to one of the three cDNA libraries analysed (*viz.* green, softening and ripening berries), indicating dramatic differences between the different stages of berry ripening. The authors

concluded that grape berry cDNA appears to be more diversified than other plant material, reflecting the large array of cellular functions carried out by berry pulp cells, or the diversity of cellular types present in the pericarp (Terrier *et al*., 2001a).

Despite these recent advances, the ripening of the grape berry – and other non-climacteric fruit – at molecular level is still poorly understood. Most of the ripening-related genes identified remain to be fully characterised and their function elucidated. Particularly, little is known about ripening-related genes which are transcriptionally activated at the onset of berry ripening (*véraison*), and whose expression has been shown to be berry-specific. Amongst these - which include genes encoding for the two thaumatin-like proteins VVTL1 and VVTL2 (Tattersall *et al*., 1997; Davies and Robinson, 2000), a chitinase (*VvChi*4 - Robinson *et al*., 1997), UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) expressed in grape berry skins but not in the flesh (Davies *et al*., 1997), an alcohol dehydrogenase (G7ADH - Or *et al*., 2000a), three putative stress response proteins (Grip 22; Grip 32 and Grip61 - Davies and Robinson, 2000), four putative cell wall proteins (Grip 3/4; Grip 13; Grip 15 and Grip 28 - Davies and Robinson, 2000), pectin methylesterase and polygalacturonase (VvPME1 and VvPG1 - Nunan *et al*., 2001) and two plasma membrane aquaporins (Picaud *et al*., 2003) - only nine genes have also been shown to be expressed specifically in the berry tissue. These are the two genes encoding for the thaumatin-like proteins VVTL1 and VVTL2, and the grapevine ripening induced proteins Grip 3/4; 13; 15; 22; 28; 32 and 61. Although not expressed in leaves, root or seeds, low levels of the *VvChi4* transcript was also detected in flowers (Robinson *et al*., 1997).
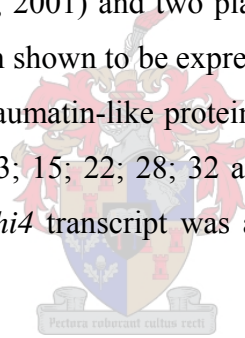
Even in tomato – the model system in which the molecular and genetic analysis of fleshy fruit development has resulted in significant gain in knowledge over recent years - only a small number of genes, which are transcriptionally activated at the onset of ripening, has been identified. These include an expansin, *LeExp*1 (Rose *et al*., 1997) and the genes E4, E8 (Lincoln and Fischer, 1988), polygalacturonase (DellaPenna *et al*., 1986) and phytoene synthase (Giuliano *et al*., 1993). However, transcription of only the three genes *LeExp*1, *E4* and *E8* was shown to be fruit-specific (Lincoln and Fischer, 1988; Cordes *et al*., 1989; Rose *et al*., 1997).

In strawberry transcription of *FaExp2*, an expansin with only 52 % sequence homology to *LeExp*1, and *FaExp*5 were shown to be ripening-related and tissue-specific. In fruit, both genes were shown to be transcriptionally activated at the onset of ripening. No mRNA of the two genes could be detected in vegetative tissues (root, stem, leaves and sepals), ovaries and green achenes (Civello *et al*., 1999; Harrison *et al*., 2001).

2.2 THE ISOLATION AND CHARACTERISATION OF RIPENING-RELATED GENES

Until recently, most studies on differential gene transcription during fruit ripening employed the construction and differential screening of cDNA libraries (Gray *et al.*, 1992; Picton *et al.*, 1993; Manning, 1998; Woodhead *et al.*, 1998; Davies and Robinson, 2000; Vrebralov *et al.*, 2002). In recent years, however, efficient, high throughput methodologies like cDNA-AFLPs and cDNA microarrays have demonstrated their usefulness in the identification ripening-related cDNAs from grapevine (Ablett *et al.*, 2000; Venter *et al.*, 2001; Terrier *et al.*, 2001a), pepper (Kim *et al.*, 2002), raspberry (Jones *et al.*, 2000), strawberry (Aharoni *et al.*, 2002) and tomato (Moore *et al.*, 2002). cDNA-AFLP (Amplified Fragment Length Polymorphism) is a RNA fingerprinting technique that evolved from a method described by Vos *et al.* (1995) for the fingerprinting of genomic DNA. The technique has the advantage that the analysis can be performed using minimal amounts of total RNA and that multiple developmental stages or tissue types can be analysed simultaneously, visualising both up- and downregulation of gene transcription and tissue-specific transcription (Kuhn, 2001). The technique is based on the use of highly stringent PCR conditions, which avoids problems encountered with reproducibility and optimisation of reaction conditions when using arbitrarily primed PCR (Vos *et al.*, 1995; Money *et al*, 1996; Bachem *et al.*, 1996; Habu *et al.*, 1997). Considerations for this sequence-based approach include 1) observations that members of multigenic families often exhibit distinct developmental patterns during berry ripening (Davies *et* al., 1999; Fillion *et al.*, 1999; Tesnière and Verriès, 2000), which emphasise the utilisation of sequence-based analysis for the unambiguous characterisation of these isogenes and, 2) that contrary to hybridisation-based approaches, sequence-based approaches are not biased towards abundant transcripts. The identification of mRNAs is thus not limited by redundancy of highly expressed mRNAs or under-representation of rare mRNAs in a cDNA library (Liang and Pardee, 1992; Wan *et a*l., 1996; Breyne and Zabeau, 2001). It is estimated that the 105 000 ESTs in the *Arabidopsis thaliana* collection is representative of only 60 % of all the genes, illustrating the extent to which cDNA libraries fail to represent all mRNAs (Richmond and Sommerville 2000). More recently, Kuhn (2001) reported that only 1.4 to 5 % of the 1443 *Arabidopsis* genes analysed in cDNA microarrays represented highly expressed genes with abundance of more than 100 – 500 transcripts per cell. The majority of the expressed genes were low abundance with levels of less than 10 – 50 transcripts per cell (Ruan *et al.*, 1998). It is argued that many important regulatory genes can thus be overlooked by hybridisation-based approaches as abundant messages are over-represented in cDNA libraries and rarely expressed genes are often missing.

Besides cDNA-AFLPs successful application in the identification of genes differentially expressed during fruit ripening, the technique has been used extensively to identify differentially regulated genes in plants and other organisms (Breyne and Zabeau, 2001; Donson *et al*., 2002).

2.3 TRANSCRIPTIONAL REGULATION OF RIPENING-RELATED GENE EXPRESSION IN FRUIT TISSUE

Fruit specific transcriptional regulation of ripening-related genes has been extensively studied in climacteric fruit. Studies focused on the 5' UTR flanking regions (promoter regions) of polygalacturonase and the fruit-specific genes 2A11, E4 and E8 of tomato; in particular on the mapping of ethylene-responsive and fruit-ripening regulatory regions (Montgomery *et al*., 1993; Deikman *et al*., 1992). Deletion analysis revealed the presence of several positive and negative regulatory elements in the 5' regions of these genes, and the presence of several fruit-specific elements in the region 1.8 kb upstream of the transcriptional start of the 2A11 gene (Van Haaren and Houck, 1993). The 3' region of this gene has been shown to play a minor role, if any, in fruit-specific transcription of the gene – suggesting that the 1.5 kb 5' region contains the necessary information for fruit-specific transcription (Van Haaren and Houck, 1991).

In other climacteric fruit, fruit- and ripening specific regulatory elements were also identified in the 5' regions of ripening-related genes *viz*. avocado cellulase (Cass *et al*., 1990), kiwifruit polygalacturonase and actinidin (Atkinson and Gardner, 1993; Lin *et al*., 1993), melon ACC-oxidase (Lasserre *et al*., 1997), and apple ACC-oxidase and PG (Atkinson *et al*., 1998). It was shown that the 5' region of the apple ACC-oxidase gene contains elements located between –1159 and –450 upstream of the transcription initiation site that direct ripening-specific gene expression in tomato fruit. An element that directs fruit-, but not ripening-specific gene transcription, was located in the region –450 to –1. Larger fragments -1159bp to -1 and -1966 to -1 showed both fruit- and ripening specificity.

Despite the increasing focus in recent years on ripening-related gene expression in non-climacteric fruit, characterisation of the 5' UTR flanking region of a fruit-specific, ripening-related gene has not been reported. Insight into the transcriptional regulation of gene expression in the fruit tissue of non-climacteric fruit, has mainly been provided by sequence analysis of the 5' UTR flanking regions of a number of genes shown to be expressed in the fruit tissue of these fruits (Table 2.1). Note that not one of these genes is fruit-specific (refer to Section 1.3, paragraph 4). For only a few genes *viz*. grapevine *dfr*, *ldox*, *Vvht*1, *SIRK* and *Vst*1, has isolation and characterisation of more than 1 kb of 5'

Table 2.1. Genes which have been shown to be expressed in fruit tissue, and of which at least 0.3 kb of 5' UTR flanking region has been isolated.

Fruit	Gene	Size of 5' UTR (kb)	NCBI Genbank Accession number	Reference
Apple	1-Aminocyclopropane-1-carboxylate (ACC)-oxidase	2.056	AF030859	Atkinson *et al*., 1998
	Polygalacturonase (PG)	2.629	AF031233	Atkinson *et al*., 1998
	*Ypr*10, encoding Mal d 1 allergen	1.28	AF074721	Pühringer *et al*., 2000
Avocado	Cellulase (*Cel*1)	1.507	X59944	Cass *et al*., 1990
Banana	ACC-oxidase	0.931	X91076	Lopez-Gomez *et al*., 1997
Grapevine	Alcohol dehydrogenase gene (GV-*Adh*1)	0.306	U36586	Sarni-Manchado, 1997; Fillion *et al*., 1999
	Alcohol dehydrogenase gene (GV-*Adh*7)	0.384	AF195867	Or *et al*., 2000a
	Chitinase (*GV-Chi*)	0.4	AJ430782	Seibicke, 2002
	Dihydroflavonol reductase (*dfr*)	2.278	AF280768	Gollop *et al*., 2002
	Glucanase (GV ß-1,3-glucanase)	0.919	AJ430781	Seibicke, 2002
	Hexose transporter gene (*Vvht*1)	2.471	AJ001062	Fillion *et al*., 1999
	Leucoanthocyanidin dioxygenase (*LDOX*)	2.4	AF290432	Gollop *et al*., 2001
	Stomatal Inward Rectifying channel K^+ (*SIRK*)	2.916	AF359522	Pratelli *et al*., 2002
	Sucrose transporter 2 (*VvSUT*2)	2.184	AF439321	Unpublished
	Stilbene synthase (*Vst*1)	1.016 1.5	Y18532	Unpublished Schubert *et al*., 1997
	UDP-glucose:flavonoid 3-O-glucosyltransferase (*F1UFGT1*)	0.459	AB047098	Kobayashi *et al*., 2001
	UDP-glucose:flavonoid 3-O-glucosyltransferase (*F1UFGT2*)	0.425	AB047099	Kobayashi *et al*., 2001
	Vacuolar pyrophosphatase (*GV-VPPase)*	1.567	AJ544719	Unpublished
Greenpepper	Ccs, encoding capsanthin/capsorubin synthase	2.007	Y14165	Bouvier *et al*., 1998
Kiwi	Actinidin protease	1.362	L07552	Lin *et al*., 1993
	Polygalacturonase	3.195	L12019	Atkinson and Gardner, 1993
Melon	ACC-oxidase (*CM-ACO*1)	0.738	X95551	Lasserre *et al*., 1997
	ACC-oxidase (*CM-ACO*3)	2.26	X95553	Lasserre *et al*., 1997
Strawberry	Pyruvate decarboxylase (*pdc*1)	0.78	AF333772	Unpublished
Tomato	*2A11*	4.752	M37631	Van Haaren and Houck, 1991; Van Haaren and Houck, 1993
	E4	1.441	S44898	Montgomery *et al*., 1993 Coupe and Deikman, 1997
	E8	2.16	AF515784	Deikman *et al*., 1992 Coupe and Deikman, 1997
	ACC-oxidase (*LEACO*1); ACC-oxidase (*LEACO*2); ACC-oxidase (*LEACO*3);	1.855 2.385 2.267	X58273 Y00478 Z54199	Blume *et al*., 1997 Barry et al., 1996
	Polygalacturonase	1.410	X14074	Bird *et al*., 1988 Nicholass *et al*., 1995
	Phytoene desaturase (*pds*)	0.564	X71023	Mann *et al*., 1994

UTR flanking region been reported (Table 2.1). Characterisation of these 5' regions was mainly based on sequence and *in silico* analysis; and expression analysis of promoter-*uid*A fusions in transgenic tobacco, grapevine or *Arabidopsis* (Schubert *et al.*, 1997; Gollop *et al.*, 2001; Gollop *et al.*, 2002; Pratelli *et al.*, 2002). The 5' regions of the genes *dfr* and *Vst*1 were also characterised by deletion analysis.

Analysis of the 5' UTR flanking region of these five genes revealed the presence of several potential regulating *cis*-elements, including hormone (abscisic acid, auxin and ethylene), light, phytochrome, and elicitor-responsive elements. Other potential regulating *cis*-elements identified include a low-temperature responsive element (*Vvht*1); a P-box - which is a sequence found in the 5' regions of several genes encoding seed storage proteins (*Vvht*1), and sugar responsive elements in the 5' regions of the genes *Vvht*1, *ldox* and *dfr*. A sequence described as a Suc-responsive element (SURE1STPAT21) and regions homologous to the Suc 2 and 3 boxes were described in the 5' regions of the grapevine genes *Vvht*1, *ldox* and *dfr* (Fillion *et al.*, 1999; Gollop *et al.*, 2001; Gollop *et al.*, 2002). Similar elements were identified in various other ripening-related genes, including tomato E8, melon ACC-oxidase (*CM-ACO*3) and strawberry *pdc*1. Sucrose boxes 2 and 3 were originally identified in the sporamin gene family of the sweet potato, which are expressed at high levels when high concentrations of sucrose are applied to stems (Hattori and Nakamura, 1988; Hattori *et al.*, 1990). These boxes also exist in the 5' region of the chalcone synthase (*chs*-A) gene from petunia, which has been shown to be induced by sucrose, glucose and fructose in transgenic *Arabidopsis* (Tsukaya *et al.*, 1991). The sucrose boxes were found to be present in the *chs* gene from different plant species, and in other sugar-responsive genes, such as patatin and the gene for proteinase inhibitor II in potato (Tsukaya *et al.*, 1991). Interestingly, the Suc-responsive element (SURE1STPAT21) was also identified in the 5' region of the Nectarin I gene of tobacco; a gene of which the expression has been shown to be nectary-specific and specific to the final stages of nectary development when nectar is actively secreted (Carter *et al.*, 1999; Mann *et al.*, 2000). Nectar has been shown to be a combination of a number of substances, amongst which sucrose, glucose and fructose are the chief substances (Carter *et al.*, 1999).

A number of putative c*is*-regions implicated in tissue- and developmental specificity of these genes have been identified. These include the sequence TATTT(T/A)AT identified in positions −773 and −1195 of the *Vvht*1 5' region; the sequence TACCAT which is a known *cis*-acting element controlling organ-specific transcription (*dfr*) and H-box related motifs also implicated in tissue-specific transcription (*Vst*1) (Schubert *et al.*, 1997; Fillion *et al.*, 1999; Gollop *et al.*, 2002). Deletion analysis revealed a sequence involved in developmental transcription of the *dfr* gene

between positions -725 and −233 of the *dfr* 5' region. Regions controlling ozone- and pathogen induction were identified in the 5' region of the grape *Vst*1 gene.

Besides the putative *cis*-regulatory elements identified in the 5' UTR flanking regions of these genes, factors like auxin, Ca^{2+}, sucrose and light have been shown to be involved in transcriptional regulation of genes expressed in fruit. Auxin has been implicated in the control of fruit ripening in both climacteric and non-climacteric fruit in general. In strawberry, auxin has been shown to regulate the expression of several genes during strawberry fruit ripening by the induction and repression of the expression of specific mRNAs and polypeptides (Manning, 1994). In grapes, application of the synthetic auxin BTOA was shown to delay grape berry ripening, in particular transcription of the ripening-related gene *grip*4 (Davies *et al.*, 1997), while sucrose, Ca^{2+} and light and were shown to induce gene transcription (Gollop *et al.*, 2001; Gollop *et al.*, 2002). Based on the induction of the grape *dfr* and *ldox* genes by light and sucrose under light, those authors suggested a close interaction between the sucrose and the light signaling pathways. Sugar is also one of the factors implicated in the nature of the signal that initiates ripening in grape berries (Davies and Robinson, 1996).

An important feature of the *Vvht*1 5' region, is its similarity with the 5' region of the grapevine alcohol dehydrogenase gene cloned by Sarni-Manchado *et al*. (1997). Similar to *Vvht*1, transcription of the GV-Adh is upregulated during ripening. Alignment of the two 5' regions revealed a 15-bp consensus sequence, which suggests a possible co-regulation of the transcription of the two genes probably due to the binding of a common transcription factor on the *cis* sequences that are shared by the 5' regions of the genes. On the contrary, no significant homology was revealed with the 5' regions of other hexose transporters.

Likewise, low homology was observed between the 5' regions of the apple ACC-oxidase and polygalacturonase genes, and corresponding sequences in other fruit (Atkinson *et al.*, 1998). Although several small motifs of potential significance and some small regions of homology to positive regulatory elements in *Arabidopsis*, petunia and tomato were revealed, no significant stretches of homology could be identified (Atkinson *et al.*, 1998). Previous work has indicated that high sequence homology of the coding region does not necessarily serve as an indication of similar expression profiles (Civello *et al.*, 1999; Tesnière and Verriès, 2000; Harrison *et al.*, 2001). In strawberry the ripening-regulated expansin, *FaExp*2, exhibits a pattern of transcription similar to that of the tomato expansin, *LeExp*1, although the two genes are not closely related. *FaExp*2 has a 96 % amino acid identity to another expansin gene, *FaExp*7; yet, the two genes are expressed at very different times during fruit development (Harrison *et al.*, 2001). Similarly, pairwise comparisons of

the grapevine alcohol dehydrogenase cDNAs, *Adh*1, *Adh*2 and *Adh*3, showed that the three cDNAs displayed strong homology in the coding region, but were highly divergent in the 5' and 3' untranslated regions (Tesnière and Verriès, 2000). The three cDNAs were shown to be differently expressed during grape berry ripening.

Interesting observations made in recent years are:

1) that despite different respiratory and ethylene production behaviour, climacteric and non-climacteric fruits share common regulatory mechanisms. This statement is based on the observation that the upstream regions of two ripening-related genes from pepper (a non-climacteric fruit) are both strongly expressed in the transgenic tomato fruit (a climacteric fruit), though the corresponding genes either did not exist in tomato, or did exist, but were not induced. Moreover, the expression patterns of the transgenes in the tomato are similar to those observed in pepper fruit development (Kuntz *et al.*, 1998);

2) that the identification of ripening-related genes, which can be used as molecular markers in probing disease resistance and sugar accumulation (Kim *et al.*, 2002), possibly infer that ripening-related genes are wound-inducible and regulated by increased sugar content;

3) that compared to some plant promoters which only require only about 200 to 300 bp for activity (Kuhlemeier *et al.*, 1987; Czarnecka *et al.*, 1989; Mitra and An, 1989; Matzke *et al.*, 1990) it appears as if developmentally regulated promoters, and particularly fruit-specific promoters, need at least 1.4 kb of 5' flanking region for full promoter activity (Bird *et al.*, 1988; Deikman *et al.*, 1988; Giovannoni *et al.*, 1989; Van Haaren and Houck, 1991; Van Haaren and Houck, 1993; Montgomery *et al.*, 1993).

2.4 FUNCTIONAL ANALYSIS OF PROMOTER ACTIVITY OF THE 5' UTR FLANKING REGIONS OF GENES EXPRESSED DURING FRUIT RIPENING

2.4.1 Analysis in Stably Transformed plants and by Transient Expression

Ideally, functional analysis of fruit-specific promoters (i.e. promoters of fruit-specific genes) should be performed in transgenic fruit of the crop in which the promoter will be utilised to regulate transgene expression. However, for most fruit crops currently used for the studying of ripening-related gene expression (such as apple, kiwifruit, melon and grapevine) functional analysis of such promoters is hampered by either 1) low genetic transformation efficiency and slow regeneration, or 2) a long reproductive cycle in apple, grapevine and kiwifruit (Lin *et al.*, 1993; Khachatourians *et*

al., 2002; Mezzetti *et al*., 2002; Galperin *et al*., 2003). Consequently heterologous systems, such as tomato and tobacco, have been widely used for the functional analysis of promoters of ripening-related genes (Table 2.2). Tomato and tobacco are regarded as model systems for plant genetic transformation and also have the advantage of a short reproductive cycle. Additional to the use of transgenic plants for expression analysis of transgenes, yeast and *Xenopus laevis* oocytes have recently been shown useful for the characterisation of three grapevine genes *viz. VvSuc*1 (Ageorges *et al*., 2000), *SIRK* (Pratelli *et al*., 2002) and two plasma membrane aquaporins (Picaud *et al*., 2003). However, no indication as to whether these systems would be useful for other applications, such as analysis of fruit-specific promoters, could be found.

In tomato, analysis of most of the fruit-specific and/or ripening-related promoters was performed in stably transformed tomato or tobacco plants (Table 2.2). Two studies on transient expression of promoter-reporter gene constructs by particle bombardment of the tomato fruit pericarp were reported (Montgomery *et al*., 1993; and Coupe and Deikman, 1997). In the study by Montgomery *et al*. (1993), the *E4*-reporter gene constructs were expressed in both transgenic plants and in a transient gene expression system. It was shown that transcription of the promoter-reporter genes in the transient gene expression system was qualitatively similar to that observed with equivalent promoter-reporter chimeric genes in transgenic tomato plants. Moreover, it was shown in both these studies that the transient expression system could be used to verify ripening-related transcription of the *E4* promoter.
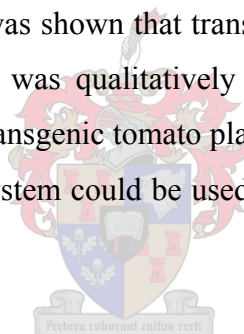
Transient assays by particle bombardment have been proven to be a powerful means for the evaluation of transgene expression in intact plant cells (Klein *et al*., 1988; Birch and Franks, 1991; Huang *et al*., 1998; Muhitch and Shatters, 1998; Nandadeva *et al*., 1999; Menossi *et al*., 2000). Although initially limited by the high cost and complexity of devices, particle gun technology was made more accessible by the development and partial optimisation of a simple and inexpensive particle bombardment device, The Particle Inflow Gun (PIG) (Finer *et al*., 1992). However, successful application of the technology appears to be limited to vegetative tissues and to fruits of which the anatomy allows preparation of sections with intact pericarp fruit cells, such as tomato (Montgomery *et al*., 1993; Coupe and Deikman, 1997). To study transient gene expression in ripe fleshy fruits, a method based on the injection of an *Agrobacterium* suspension into fruits was reported by Spolaore *et al*. (2001).

Table 2.2. Systems used for functional analysis of promoters shown to be transcriptionally active in fruit tissue.

Crop from which promoter was isolated	Promoter	Reporter gene used	Transgenic tissue in which expression of the promoter-reporter gene fusion was studied	Reference
Apple	ACC-oxidase and polygalacturonase	*uid*A	Tomato	Atkinson *et al*., 1998
	*Ypr*10	*uid*A	Tobacco	Püringer *et al*., 2000
Grapevine	*dfr*	*uid*A	Red cell suspensions derived from red fruits & grapevine plantlets and plants	Gollop *et al*., 2002
	ldox	*uid*A	Red fruit cell suspension derived from red fruits & grapevine	Gollop *et al*., 2001
	SIRK	*uid*A	Grapevine & *Arabidopsis*	Pratelli *et al*., 2002
	*Vst*I	*uid*A	Tobacco	Schubert *et al*., 1997
Green pepper	*ccs* and *fib*	*uid*A	Tomato	Kuntz *et al*., 1998
Melon	ACC-oxidase (*CM-ACO*1 & *CM-ACO*3)	*uid*A	Tobacco	Lasserre *et al*., 1997
Kiwifruit	actinidin	*uid*A	Tobacco	Lin *et al*., 1993
Tomato	2A11	*uid*A	Tomato	Van Haaren and Houck, 1991
	E4	*uid*A; LUS	Tomato	Montgomery *et al*., 1993
	E8	LUS	Tomato	Coupe and Deikman, 1997
	ACC-oxidase	*uid*A	Tobacco and Tomato	Blume and Grierson, 1997
	Polygalacturonase	CAT	Tomato	Bird *et al*., 1988

In grapevine, studies on the functional analysis of promoters were performed in cell suspensions derived from grape berries and/or stably transformed grapevine, tobacco or *Arabidopsis* plants (Table 2.2). Since none of these promoters were fruit-specific, expression of the promoter-reporter gene could be monitored in the vegetative tissues of transgenic plants. Transient expression assays in somatic embryos transformed by incubation in A*grobacterium* suspension, were reported by Li *et al*. (2001). Transient expression of the promoter-reporter gene fusion was evident two days after transformation.

Despite the relatively high transformation efficiency, rapid regeneration and short reproductive cycle of strawberries, functional analysis of ripening-related or fruit-specific promoters in transgenic strawberry is not common. The strawberry fruit is regarded as a false fruit, due to its non-ovarian origin. The achenes, which contain the pericarp tissue, are the true strawberry fruits and are

therefore the sites at which expression of fruit-specific genes can be expected. In their study, Matthews *et al*. (1995) studied the tomato fruit-specific *E4* promoter in strawberry transformation experiments, but were unable to demonstrate fruit-specific expression in transgenic strawberries. Strawberry did however prove to be a suitable system to study the tissue-specific regulation of the petunia *FBP*7 (floral binding protein7) promoter (Schaart *et al*., 2002). Activity of the promoter, which showed a high activity in the receptacles of petunia flowers, was shown to be specific to the floral and fruit tissues of transgenic strawberries. Bearing in mind that strawberry is in fact an outgrown receptacle, it was concluded that the tissue-specific regulation of the *FBP*7 promoter was conserved in transgenic strawberry fruit.

2.4.2   Reporter Genes used for Promoter-Reporter Gene Fusions

To date, most of the studies on the functional analysis of ripening-related or fruit-specific promoters were conducted using the β-glucoronidase (GUS)-encoding *uidA* gene as reporter gene (Table 2.2). Besides GUS, only two other reporter genes were used for expression analysis in stably transformed plants *viz*. the chloramphenicol acetyl transferase (*CAT*) reporter gene used by Bird *et al*. (1988) and the luciferase (LUS) reporter gene (Montgomery *et al*., 1993; Coupe and Deikman, 1997).

In 2001, Li *et al*. reported the use of the EGFP (enhanced green fluorescent protein) gene as reporter gene for transient expression assays in grapevine. The green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*, emits bright green fluorescence upon excitation with ultraviolet light, offering the advantage that detection requires no additional substrates and no invasive procedures. Transgene expression can thus be continuously monitored in a non-destructive manner. GFP has proven extremely useful as a reporter of plant gene expression in living cells and has since become a standard reporter in many biological systems (Sullivan and Kay, 1998; Abelson and Simon, 1999; Elliot *et al*., 1999; Garabagi and Strommer, 2000; Mankin and Thompson, 2001). The use of GFP for promoter-reporter gene fusions in the analysis of ripening-related promoters in transgenic fruit, should prove very useful.

2.5 CONCLUSION

The grape berry is one of the main targets for the genetic manipulation, as fruit quality is a prime determinant for the production of good wine. The availability of promoters to regulate transgene transcription in a fruit-specific and ripening-related manner, remains one of the main obstacles to the achievement of this goal. The isolation of such promoters is dependent on the identification of genes which are expressed in a fruit-specific and ripening-related manner. Although numerous ripening-related genes have been cloned from tomato and other fleshy fruits in recent years, only a

few genes of which the transcription is fruit-specific and corresponds with the onset of ripening, have been reported. Amongst these, the promoter region of only the tomato *E4* and *E8* genes have been isolated and characterised.

To study ripening-related gene transcription and identify differentially transcribed genes, a variety of technologies ranging from library screening, differential display and microarray technology, has been successfully applied. When compared to the other techniques, cDNA-AFLP technology offers several advantages *viz.* high through-put, high reproducibility, relative simplicity and minimal amounts of total RNA being used.

Due to low transformation efficiency and poor regeneration of most fruit crops, functional analysis of these promoters has been mainly performed in heterologous systems. Most of these promoters were not fruit-specific; therefore analysis of promoter-reporter gene expression could be evaluated in vegetative tissues. Transient transgene expression in the pericarp of tomato was used as a basis to evaluate the functionality of the tomato *E4* and *E8* promoters. This represents the only study to date where fruit tissues have been used for transient gene expression analyses. The only fruit specific promoter that has been analysed in stably transformed plants is the tomato *E4* promoter in tomato tissue.

Although not much used to date, the GFP reporter gene seems suitable for expression analysis of promoter-reporter gene expression; a good alternative to the much used *uidA* reporter gene.

More generally, cloning and characterisation of a fruit-specific, ripening-related promoter from grape berries will contribute to the current understanding of grape- and non-climacteric fruit ripening, and ultimately towards the final elucidation of the nature of the signal that initiates ripening in grape berries. More importantly, it will offer the possibility to regulate transgene transcription and overcome one of the hurdles in the genetic manipulation of fruit metabolism.

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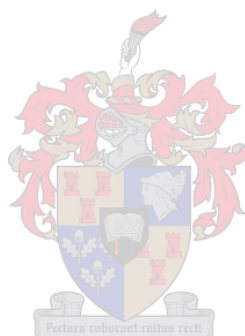
CHAPTER 3

Ripening-related gene transcription during fruit ripening in
Vitis vinifera L. cvs. Cabernet Sauvignon and Clairette blanche.

Research Results

Ripening-related gene transcription during fruit ripening in *Vitis vinifera* L. cvs. Cabernet Sauvignon and Clairette blanche.

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3.1 ABSTRACT

The gene transcription patterns in the ripening fruits of a good quality wine cultivar (Cabernet Sauvignon) and a poor quality wine cultivar (Clairette blanche) were studied using cDNA-AFLP fingerprinting. Total RNA from immature (14-weeks post flowering) and mature (18-weeks post flowering) berries was used to study ripening-related gene transcription in the post-*véraison* stages of berry ripening. A total of 1 276 fragments were analysed, of which 175 appeared to be ripening related. Average pairwise difference of the fragments amplified from immature and mature Clairette and Cabernet berries, revealed a high level of similarity between the two cultivars. Seventy percent of the ripening-related fragments were cultivar-specific. The number of cultivar specific and/ or ripening-related fragments amplified, depended on the selective nucleotides of the primers used in the cDNA-AFLP analysis. Reverse slot blot and Northern blot analysis confirmed that the transcription of the identified genes were ripening-related.

Key words:
Grapevine, ripening-related; gene transcription; cDNA-AFLP, fruit ripening; fruit quality

3.2 INTRODUCTION

Grape berry ripening is characterised by dramatic changes in characteristics that determine the quality of the final product (Hawker 1969a and b; Ruffner, 1982; Kanellis and Roubelakis-Angelakis, 1993). In climacteric fruit - such as tomato - genes involved in these biochemical and physiological changes, whose expression can possibly be manipulated to improve fruit quality, has been extensively studied (Aharoni *et al*., 2002; Moore *et al*., 2002; White, 2002). On the contrary, little is known about the ripening-related expression of genes in non-climateric fruit - such as grapevine and strawberry. In grapevine, a number of ripening-related genes were identified by the characterisation of candidate genes (Boss *et al*., 1996; Tattersall *et al*., 1997; Davies *et al*., 1999; Nunan *et al*., 2001; Terrier *et al*., 2001b), but often unexpected pathways were also activated during berry ripening (Tesnière and Verriès, 2000). To date however, most ripening-related genes reported in grapevine have been identified through sequence analysis and hybridisation-based differential screening of cDNA libraries (Davies and Robinson, 2000; Ablet *et al*., 2000; Terrier *et al*., 2001a) or the gel-based RNA fingerprinting technique cDNA-AFLPs (Venter *et al*., 2001).

In an attempt to identify genes involved in the developmental processes related to berry quality, ripening-related gene transcription in two cultivars with different wine making qualities, was studied. Cabernet Sauvignon - the world's most renowned grape variety for the production of fine red wine, and Clairette blanche - a decidedly old-fashioned variety known for the production of flabby white wine, was selected for the analysis. Both cultivars were grown at the ARC-Nietvoorbij grapevine collection block in Stellenbosch. Furthermore, both cultivars flower late (3rd week and 4th week of October) and mature late in the season (2nd and 3rd week of March), minimising the potential effect of different environmental factors on grape berry ripening and ripening-related gene transcription. Given that the most dramatic changes in the characteristics that determine the quality of the final product, occurs as the fruit enters into the ripening phase, this study focused on ripening-related gene transcription in the post-*véraison* stages of ripening. The term "post-*véraison*" refers to the stages of ripening following the change in berry skin colour, and the onset of sugar accumulation and berry softening.

Gene transcription in the ripening berries was studied using cDNA-AFLP fingerprinting. Amplified Fragment Length Polymorphism (AFLP) is a powerful technique for the fingerprinting of genomic (Vos *et al*., 1995) and complementary DNA (cDNA) (Money *et al*., 1996; Bachem *et al*., 1996; Habu *et al*., 1997). The technique represents an ingenious combination of restriction fragment length polymorphism (RFLP) analysis and polymerase chain reaction (PCR), resulting in highly informative fingerprints. cDNA-AFLP analysis consists of four steps: (1) synthesis of cDNA, (2)

production of primary template by restriction digestion of cDNA with two restriction enzymes and ligation of adaptors to the termini of these cDNA fragments, (3) pre-amplification with primers corresponding to the adaptors ligated to the cDNA fragments, and (4) selective amplification of the cDNA fragments with primers extended with one or more specific bases. A fingerprint is produced by radioactive labeling of one of the primers used for selective amplification, polyacrylamide gel electrophoresis of the amplification product and visualisation of the amplification products by autoradiography (exposure to X-ray film). A step-by-step protocol is present by Bachem *et al*. (1998).

Considerations for the cDNA-AFLP approach included 1) the extensive use of AFLPs to identify differentially-regulated genes in plants and other organisms (Breyne and Zabeau, 2001; Donson *et al*., 2002); 2) the observations that members of multigenic families often exhibit distinct developmental patterns during berry ripening (Davies *et* al., 1999; Fillion *et al*., 1999; Tesnière and Verriès, 2000), which emphasises the utilisation of sequence-based analysis for the unambiguous characterisation of these isogenes and, 3) that contrary to hybridisation-based approaches, sequence-based approaches are not biased towards abundant transcripts (Breyne and Zabeau, 2001). In sequence-based approaches, identification of mRNAs is not limited by redundancy of highly expressed mRNAs or under-representation of rare mRNAs in a cDNA library. It is estimated that the 105 000 ESTs in the *Arabidopsis thaliana* collection is representative of only 60 % of all the genes, illustrating the extent to which cDNA libraries fail to represent all mRNAs (Richmond and Sommerville 2000). More recently, Kuhn (2001) reported that only 1.4 to 5 % of the 1443 *Arabidopsis* genes analysed in cDNA microarrays represented highly expressed genes with abundance of more than 100 to 500 transcripts per cell. Most members in this class were well-characterised housekeeping or tissue-specific genes. The majority of the expressed genes were low abundance with levels of less than 10 to 50 transcripts per cell (Ruan *et al*., 1998). Many important regulatory genes can thus be overlooked by hybridisation-base approaches as abundant messages are over-expressed in cDNA libraries and rarely expressed genes are often missing.

Here we illustrate the usefulness of cDNA-AFLPs for the characterisation of ripening-related gene transcription during grape berry ripening. In addition, the results suggest that gene transcription in ripening berries of the two cultivars Cabernet Sauvignon and Clairette blanche, is remarkably similar. Nevertheless, obvious differences in ripening-related gene transcription of the two cultivars were identified.

3.3 RESULTS

3.3.1 Total RNA and mRNA isolation

The average total RNA yield from ripening Clairette and Cabernet berries was 25±5 and 8±2 μg.g^{-1} fresh weight for "immature" (14-wpf) and "mature" (18-wpf) berries respectively. In both immature and mature berries the poly(A)$^+$ mRNA comprised 0.85 % of the total RNA.

3.3.2 cDNA-AFLP analysis

Approximately fifty discrete fragments, ranging from 80 to 600 base pairs, were visualised for each of the 25 *Mse*I and *Pst*I primer combinations used (Table 3.1). A total of 1 276 fragments were visualised. The high number of fragments for primer combinations *Mse*I (+3) and *Pst*I (+0) made accurate scoring of the fragments difficult and these were therefore not included in further analysis. cDNA-AFLP fingerprints generated from the duplicate cDNA samples were identical. No amplification products could be visualised in the reactions using total RNA as template.

Table 3.1 Primer combinations used, and the number of fragments visualised by cDNA-AFLP analysis of ripening Clairette blanche and Cabernet Sauvignon berries. The number of differentially amplified fragments identified by the analyses is indicated. *Mse*I primers used for selective amplification included *Mse*I (+CAA), (+CAC), (+CAG), (+CAT) and (+CTG).

	Number of cDNA fragments visualised (average number of fragments per primer combination)	Number of differentially amplified cDNA fragments (expressed as a percentage of the total number of differentially amplified fragments)
*Mse*I (+3) + *Pst*I (+ 0)	357 (71)	0[#]
*Mse*I (+3) + *Pst*I (+ C)	315 (63)	24 (7,6 %)
*Mse*I (+3) + *Pst*I (+ G)	226 (45)	67 (29,6 %)
*Mse*I (+3) + *Pst*I (+ GT)	198 (40)	46 (23,2 %)
*Mse*I (+3) + *Pst*I (+ GTA)	180 (36)	38 (21,1 %)
Total	**1 276**	**175**

[#]The high number of fragments visualised per gel impeded on the accurate scoring of the fragments, and these fragments were omitted from further analysis.

Average pairwise differences revealed a 16 % difference in the fragments amplified from mature and immature Clairette berries, and 19 % in the case of Cabernet, respectively (Fig. 3.1). The average pairwise difference between the two cultivars was 35 %.

One hundred and seventy five ripening-related fragments were identified (Table 3.1 and 3.2). Only 52 of these (30 %) were similarly expressed in Clairette and Cabernet, of which 60 % were more abundant in the mature berries (Table 3.2, printed in italics). Most of the remaining 123 fragments

were cultivar specific and up-or down-regulated during fruit ripening (Table 3.2, indicated by the arrowheads). To determine whether ripening-related fragments were in general up- or down-regulated during berry ripening, the score of "1" was multiplied by the "number of fragments" (Table 2, indicated by the totals in parenthesis). It was shown that in Clairette, most of the ripening-related fragments were up-regulated during berry ripening. In Cabernet, however, most of the ripening-related fragments were down-regulated.
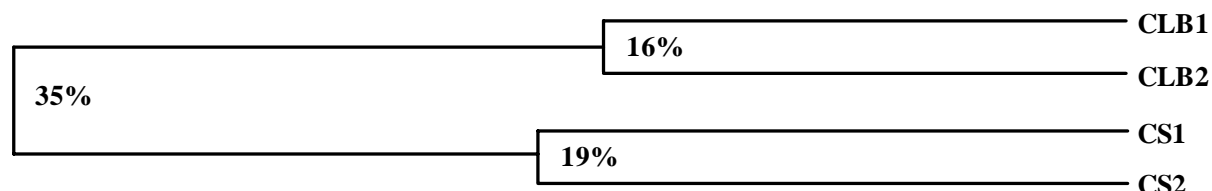
Figure 3.1 UPGMA tree of the average pairwise difference between cDNA fragments amplified from immature and mature Clairette blanche and Cabernet Sauvignon berries. CLB1 and CLB2: Clairette blance immature and mature berries, respectively; CS1 and CS2: Cabernet Sauvignon immature and mature berries, respectively. Average pairwise difference is expressed as percentage of the total number of cDNA fragments scored.

Table 3.2 The number of differentially amplified fragments identified by cDNA-AFLP analysis, and their expression in ripening Clairette blanche and Cabernet Sauvignon berries. CLB1 and CLB2: "immature" and "mature" Clairette berries: CS1 and CS2: "immature" and "mature" Cabernet berries. Presence or absence is indicated by the "1" or "0". Fragments similarly regulated in both cultivars are printed in *italics*. Arrowheads indicate fragments not present in the one cultivar, but ripening-related in the other. The number in parenthesis was calculated by multiplying the score of "1" by the "number of fragments". This was only performed in cultivars where the fragment(s) were ripening-related.

Number of fragments	Comment	Clairette blanche		Cabernet Sauvignon	
		CLB1	CLB2	CS1	CS2
21	*Down-regulated in both cultivars*	*1 (21)*	*0*	*1 (21)*	*0*
31	*Up-regulated in both cultivars*	*0*	*1 (31)*	*0*	*1 (31)*
➤ 20	Not present in Clairette, up-regulated in Cabernet	0	0	0	1 (20)
➤ 23	Not present in Clairette, down-regulated in Cabernet	0	0	1 (23)	0
6	Constitutive in Clairette, up-regulated in Cabernet	1	1	0	1 (6)
19	Constitutive in Clairette, down-regulated in Cabernet	1	1	1 (19)	0
➤ 16	Not present in Cabernet, up-regulated in Clairette	0	1 (16)	0	0
➤ 27	Not present in Cabernet, down-regulated in Clairette	1 (27)	0	0	0
10	Constitutive in Cabernet, up-regulated in Clairette	0	1 (10)	1	1
2	Up-regulated in Clairette; down-regulated in Cabernet	0	1 (2)	1 (2)	0
175	← **Totals** →	**(48)**	**(59)**	**(65)**	**(57)**

Primer combination effected the number of differentially amplified fragments that could be identified (Table 3.1). Most of the differentially amplified fragments were identified using the primer combinations *Mse*I (+3) and *Pst*I (+G), while the primer combinations *Mse*I (+3) and *Pst*I (+C) yielded the lowest number of differentially amplified fragments. The higher the number of selective nucleotides, the lower the number (and percentage) of differentially amplified fragments identified.

Some primer combinations resulted in the visualisation of a larger number of ripening-related fragments, while others amplified a large number of cultivar-specific fragments. For instance, the use of primer combinations *Pst*I (+C) and *Mse*I (+CAA; +CAC; +CAG; +CAT) did not result in the visualization of a large number of ripening-related fragments, but instead a large number of cultivar-specific fragments. The use of primer combinations *Pst*I (+G and +GTA) and *Mse*I (+CAC; +CAG; +CAT) resulted in the visualisation of the highest number of ripening-related fragments in Clairette and Cabernet (Fig. 3.2).
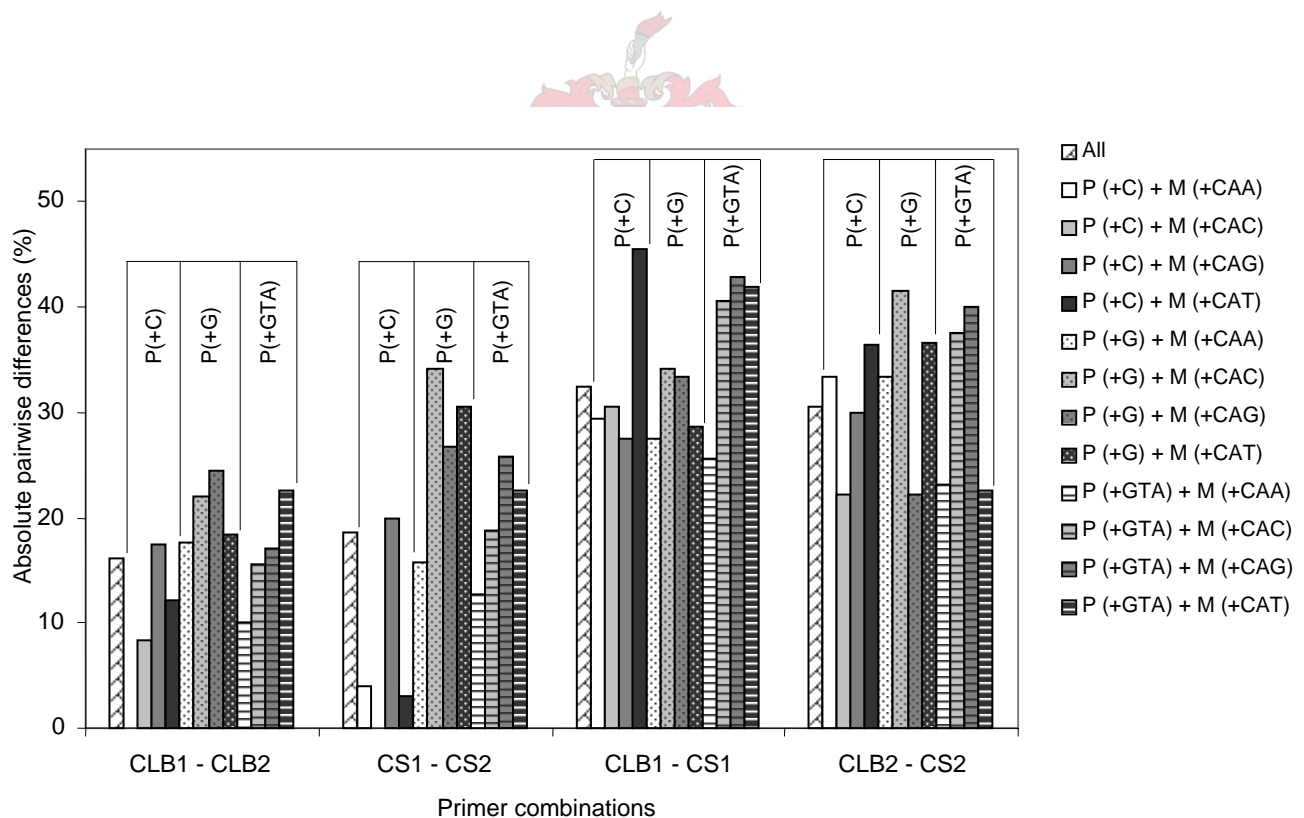
Figure 3.2 Absolute pairwise difference of fragments amplified from cDNA isolated from ripening Clairette blanche (CLB1-CLB2) and Cabernet Sauvignon (CS1-CS2) berries. The twelve primer combinations used in the selective amplification procedure include *Pst*I (+C), (+G), and (+GTA) and *Mse*I with three selective nucleotides (+CAA), (+CAC), (+CAG) and (+CAT). Differences between fragments amplified from cDNA isolated from immature and mature berries are indicated by CLB1-CLB2 (Clairette blanche) and CS1-CS2 (Cabernet Sauvignon), while differences between the two cultivars are indicated by CLB1-CS1 and CLB2-CS2. Absolute pairwise difference is expressed as a percentage of the total number of cDNA fragments scored.

3.3.3 Transcription analysis of ripening-related cDNA fragments

All fragments, except for fragments selectively amplified using the primer *Mse*I (+CAT), excised from dehydrated gels could be re-amplified.

Reverse slot blot analysis

A subset of 23 fragments excised from the dehydrated gels was subjected to reverse slot blot analysis. To compensate for the background signal produced by the negative control, only sequences with stronger hybridisation signals were considered for further analysis. Of these, 15 of the Clairette cDNA sequences were shown to be differentially transcribed during berry ripening (Fig. 3.3A). All 15 were characterised by high levels of transcription in the immature berries. Five of the sequences appeared to be constitutively expressed (Fig. 3.3A). None of these fragments were expressed in leaf tissue.

Northern blot analysis

Differentially expressed cDNA fragments were subjected to Northern blot analysis to verify their ripening-related transcription. Five fragments (C2, D1, D3, D9.2 and G2) were characterised by higher levels of the transcript in immature berries, while no mRNA could be detected in leaf tissue (Fig. 3.3B). The size of the five transcripts ranged from 1 to 1.5 kb.
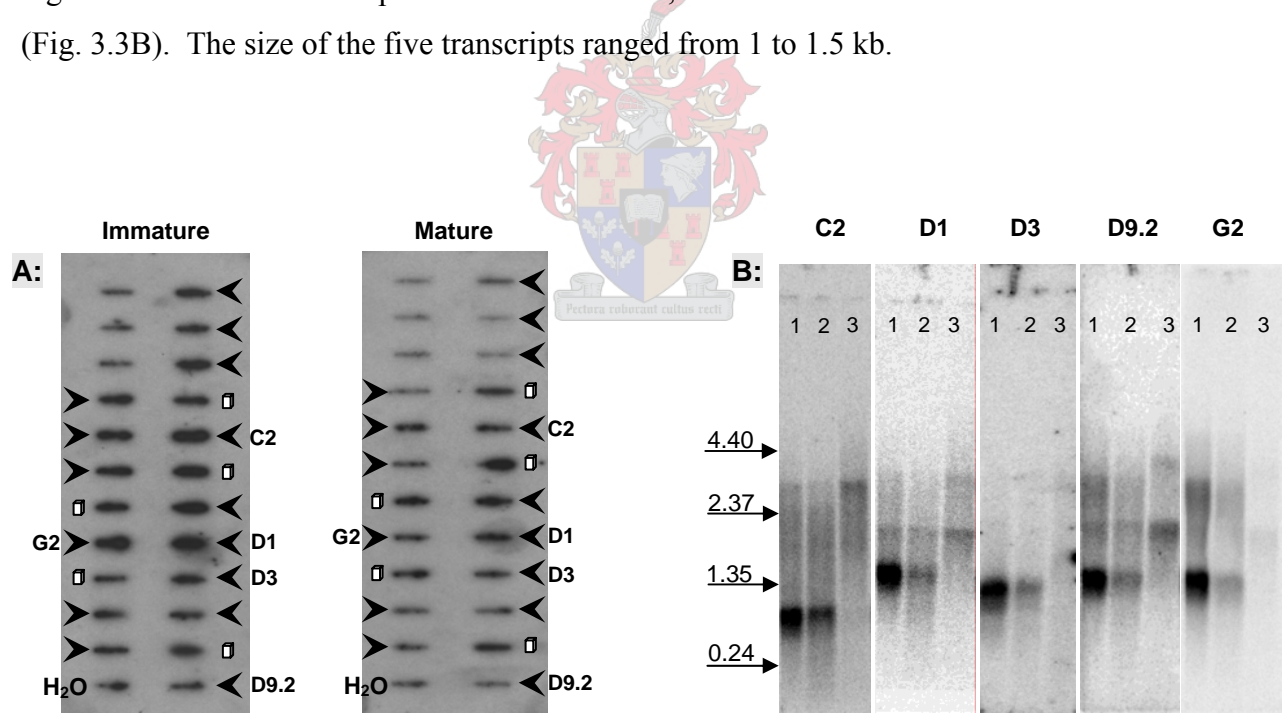
Figure 3.3 Transcription analysis of fragments differentially amplified from immature and mature Clairette blanche berries by cDNA-AFLP analysis. **A**: Reverse slot blot analysis was performed by probing duplicate membranes with cDNA probes synthesised from total RNA from immature and mature berries. The negative control (filtered water) is shown in the left, bottom well. Fragments differentially transcribed during berry ripening are indicated by the arrowheads, while fragments which appear to be constitutively expressed, are indicated by the blocks. The five fragments identified for further analysis (C2; D1; D3; D9.2 and G2) are indicated. **B**: Ripening-related transcription of the five fragments C2; D1; D3; D9.2 and G2. Lanes 1-3: mRNA from immature (lane 1), mature berries (lane 2) and leaf tissue (lane 3). Molecular size (kb) is indicated by the arrows.

3.4 DISCUSSION

The method of cDNA-AFLP analysis allows the identification of differentially expressed transcripts. In this study, AFLP analysis of cDNA from 14- and 18-weeks post flowering (wpf) Clairette blanche and Cabernet Sauvignon berries led to the identification of several transcripts differentially expressed during the post-*véraison* stages of berry ripening. Differences in the mRNA fingerprints produced from 14-wpf ("immature") and 18-wpf ("mature") berries, were referred to as "ripening-related gene transcription".

Complementary DNA was synthesised directly from total RNA, circumventing the isolation of mRNA as previously described (Money *et al*., 1996; Bachem *et al*., 1996; Habu *et al*., 1997). Considering that mRNA comprised 0.85 % of the total RNA isolated from ripening berries, and that first- and second-strand cDNA synthesis has been shown respectively about 12 to 50 %, and 80 % effective (Universal Riboclone® cDNA synthesis system Technical Manual), it implies that cDNA-AFLP synthesis was performed using 5.1 to 17 ng of double-stranded cDNA as template. No amplification products could be visualised in reactions using total RNA as template, indicating that the RNA samples were free of genomic DNA.

Amplification using the primer combinations *Mse*I (+3) and *Pst*I (+0) resulted in the visualisation a number of fragments, much higher than reported for wheat (Money *et* al., 1996). In fact, in this study it was shown that amplification using primer combinations with *Pst*I (+GTA) resulted in the visualisation of an average of 36 fragments per gel. If considering that first-strand cDNA synthesis was primed using the anchored primer VT$_{12}$GACGTCTGA, amplification using a *Pst*I primer with selective nucleotides other than a "T" would discriminate against cDNA fragments only containing the incorporated *Pst*I recognition site. It thus seems as if grapevine has a relative high proportion of cDNAs with *Pst*I sites and that dependence upon the presence of this 6-bp recognition site, is not a limitation in cDNA-AFLP analysis of grapevine.

This study confirmed the usefulness of cDNA-AFLPs to study grapevine ripening-related gene transcription, and for the identification of genes differentially transcribed during grape berry ripening. Considering that the two cultivars, Cabernet Sauvignon and Clairette blanche differ in many other aspects besides berry and wine-making qualities, it was surprising to find that gene transcription during berry ripening in the two cultivars is remarkably similar. Nevertheless, obvious differences in the ripening-related gene transcription of the two cultivars were identified. It is these

differences that should be targeted to identify genes related to the phenotypical differences between the two cultivars, and to identify genes possibly involved berry quality.

cDNA-AFLPs is a sequence-based approach; therefore the differences in ripening-related gene transcription of the two cultivars will also reflect single nucleotide polymorphisms (SNPs). Although some SNPs may only be related to genetic distance between the two cultivars, it may also be indicative of different members of a multigene family. In grapevine, members of multigenic families often exhibit distinct patterns of regulation during berry ripening (Davies et al., 1999; Fillion et al., 1999; Tesniere and Vierres, 2000), which emphasises the utilisation of a sequence-based approach for the unambiguous characterisation of these isogenes. Further characterisation of differentially-amplified sequences by sequence- and hybridisation-based analysis in both cultivars, will clarify the issue.

Cloning and sequence analysis of differentially expressed fragments were not within the objectives of the study. The five ripening-related cDNA fragments shown in Figure 3.3B are however considered ideal candidates for further characterisation and possibly the isolation of fruit-specific, ripening-related genes. Cloning and sequence analysis of the five fragments will possibly lead to the identification of the corresponding genes and their function during grape berry ripening.
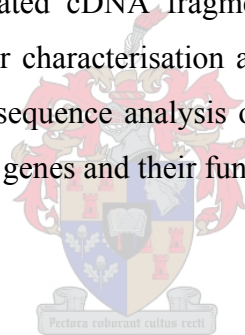
3.5 MATERIALS AND METHODS

3.5.1 Plant Material:

Ripening Cabernet Sauvignon and Clairette blanche berries were collected from the ARC-Nietvoorbij collection block in Stellenbosch. "Immature" berries were collected 14-weeks post flowering (14-wpf), which was one week post-*véraison*. The dark red colour of the Cabernet Sauvignon berries and the yellowish-green colour of the 14-wpf Clairette blanche berries, was consistent with the post-*véraison* stage of berry ripening, which is characterised by the loss of chlorophyll from the skin and the accumulation of anthocyanins. "Mature" berries were collected 18-wpf, just prior to commercial harvest (sugar content: Clairette blanche: 21.4 degree Balling; Cabernet Sauvignon, 22.8 degree Balling). Berries were deseeded, frozen in liquid N_2 and stored at –80 °C until further use.

3.5.2 Methods:

3.5.2.1 Total RNA isolation and cDNA synthesis

Total RNA was isolated from ripening berries and leaf tissue using a modified sodium-perchlorate method (Venter *et al.*, 2001). Total RNA, 5 μg from each sample, was *DNase*I treated using the MessageClean kit (GenHunter Corporation) and subjected to first strand synthesis using an oligonucleotide 5'-AGTCTGCAGT$_{12}$V-3', where V denotes A, C or G (Money *et al.*, 1996). Second strand synthesis was performed using the Universal Riboclone® cDNA synthesis system (Promega Corporation). Duplicate cDNA samples were prepared from RNA obtained from independent RNA isolations.

3.5.2.2 cDNA-AFLP analysis

cDNA-AFLP analysis was performed according to Venter *et al.* (2001). Twenty-five combinations of *Pst*I and *Mse*I primers, containing up to three selective nucleotides each, was used for selective amplification (Table 1). To confirm that total RNA samples were free of genomic DNA, AFLP analysis was performed using an aliquot of the total RNA samples as template. To verify the reproducibility of the technique, duplicate cDNA samples were subjected to AFLP analysis using three primer combinations viz. *Mse*I (+CAA) and *Pst*I (+0); *Mse*I (+CAT) and *Pst*I (+C); *Mse*I (+CAC) and *Pst*I (+GTA).

Following visualisation of the amplification products, the amplified cDNA fragments were scored manually, "present" or "absent". Absolute pairwise differences of the cDNAs amplified were determined using PAUP version 4.0b10 for Macintosh. Differentially amplified fragments were

excised from the dehydrated polyacrylamide gels and re-amplified as described in Venter *et al.* (2001).

3.5.2.3 Reverse slot blot analysis

A 24 well slot blot manifold was used for this purpose (Sigma-Aldrich Chemie). Preparation and application of the cDNA samples (5 µl of each re-amplification product) to the positively charged nylon membrane (Roche Diagnostics Mannheim) were performed according to Ausubel *et al.* (1992). Filtered water (0.22 µm Cameo 25AS cellulose acetate syringe filter, Osmonics) was applied to the membrane as negative control. Two identical membranes were prepared for each of the cultivars.

Single strand cDNA probes were synthesized by reverse transcription of 5 µg total RNA from each sample. Reverse transcription was performed using an anchored oligo-dT primer and Superscript^{TM} II (Invitrogen Life Technologies). Procedures were according to the protocol supplied with the reverse transcriptase, except for the replacement of the dNTP solution with dNTPs (-dCTP), final concentration 75 µM, and the addition of 50 µCi [α-^{32}P] dCTP (3000 Ci.mmol^{-1}) (Amersham Pharmacia Biotech). The reaction volume was adjusted to 30 µl. Hybridisation was performed using cDNA probes of equal specific activity (1 x 10^7 cpm.µg^{-1} DNA) and Rapid-hyb buffer (Amersham Pharmacia Biotech). Procedures for hybridisation and stringency washes were according to the manufacturer's instructions. Hybridisation was visualised by autoradiography (Kodak BioMax MS film, intensifying screens, room temperature, overnight). Differences in hybridisation intensities were visually determined. Visual differences were considered significant when the hybridisation signal was (1) clearly stronger than the background signal produced by the negative control, and (2) clearly stronger than the hybridisation signal in the sample to which it was compared.

The membranes were stripped using boiling 0.1 % (w/v) SDS before probed with cDNA from leaf material.

3.5.2.4 Messenger RNA isolation

Messenger RNA was isolated from Clairette blanche berries and leaf material from total RNA using PolyATtract® mRNA isolation systems (Promega Corporation) and quantified fluorometrically using the Ribogreen^{TM} RNA Quantitation kit (Molecular Probes) and the Bio-TEK FL600 Microplate Fluorescence reader (BIO-TEK® Instruments Inc.).

3.5.2.5 Northern blot analysis

The re-amplified, uncloned fragments were radioactively labelled as described by Venter *et al.* (2001). Messenger RNA (50 ng per lane) was denatured, size fractionated (1.2 % agarose, 3 V/cm; 2 h) and transferred to positively charged nylon membrane (Roche Diagnostics Mannheim) by alkaline downward capillary blotting (Ingelbrecht *et al.*, 1998). Duplicate membranes were prepared to accommodate analysis of all the selected cDNA fragments simultaneously. RNA was cross-linked to the membrane (2.5 min at 120 mJcm^{-1}) using an ultraviolet crosslinker (ULTRA.LUM, Scientific Associates). The membranes were left to dry before subjected to hybridisation procedures in a 50 % formamide-containing hybridisation buffer (ULTRAhyb™ Ultrasensitive Hybridisation buffer, Ambion) at 42 °C. Stringency washes were performed according to the manufacturer's recommendations: 2 x 10 min in 2xSSC + 0.1 % (w/v) SDS at 42 °C, and 2 x 15 min in 0.1xSSC + 0.1 % (w/v) SDS at 42 °C. Hybridisation was visualised by phosphor-imaging using a Cyclone™ Phosphorimager (Packard Instrument Co.) and an AlphaImager™2000 documentation and analysis system (Alpha Innotech Corporation). Differences in hybridisation intensities were visually determined, and regarded as significant as stated for reverse slot blot analysis.

3.6 ACKNOWLEDGEMENTS
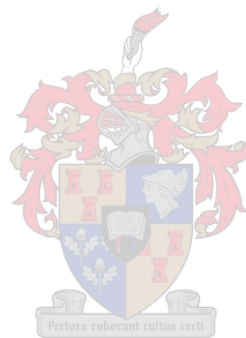
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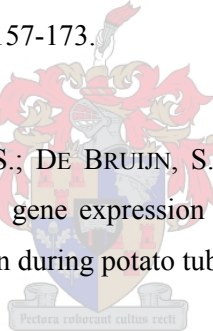
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CHAPTER 4

Cloning of a specific ripening-related gene from the multiple of ripening-related genes identified from a single band excised from a cDNA-AFLP gel.

Research Results

Cloning of a specific ripening-related gene from the multiple of ripening-related genes identified from a single band excised from a cDNA-AFLP gel.

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4.1 ABSTRACT

High-throughput methods such as amplified fragment length polymorphism (AFLP) analysis of complementary DNA (cDNA) and cDNA microarrays are useful for identifying ripening-related cDNAs from grapevine. Here we describe the identification and cloning of a gene that is transcriptionally activated at the onset of grape berry ripening. In addition, we describe the presence of multiple ripening-related genes in a single band excised from a cDNA-AFLP gel and the additional steps implemented to identify a specific ripening-related gene. In total, seven cDNAs were identified in the band excised from the cDNA-AFLP gel. All seven cDNAs were shown to be ripening regulated during berry development, though most were characterised by low levels of expression during berry ripening. These results highlight the limitation placed on the isolation of a specific sequence from a cDNA-AFLP gel and the steps that can be taken to overcome this limitation.

Key words: cDNA–AFLP analysis, differential amplification, fruit ripening, grapevine, ripening-related gene transcription

4.2 INTRODUCTION

Analysis of differential gene transcription is one of the cornerstones for modern molecular biology. It forms the basis for unravelling the control of plant growth and development, and allows for the identification of specific control points of metabolism. In addition, it is an important prerequisite for the identification of specific promoter elements, which in turn is invaluable in gaining insight into the regulation of gene transcription and the possibility to control gene transcription in many areas.

Techniques for isolating differentially expressed genes include the now classical approaches of differential and subtractive hybridisation (Sargent, 1987; Gray *et al.*, 1992; Woodhead *et al.*, 1998; Davies and Robinson, 2000), and the polymerase chain reaction (PCR)-based approaches for the selective amplification of complementary DNA (cDNA). The main advantage of PCR-based approaches is their ability to rapidly and simultaneously display mRNAs that are expressed in various eukaryotic cells or tissues, in different stages of development, or under altered conditions. Differential display reverse transcription PCR (DDRT-PCR), the first *in vitro* technique for the determination of transcript patterns, was developed in 1992 (Liang and Pardee, 1992). It has since been widely applied to identify and clone a large number of genes that are differentially expressed (Liang *et al.*, 1993; Aiello *et al.*, 1994; Liu and Raghothama, 1995; Oh *et al.*, 1995; Wilkinson *et al.*, 1995; Baldwin *et al.*, 1999). The technique however tends to give high rates of false positives (Debouck, 1995), primarily attributed to the presence of multiple DNA fragments in one particular band (Bauer *et al.*, 1993; Li *et al.*, 1994; McClelland *et al.*, 1995; Men and Gresshoff, 1998).

To counteract this high rate of false positives, several improved PCR-based methods have been described (Kawamoto *et al.*, 1999; Shimkets, *et al.*, 1999; Sutcliffe *et al.*, 2000). The method currently most widely used for the transcription analysis of multigene families, is a method developed by Fischer *et al.* (1995), who combined DDRT-PCR and amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995). This technique, cDNA-AFLP analysis, has in recent years been used extensively to identify differentially regulated genes in plants and other organisms (Bachem *et al.*, 1996; Breyne and Zabeau, 2001; Donson *et al.*, 2002), and has proven itself highly reproducible and more reliable than DDRT-PCR.

Here, a putative ripening-related cDNA fragment, designated C2, was differentially amplified from ripening grape berries by cDNA-AFLP analysis. It is shown that the single band excised from the cDNA-AFLP gel contains multiple ripening-related sequences. Steps for the identification of a specific ripening-regulated gene, are described.

4.3 MATERIALS AND METHODS

4.3.1 Plant Material:

The initial analysis was performed using *Vitis vinifera* L. cv. Clairette blanche berry and leaf material collected in January and February 1999 at the ARC-Nietvoorbij collection block, Stellenbosch. The berries were collected 14-weeks post flowering (wpf), which was one week post-*véraison*, and just prior to commercial harvest, 18-wpf. Ripening Merlot berries were collected from September 2000 to March 2001 at Grondves, an experimental farm of the KWV in Stellenbosch. These berries were collected 2-wpf and there after on a two-weekly basis until berries reached full maturity, 16-wpf. The samples were designated M1 to M8. Based on the change of the berry colour from yellow to red, *véraison* was 10-wpf. *Véraison* is defined as the onset of berry ripening, indicating the onset of berry softening and sugar accumulation. Berries were deseeded, frozen in liquid N_2 and stored at $-80\ °C$ until further used.

4.3.2 Methods:

4.3.2.1 Isolation of the particular differentially-expressed cDNA fragment

The putative ripening-related cDNA fragment, designated C2, was identified from 14-wpf Clairette blanche berries by cDNA-AFLP analysis. Complementary DNA was synthesised from total RNA isolated from 14- and 18-wpf berries. The resulting cDNA was digested using the restriction endonucleases *Mse*I and *Pst*I, and subjected to cDNA-AFLP analysis as described in Venter *et al*. (2001). Amplification products were denatured, size fractionated (5 % (m/v) polyacrylamide; 80 W; 100 min), and differentially amplified fragments excised from the dehydrated polyacrylamide gel, according to the procedures described in Venter *et al*. (2001).

4.3.2.2 Re-amplification, cloning and sequence analysis

Re-amplification (30 cycles) was performed according to Habu *et al*. (1997) using the primers used for selective amplification, *Mse* I (+CAT) and *Pst* I (+C). For cloning procedures 5µl of this re-amplified product was used for ligation into pGEM®-T Easy Vectors (Promega Corporation). Ligation and transformation of *Escherichia coli* strain DH10B cells were performed according to the supplier's protocol (Promega Corporation). Nucleotide sequences were obtained by using the Applied Biosystems PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit, following the manufacturer's instructions. Samples were analysed by capillary electrophoresis in an Applied Biosystems automatic sequencer model ABI 3100. Sequence analysis of the re-amplified, uncloned PCR product was performed by subjecting the agarose gel purified re-amplified PCR product to sequence analysis, using the selective amplication primers to prime the sequencing

reaction. Sequences not corresponding to the selective nucleotides of the primers used for selective amplification were omitted from further analysis.

Homology searches were performed using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al*., 1990). All ambiquities, vector and primer sequences were removed before nucleotides sequences were submitted for search amongst entries in the National Centre for Biotechnology Information (NCBI) non-redundant and expressed sequence tag genetic databases (Genbank). The significance of the homology was based on the Score and E- value. A minimum score of 50 was set as an indication of significant homology.

4.3.2.3 Total RNA and mRNA isolation

Total RNA was isolated from Merlot berry- and Clairette blanche berry and leaf material according to Venter *et al*., 2001. Messenger RNA was isolated from total RNA using PolyATtract® mRNA isolation systems (Promega Corporation) and quantified fluorometrically using the Ribogreen™ RNA Quantitation kit (Molecular Probes) and the Bio-TEK FL600 Microplate Fluorescence reader (BIO-TEK® Instruments Inc.).

4.3.2.4 Northern blot analysis

Messenger RNA (50 ng per lane) was transferred to positively charged nylon membrane (Roche Diagnostics Mannheim, Germany) by alkaline downward capillary blotting (Ingelbrecht *et al*., 1998). RNA cross-linking, hybridisation (using ULTRAhyb™ ultrasensitive hybridization buffer) and washing procedures were carried out as described by the manufacturer (Ambion). The fragments were radioactively labelled as described by Venter *et al*. (2001). Hybridisation was visualised by phosphor-imaging using a Cyclone™ Phosphorimager (Packard Instrument Co.) and an AlphaImager™2000 documentation and analysis system (Alpha Innotech Corporation). Differences in hybridisation intensities were visually determined, and regarded as significant as stated for reverse slot blot analysis.

4.3.2.5 Cloning of differentially hybridised cDNAs

For cloning of the appropriate differentially hybridised cDNAs, the area of the nylon membrane displaying differential hybridisation was excised. The excised membrane was then subjected to procedures as described by Li *et al*. (1994) to strip cDNAs hybridised to the membrane. An aliquot (2.5 µl) of this supernatant was used for re-amplification. The PCR product was purified using QIAGEN PCR purification cleanup kit (QIAGEN Inc.), quantified fluometrically using a Hoefer DyNAQuant 200 fluorometer, and ligated (3 ng) into the pGEM®-T easy vector.

4.3.2.6 Reverse Northern blot analysis

Plasmid DNA of each of the clones was dot blotted unto four duplicate positively charged nylon membranes (Roche Diagnostics Mannheim). The equivalent of 300 ng insert plasmid DNA was applied to the four membranes. The membranes were air-dried. Plasmid DNA was denatured (2 min in 1.5 M NaCl, 0.5 M NaOH), neutralised (5 min in 1 M Tris pH 7.4, 1.5 M NaCl) and rinsed for 30 sec in 0.2 M Tris, pH 7.5, 2 x SSC (0.3 M NaCl; 0.03 M Tri-sodium citrate pH 6.8; citric acid). Membranes were air-dried on filterpaper before the plasmid DNA was crosslinked to the membrane (2.5 min at 120 mJcm^{-1}) using an ultraviolet crosslinker (ULTRA.LUM, Scientific Associates). The membranes were left to dry before used for hybridisation.

Four single stranded cDNA probes *viz*. M1-M2 (2- to 4-wpf); M3-M4 (6- to 8-wpf); M5-M6 (10- to 12-wpf) and M7-M8 (14- to 16-wpf) were synthesized. Each of the four probes was synthesized from a total of 1 µg mRNA, comprising of 500 ng of mRNA from berries of each of the two ripening stages. Reverse transcription was performed using Superscript^{TM} II (Invitrogen Life Technologies). Procedures for reverse transcription were according to the protocol supplied by the manufacturer except for two modifications *viz*. 1) the replacement of the dNTP solution with dNTPs (-dCTP), final concentration 75 µM, and 2) the addition of 50 µCi [α-^{32}P] dCTP (Amersham Pharmacia Biotech).

Hybridisation was performed using Rapid-hyb buffer (Amersham Pharmacia Biotech). Hybridisation and washing procedures were according to the manufacturer's instructions. Complementary DNA (cDNA) probes of equal specific activity (1 x 10^8 cpm.ug^{-1} DNA) were used to probe the four duplicate membranes. Hybridisation was visualised by phosphor-imaging (refer Section 4.3.2.4).

4.4 RESULTS

4.4.1 Isolation and cloning of the cDNA corresponding to the ripening-related gene identified by cDNA-AFLP analysis

Sixty-three discrete fragments, ranging from 80 to 600 base pairs, were amplified from the ripening Clairette blanche berries by cDNA-AFLP analysis using primers with selective nucleotides *Mse* I (+CAT) and *Pst* I (+C). The putative ripening-related cDNA fragment (C2), was differentially amplified from 14-wpf Clairette blanche berries (Fig. 4.1). The putative ripening-related fragment was excised and re-amplified. Northern blot analysis using the re-amplified, uncloned C2 product, confirmed the ripening-related transcription demonstrated by the cDNA-AFLP analysis (Fig. 4.2).
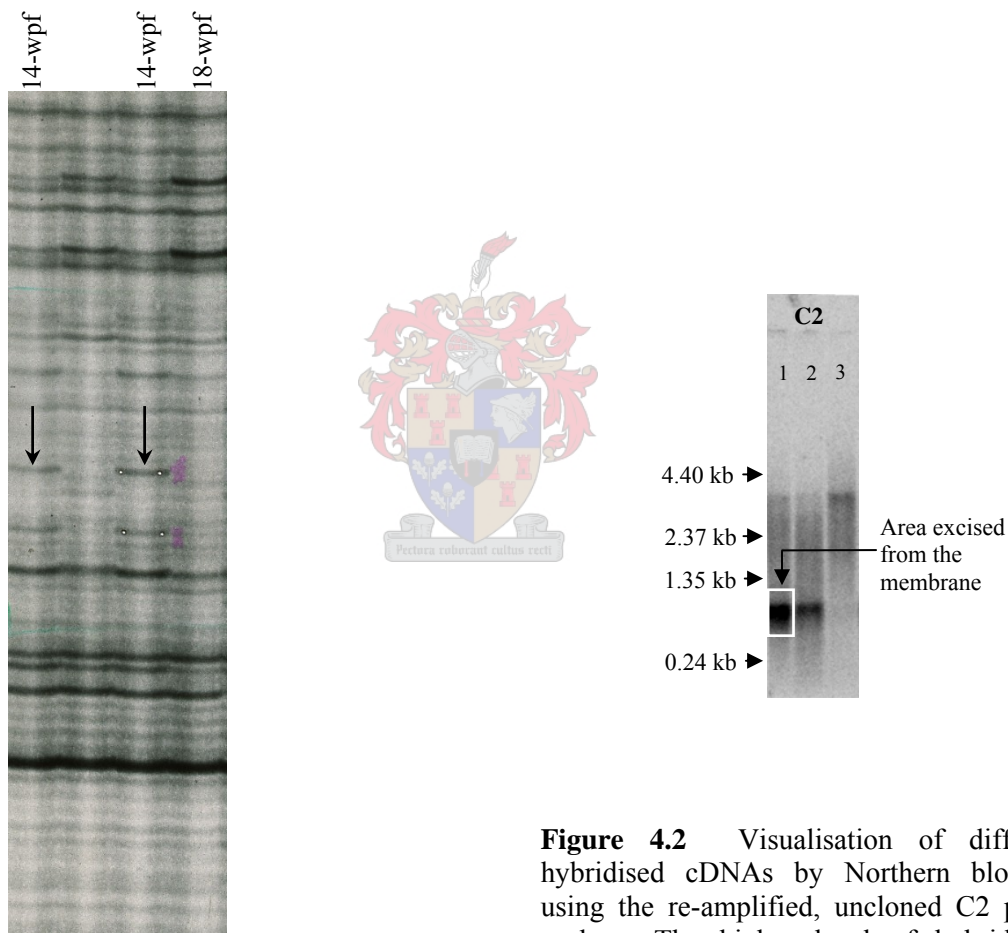
Figure 4.1 Portion of the cDNA-AFLP gel autoradiogram showing the differential amplification of the putative ripening-related cDNA fragment, C2, from 14-wpf and 18-wpf Clairette blanche berries. The C2 fragment is indicated by the arrow. The first lane on the left represents the duplicate 14-wpf sample which was included to verify the reproducibility of the analysis.

Figure 4.2 Visualisation of differentially-hybridised cDNAs by Northern blot analysis using the re-amplified, uncloned C2 product as probe. The higher level of hybridisation to mRNA from 14-wpf berries, (lane 1) confirms the ripening-related transcription demonstrated by the cDNA-AFLP analysis. The area of the membrane representing the differentially-hybridised cDNAs (indicated by the arrow) was excised and subjected to procedures to strip the hybridised cDNAs from the membrane. The position of the size standards are indicated by the arrowheads (kb). Lane 2, 18-wpf berries; lane 3, leaf tissue.

After cloning of the C2 fragment, two random colonies (C2-2 and C2-24) were picked for sequence analysis. It revealed that the nucleotide sequences of C2-2 and C2-24 were distinctly different. In addition, it was shown that C2-24 was identical to the nucleotide sequence obtained from the re-amplified, uncloned C2. Homology searches revealed high sequence identity of both these sequences with mRNA sequences from grape berries (Table 4.1). However, ripening-related transcription of the two sequences could not be confirmed by Northern blot analysis, in fact, no hybridisation of the two sequences to mRNA from ripening Clairette blanche berries or leaf material could be observed.

Table 4.1 Characterisation of the seven cDNA sequences identified from the single band, C2, excised from the cDNA-AFLP polyacrylamide gel.

Clone (size of the insert in base pairs, bp)	Homology (A minimum score of 50 was set as indication of significant homology)	Significance of identity (Score and E-value)
C2-2 (123 bp)	mRNA sequence from stressed berries of grapevine var. Chardonnay, accession nr. CB006924.	Score 223, E value 5e-56)
C2-24 (123 bp)	mRNA from grapevine Cabernet Sauvigon berry Stage I, accession nr. CB973539.	Score 236, E value 7e-60
C2-5 (120 bp)	No sequences with significant homology identified.	-
C2-6 (122 bp)	mRNA sequence from grapevine stressed leaves, accession nr. CD721029; mRNA sequence from grapevine buds, accession nr. CF514173.	Score 217, E value 7e-54
C2-17 (128 bp)	Grapevine ripening induced protein *grip*15 mRNA, accession nr. AJ237984.	Score, 234; E-value, 7e-60
C2-23 (127 bp)	No sequences significant homology identified.	-
C2-73 (126 bp)	mRNA sequences from young grape root, accession nr. CF605780, and grape berries and petioles developmental stage *véraison*, accession nrs. CF602951 and CF519271; Grape ripening induced protein (GASR) mRNA. Berries harvested at onset of ripening.	Score, 206, E-value, 7e-51 Score, 200, E-value, 1e-49

To clone the differentially hybridised cDNA(s) visualised in Figure 4.2, the hybridised cDNA(s) were stripped from the membrane and cloned. Eighteen clones were picked for sequence analysis. From these, eight additional sequences were identified. Three of the sequences did not contain terminal sequences corresponding to the selective nucleotides of the primers used for amplification, and were omitted from further analysis. Almost half of the remaining clones (45 %) contained a sequence identical to that of clone C2-24. Abundance of the other clones was low: C2-5 and C2-23 (10 % representation) and clones C2-6; C2-17 and C2-73 (5 % representation) (Table 4.1 and Fig. 4.3). The size of the five sequences varied from 120 to 128 bp (Table 4.1). Homology searches revealed significant sequence identify of C2-6 with mRNA sequences from grapevine leaves and buds. Both C2-17 and C2-73 revealed high sequence identity with grapevine ripening-related

mRNAs (Table 4.1). No sequences with significant homology to C2-5 and C2-23 could be identified. The seven cDNA sequences were deposited in Genbank , accession numbers CN069438 to CN069444.

4.4.2 Reverse Northern blot analysis

Transcription analysis of the seven cDNA sequences was performed using reverse Northern blot analysis. Contrary to the Northern blot analysis, which did not show any hybridisation of C2-2 and C2-24 to mRNA from ripening berries, the presence of the two sequences in ripening berries was confirmed by reverse Northern blot analysis (Fig. 4.3). Like for C2-5, reverse Northern blot analysis revealed that C2-2 and C2-24 were transcribed at very low levels in 6- to 12-wpf Merlot berries. All seven sequences identified were shown to be ripening-regulated during berry development, mimicking the differential amplification visualised by cDNA-AFLP analysis and the ripening-related transcription visualised by the re-amplified, uncloned product (Fig. 4.2). For all seven sequences, immature berries (2- to 4-wpf) were characterised by no or low levels of the transcript, followed by a rapid accumulation of the transcripts in pre-*véraison* (6- to 8-wpf) or post-*véraison* (10- to 12-wpf) berries. Levels of the transcript declined towards the final stages of ripening, with no or much lower levels of transcription visualised in 14- to 16-wpf berries. Only two cDNAs, clones C2-17 and C2-73, were found to be abundantly expressed – possibly accounting for the high level of the transcript in 12- and 16-wpf berries visualised by the re-amplified, uncloned product (Fig. 4.2).
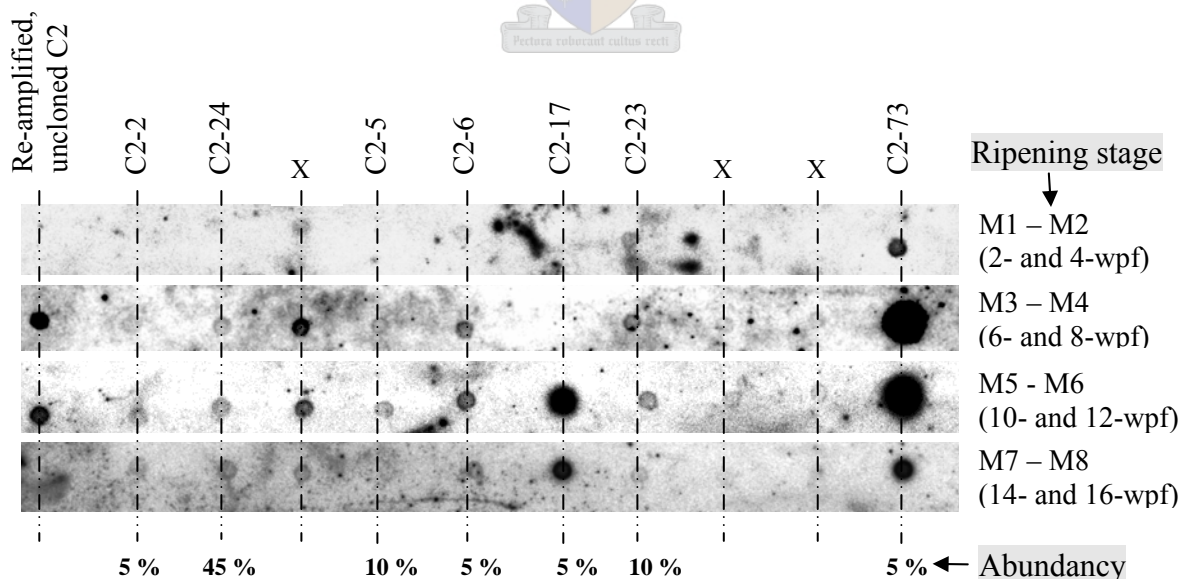
Figure 4.3 Reverse Northern blot analysis to display ripening-related transcription and levels of transcription of the re-amplified, uncloned fragment C2, and the ten sequences identified from the re-amplified, uncloned fragment C2. Plasmid DNA, equivalent to 300 ng of the insert, was blotted unto duplicate membranes and probed with the four cDNA probes M1-M2, M3-M4, M5-M6, M7-M8. Abundancy: refers to the number of clones containing the specific sequence, as a percentage of the twenty clones subjected to nucleotide sequence analysis. X: refers to the three cDNAs which was omitted from further analysis since their terminal sequences did not respond to the selective nucleotides of the primers used for amplification.

According to the ripening-related transcription and significant homology of some of the sequences with entries in Genbank, clones C2-2, C2-5, C2-24, C2-17, C2-23 and C2-73 correspond to different genes.

4.4.3 Northern blot analysis

Clone C2-17 was identified for further characterisation, based on the relative high levels of the transcript and the onset of gene transcription 10- to 12-wpf. Northern blot analysis confirmed the ripening-related accumulation of the transcript as revealed by reverse Northern blot analysis. Moreover it indicated that the gene is transcriptionally activated at *véraison* (Fig. 4.4).
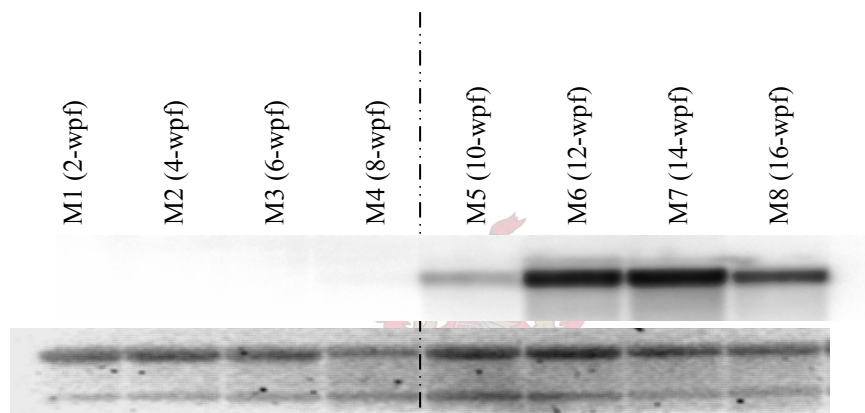
Figure 4.4 Northern blot analysis to illustrate ripening-related transcription of clone C2-17 during Merlot berry ripening, 2- to 16-wpf. *Véraison* is indicated by the dashed line. The bottom panel illustrates approximately equal amounts of intact total RNA used for the analyses.

4.5 DISCUSSION

In this study, a gene which is transcriptionally activated at the onset of grape berry ripening (*véraison*), was cloned following cDNA-AFLP analysis and the implementation of additional steps to identify a specific ripening-related gene. This gene, C2-17, is an ideal candidate for studying ripening-related gene transcription during the post-*véraison* stages of grape berry ripening and for the isolation of a late-ripening-specific promoter to achieve transgene transcription in genetically modified grapevine.

The study shows that despite the identification of multiple cDNAs in the single band excised from the cDNA-AFLP gel, the implementation of the additional steps led to the identification of a specific ripening-related gene, and six other genes differentially expressed during grape berry ripening. The presence of multiple sequences in a single band excised from a DDRT-PCR gel is well documented (Callard *et al*., 1994; Wan *et al*., 1996; Zegzouti *et al*., 1997). Although the origin of these additional sequences remains unclear, the sequences are usually regarded as "false positives". The three main sources of these "false positives" are considered as 1) artifactual differences created in the original RNA populations by non-standardised extraction procedure, 2) "false positives" introduced through PCR re-amplification of differential display cDNA, and 3) identical-sized cDNA fragments that co-migrate with the band of interest on display gels (Miele *et al*., 1998).

In this study, all seven sequences identified from the single excised band were shown to be ripening-related. Although only two of these are considered to account for the ripening-related transcription visualised by the re-amplified, uncloned product, none of the other five sequences is considered as "false positives". Considering the mRNA complexity of a cell (20 000 to 30 000 distinct mRNAs per cell) (Wan *et al*., 1996) and the high probability of *Mse*I restriction sites in the AT-rich 3' untranslated regions of the mRNAs, the seven sequences identified from the single excised band are regarded as identical-sized cDNA fragments that co-migrate with the band of interest on the cDNA-AFLP gel. Despite the high abundance of clone C2-24 amongst the total number of clones analysed in the study, reverse Northern blot analysis showed that the sequence is characterised by a low level of transcription during grape berry ripening. This contradiction can possibly be attributed to phenomena like preferential amplification or cloning efficiency (Liang and Pardee, 1992).

In this study, reverse Northern blot analysis was shown to be an effective tool for the transcription analysis of the seven cDNA sequences and the identification of cDNAs accounting for the levels of transcription visualised by the uncloned, re-amplified product. The procedure does require

substantial quantities of poly(A)$^+$ RNA, which might be a problem in situations where large quantities of material are not available and where low-copy number transcripts are involved.

Unlike previously stated (Debouck, 1995) use of the re-amplified, uncloned product proved to be useful. Not only was it useful to confirm the presence of differentially-regulated cDNAs in the excised band, but the levels of transcription visualised by the uncloned product were used to identify the cDNA corresponding to the differentially regulated gene identified by the analyses. In this study the ripening-related transcription visualised by the re-amplified, uncloned fragment was used as motivation for further investigation.

Contradictory to some reports (Linskens *et al.*, 1995; Wang and Feuerstein, 1995; Martin *et al.*, 1998), "direct" sequencing of the uncloned, re-amplified product was not of any help in the identification of the appropriate differentially-expressed cDNA. Sequence analysis produced a clear sequence, creating the impression of homogeneity; not the presence of multiple different sequences. Since the nucleotide sequence of the uncloned product corresponded to that of clone C2-24, which represented 45 % of the total number of clones sequenced, sequence analysis of the unclonded, re-amplified product was only helpful in the identification of the most abundant sequence.

With cDNA-AFLP analysis it would be possible to eliminate most (possibly all) of the additional sequences from the PCR product by increasing the number of selective nucleotides of the primers used for selective amplification. Bachem and co-workers (1996) showed that simple and rapid verification of band identity could be achieved by using primers with three selective nucleotides for amplification. When the sequence of the target sequence is unknown, this will require an additional four reactions for each extra selective nucleotide. If extensive sets of primers are not available to accommodate this approach, time and costs involved in primer synthesis might impede on the usefulness of this approach.

The results presented in this study serves as confirmation of two of the aspects considered as advantages of the cDNA-AFLPs approach. Firstly, except for the three cDNA sequences which were omitted from further analysis, all cDNA sequences identified contained terminal sequences corresponding to the primers and selective nucleotides used for amplification. In addition, the studied showed that the ripening-related transcription of the seven cDNAs cloned from the band excised from the cDNA-AFLP gel, corresponds with the ripening-related transcription of C2 demonstrated by the cDNA-AFLP analysis *viz.* a high level of transcription in maturing berries, followed by a decrease in the level of the transcript in fully matured berries. These results illustrate the stringency and fidelity of the analyses as performed in this study. Secondly, since the majority

of the seven genes identified are characterised by low levels of the transcript in the ripening grape berry, it confirms that sequence-based approaches are not biased towards abundant transcripts (Breyne and Zabeau, 2001).

Here, the capturing of differentially hybridised cDNAs from the uncloned, re-amplified product and the subsequent cloning and reverse Northern blot analysis were shown to be an effective approach for the identification of the cDNAs corresponding to the differentially-regulated genes visualised by cDNA-AFLP analysis. It is proposed that cDNA populations amplified by differential display are more complex than currently anticipated, and that this should be considered as a factor contributing to the incidence of the "false positives" which differential display is so often criticised for.
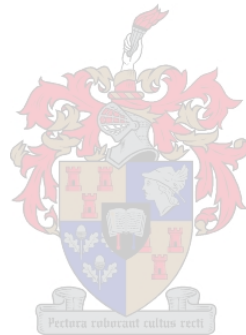
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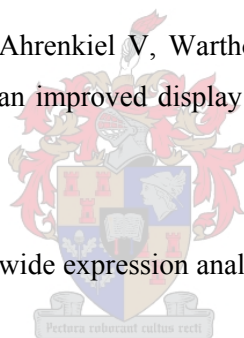
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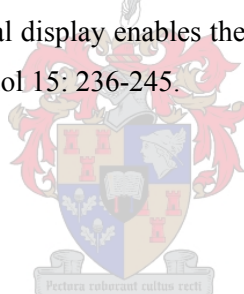
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Characterisation of the gene encoding the Merlot ripening-induced protein 1 (mrip1): evidence that this putative protein is a distinct member of the plant proline-rich protein family.

Research Results

Characterisation of the gene encoding the Merlot ripening-induced protein 1 (mrip1):

evidence that this putative protein is a distinct member of the

plant proline-rich protein family.

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5.1 ABSTRACT

Proline-rich proteins (PRPs) are considered to be involved in cell wall composition and structure. Despite this proposed role and the fact that cell wall modification is one of the most important changes during fruit ripening, very little is known about PRPs involved in fruit ripening. In this study a gene encoding a PRP associated with ripening (*mrip*1, Merlot ripening-induced protein 1) was isolated from *Vitis vinifera* cv. Merlot. It was shown that the gene is specifically transcribed in the fruit tissue, seed and bunchstems of grapes, from 10-weeks post flowering (*véraison*) to the final stages of berry ripening. Sequence analysis revealed that *mrip*1 encodes a distinct member of the plant PRP family. Most obvious is the central region of mrip1, which is comprised of eight consecutive repeats of 19 amino acid residues each. In comparison with other grapevine PRPs, mrip1 revealed single amino acid differences and deletion of one of the 19 amino acid residues repeats, all in the central region of mrip1. *In situ* hybridisation studies showed that accumulation of the *mrip*1 transcript in the ripening berry is limited to the mesocarp and exocarp cells of the ripening grape berry. No transcript with high sequence similarity to *mrip*1 could be detected in ripening strawberry or tomato fruit.

Key words: Grapevine, ripening-related, proline-rich protein, fruit ripening, *in situ* hybridisation, protein primary structure

5.2 INTRODUCTION

Fruit development and ripening are associated with softening and textural changes of the fruit tissue, which is generally accepted as being brought about by changes in cell wall composition and structure [1-4].

A component suggested to be involved in the modification and structure of the cell wall is proline-rich proteins (PRPs) [5–10]. This group of proteins constitutes one of the major sub-groups of hydroxyproline-rich glycoproteins (HRGPs) [5, 6]. HRGPs are characterised as extracellular, structural components of the plant cell wall, while PRPs specifically are considered to be involved in cell wall rigidification associated with the cessation of cell expansion [3, 7-10]. Once cell expansion has been completed, the primary cell wall is locked into shape. Extensins, the best-studied sub-group of the HRGPs, are probably an important component of this locking mechanism [8]. The PRPs, which accumulate later in cell development, are apparently inserted to lock the extensions, thereby forming a heteropeptide framework [7]. Based on the predicted isoelectric points of PRPs (pI 8.8 to 10) the PRPs may interact with the acidic pectin network within the cell wall [11-13].

Despite the proposed role of PRPs in cell wall modification and structure, and the fact that cell wall metabolism is regarded as the main factor in fruit softening and texture, surprisingly little is known about PRPs from ripening fruit. To date, only five fruit PRPs have been reported. These are TPRP-F1, a PRP highly expressed in young tomato fruit [14] and the four grapevine ripening-induced PRPs, *viz. Grip*3, 4, 13 and 15 [15]. Accumulation of latter transcripts coincides with the onset of berry ripening (*véraison*), and is specific to the berry. Although developmentally-regulated during fruit ripening, the tomato PRP and the grapevine PRPs share no sequence identity.

Proline-rich proteins are characterised by unique amino acid compositions and distinct patterns of regulation and localisation, implicating their particular roles in the development, structure and function of the cell walls of particular cells [12, 15–22]. In grapevine, the PRPs *Grip* 3, 4, 13 and 15 are characterised by several repeats of the pentapeptide motif PEHKP. Although this motif and a motif very similar to it (PVHKP) could be identified in some PRPs, no significant homology to any of the currently known PRPs is evident (15, 16, 18, 23-26].

This raised the question as to whether the unique amino acid composition and regulation of the grapevine PRP would indeed be indicative of distinct patterns of localisation, implicating a particular role in the development, structure and function of the cell walls of specific cells. Furthermore, with grapevine PRPs so clearly different from the currently known members of the

PRP family, we were also interested to know how grapevine PRP would relate to the three groups into which PRPs have been classified based on the distribution of the proline repeats, cysteine content and hydrophobic features [27].

Here we describe the use of a 128 bp ripening-related cDNA-AFLP fragment to isolate a gene encoding a ripening-induced PRP (*mrip*1) from Merlot. Transcription of *mrip*1 was abundant in berries 10 to 16-weeks post flowering (wpf), and limited to particular cell types.
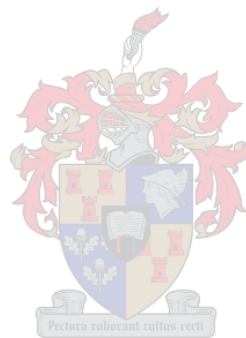
5.3 MATERIALS AND METHODS

5.3.1 Plant material:

Grape berries (*Vitis vinifera* L. cv. Merlot) were sampled during the 2000/2001 growing season at Grondves, an experimental farm of the KWV in Stellenbosch. Berries were collected at 2-week intervals, starting 2-weeks post flowering (wpf) until berries reached full maturity, 16-wpf. These samples were designated M1 – M8. Berries were deseeded and the seeds pooled into two samples *viz.* young seeds (collected from berries M2 – M4) and old seeds (collected from berries M6 – M8), with each of the ripening stages equally represented in the final sample. Stalk (bunchstem) and leaf material (young and old) was collected from the same vines. Due to the disruptive nature of the collection procedure, root material was not collected from these vines but from hydroponically grown Merlot cuttings. All material was immediately quick-frozen in liquid N_2 and stored at –80 °C until further used.

Strawberry fruit and leaf material (*Fragaria x ananassa* cv. Selekta) was collected from field grown plants. Berries were collected at three stages *viz.* immature green (7 days after anthesis), half white and half red (36 days after anthesis) and mature full red (42 days after anthesis). Leaf material was collected from fully grown leaves. Mature full red tomatoes (*Lycopersicom esculentum* cv. Belmonty) were collected from tunnel grown plants.

5.3.2 Methods:

5.3.2.1 Sugar extraction and analysis

Berry tissues were homogenised in liquid nitrogen and extracted by suspending 50 mg of frozen material in 1 ml of boiling 100 mM Tris, pH 8 and incubating the suspension at 70 °C overnight. Insoluble material was removed by centrifugation. The supernatant was filtered (0.45 μm Millex-HV filters, Millipore) and used for the preparation of ten-fold dilutions. Sugars were fractionated by HPLC using a Shimadzu PV 10AD system. Sugars were separated for 10 min on a RCX-10 column (Hamilton) using 60 mM NaOH as the mobile phase and a flow rate of 2 ml.min^{-1}. Sugars were quantified by differential refractometry (Pulse Detector, Waters).

5.3.2.2 Nucleic acid isolation and cDNA synthesis

Frozen tissue was ground to a fine powder in liquid nitrogen. Total RNA was extracted from all tissues using a guanidine thiocyanate solution and isolated by cesium chloride ultra centrifugation. In short: 6 g of homogenised tissue was thawed in 30 ml of guanidine thiocyanate solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5 % sarkosyl and 0.1 M β-mercaptoethanol). Polyvinylpyrrolidone (PVP-40) was added to a final concentration of 1 % (w/v). The mixture was

thoroughly mixed before the cell debris was collected by centrifugation (17000 x g for 30 min at 20 °C). The supernatant was layered unto a 8 ml 5.7 M cesium chloride cushion and subjected to ultra centrifugation (27000 g for 20 h at 20 °C) using a Beckman SW-28 rotor. The RNA pellet was resuspended in 500 µl of filtered water (0.22 µm Cameo 25AS cellulose acetate syringe filter, Osmonics, USA) from which the RNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v), and once with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). The RNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice-cold ethanol to the aqueous phase and incubating at –20 °C overnight. Finally, the RNA was pelleted by centrifugation (17000 x g for 30 min at 4°C). The RNA was washed with 70 % (v/v) ethanol, air-dried, resuspended in 250 µl filtered water and quantified spectrophotometrically.

Messenger RNA was isolated from total RNA using PolyATtract® mRNA isolation systems (Promega Corporation) and quantified fluorometrically using the Ribogreen[TM] RNA Quantitation kit (Molecular Probes) and the Bio-TEK FL600 Microplate Fluorescence reader (BIO-TEK® Instruments Inc.). Complementary DNA was synthesised by reverse transcription of 1 µg mRNA using Superscript[TM] II (Invitrogen Life Technologies) according to the protocol supplied by the manufacturer.

Genomic DNA was isolated from grapevine leaves based on a method described by Steenkamp *et al*. [28]. Modifications to this method included the addition of 2 % (w/v) insoluble PVPP (Sigma-Aldrich Chemie) to the extraction buffer, the use of 3 g of leaf material per 20 ml of extraction buffer and incubation of the crude extract at 65 °C. After extraction and precipitation, the DNA was spooled from the solution and transferred to 30 ml of wash buffer. The solution was incubated at 65 °C for 8 min where-after the wash buffer was decanted and the DNA freeze-dried. The DNA was resuspended in 1.4 ml TE (10 mM Tris-HCl, 1 mM EDTA, pH7.4). To digest contaminating RNA, the DNA solution was incubated in the presence of RNaseA (final concentration 10 µg.ml^{-1}) for a further 30 min at 37 °C.

5.3.2.3 Isolation of *mrip*1 genomic and cDNA clones

A 128 bp ripening-related cDNA, clone C2-17, was isolated from Clairette blanche berries by cDNA-AFLP analysis (Chapter 3 and 4). Complementary DNA for the cDNA-AFLP analysis was synthesised from total RNA isolated from 14- and 18-wpf berries, digested using *Mse*I and *Pst*I, and subjected to cDNA-AFLP analysis as described in Venter *et al*. [29]. The 128 bp fragment was differentially amplified from cDNA from 14- and 18-wpf berries using primers with selective nucleotides *Mse*I (+CAT) and *Pst*I (+C).

A Merlot 14-wpf cDNA library was constructed using cDNA synthesised from 1 µg of mRNA isolated from 14-wpf Merlot berries. Complementary DNA was synthesised using a cDNA synthesis system according to the manufacturer's instructions (Univeral Riboclone cDNA Synthesis Kit, Promega Corporation). *Eco*RI adaptors were ligated to the cDNA and the resulting fragments size fractionated and then cloned into pre-digested Lambda ZAP II/*Eco*RI/CIAP phage arms and packaged (Gigapack II Gold, Stratagene).

One thousand one hundred and fifty two clones of the Merlot 14-wpf berry cDNA library (λZAPII; Stratagene) (4.5 x 10^5 plaque-forming units ml^{-1}, unamplified) were screened using the 128 bp cDNA-AFLP fragment as probe. Hybridisation (using Rapid-hyb buffer) and washing procedures were according to the manufacturer's recommendations (Amersham Pharmacia Biotech).

To amplify the 5' end of the *mrip*1 cDNA, first-strand cDNA was synthesised from 500 ng mRNA isolated from 12-wpf Merlot berries. An oligo (dT) primer was used to prime the reverse transcription reaction. The cDNA was quantified fluorometrically and 80 ng of cDNA/RNA hybrid subjected to dC-tailing using NEB Terminal Transferase (New England Biolabs Inc.). Modifications to the protocol supplied by the manufacturer included denaturing of the cDNA/RNA hybrid prior to the tailing reaction (94 °C, 2 min and quick chill on ice), the use of 20 U Terminal Transferase and dCTP at a final concentration of 40 µM. The reaction mixture was incubated at 37 °C for 15 min before the Terminal Transferase was denatured and the dC-tailed cDNA/RNA hybrid purified using QIAGEN's PCR purification kit. Complementary DNA (2 µl) was amplified by PCR in a 50 µl reaction solution containing 300 nM of a *mrip*1 specific reverse primer (5'-GTG GCT TGT GGG GAG GAG TTG GAG GAG-3'), 300 nM of an oligo (dG) primer (5'-ATA GAA TTC CCG GGG GGG GGG GG-3') [30], 150 nM dNTPs, 1.5 mM MgCl$_2$ and 5 U of *Taq* DNA polymerase (Invitrogen life technologies, UK). The PCR conditions included one cycle of 94 °C for 3 min; 56 °C for 2 min and 72 °C for 10 min for second strand synthesis and forty cycles of 94 °C for 50 sec; 60 °C for 1 min and 72 °C for 1 min for amplification. The resulting PCR product was cloned into a pGEM®-T Easy vector (Promega Corporation).

Inverse PCR was performed using 150 ng of genomic DNA digested by the restriction enzymes *Apa*I, *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Nhe*I, *Not*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*II, *Xba*I and *Xho*I. The digested DNA was re-ligated in a 400 µl reaction volume at 16 °C overnight and precipated in five volumes of 5 mM MgCl$_2$ in 70 % ethanol. The precipitated DNA was collected by centrifugation (13 000 g x 30 min x 4 °C), resuspended in 22 µl filtered water and quantified spectrophotometrically. *Mrip*1 specific fragments were amplified in a 50 µl reaction solution containing 150 ng of re-ligated genomic DNA and two gene specific primers, iPCRrev (5'-CCA CCA TTC CAA GCA ACA ATA CTA GCA AGC-3') and iPCRfrw (5'-GAT AGT GTT CTA TGT

ATG AGT CTC TAC GAC GCC CTG –3'). Elongase enzyme mix (Invitrogen Life Technologies) was used for amplification. Reaction mixtures were prepared according to the protocol supplied by the manufacturer using primers at a final concentration of 200 nM. Thermocyling was performed in a Applied Biosystems GenAmp9600 Thermocycler using cycling conditions suggested by the Expand Long Template PCR system (Roche Molecular Biochemicals). Elongation times were set at 10 min (60 sec per kb of target). PCR products were digested with the respective restriction enzymes used for preparation of the template genomic DNA, blunt ended, phosphorylated and cloned into dephosphorylated, *Sma*I digested pBlueScript vector (Stratagene).

5.3.2.4 Northern and Southern blot analysis

Radio-labeled DNA probes were synthesised from 75 ng of plasmid DNA according to procedures described by Venter *et al*. [29]. Prior to labeling, cloned fragments were excised from the vector and purified by agarose gel electrophoresis. Probes with specific activities of 1×10^8 to 1×10^{10} cpm.ug^{-1} DNA were used for hybridisation.

Total RNA (10 μg per lane) was denatured, size fractionated (1.2 % agarose, 3 V/cm; 2 h) and transferred to positively charged nylon membrane (Roche Molecular Biochemicals) by upward capillary blotting in 10xSSC (1.5 M NaCl; 0.15 M Tri-sodium citrate pH 6.8; citric acid). RNA was cross-linked to the membrane (2.5 min at 120 mJcm^{-1}) using an ultraviolet crosslinker (ULTRA.LUM, Scientific Associates). The membranes were left to dry before subjected to hybridisation procedures in a 50 % formamide-containing hybridisation buffer (ULTRAhyb™ Ultrasensitive Hybridisation buffer, Ambion) at 42 °C. Stringency washes were performed according to the manufacturer's recommendations: 2 x 10 min in 2xSSC + 0.1 % (w/v) SDS at 42°C, (low stringency) and 2 x 15 min in 0.1xSSC + 0.1 % (w/v) SDS at 42 °C (high stringency). Hybridisation was visualised by phosphor-imaging using the Cyclone™ Phosphorimager (Packard Instrument Co.) and an AlphaImager™2000 documentation and analysis system (Alpha Innotech Corporation).

For Southern blot analysis genomic DNA (8 μg per lane) was digested using restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Nhe*I, *Pst*I and *Xba*I, size fractionated (0.8 % agarose, 3 V/cm, 2 h) and then denatured (1.5 M NaCl; 0.5 M NaOH) and neutralised (1 M Tris pH 7.4; 1.5 M NaCl). The gel was rinsed in 10xSSC before the digested genomic DNA was transferred and cross-linked to a positively charged nylon membrane according to procedures described for Northern blot analysis (refer previous paragraph). Hybridisation procedures were performed in Rapid-hyb buffer (Amersham Pharmacia Biotech) at 65 °C. Stringency washes were performed at 65 °C: 2 x 10 min

in 2xSSC + 0.1 % (w/v) SDS, and 2 x 15 min in 0.1xSSC + 0.1 % (w/v) SDS. Hybridisation was visualised by phosphor-imaging.

5.3.2.5 *In situ* hybridisation

In situ hybridisation was performed using berries 2-, 8- and 14-wpf (stages 1, 4 and 7). The roots and leaves were collected from grape plants cultured *in vitro* on medium containing Murashige and Skoog [31] (MS) basal medium with 3 % (w/v) sucrose and 0.2 % (w/v) Gelrite under a 16 h photoperiod at 22 °C. Tissue was fixed in paraformaldehyde, dehydrated in a graded ethanol series, cleared in xylene and infiltrated with Paraplast X-tra (Sigma-Aldrich Chemie). *In situ* hybridisation experiments were carried out on 10 μm dewaxed sections pretreated with 0.125 mg.ml^{-1} pronase (Sigma-Aldrich Chemie) in 0.05 M Tris-HCl pH 7.5, 0.005 M EDTA for 10 min at room temperature, 0.2 % (w/v) glycine in phosphate-buffered saline (PBS) for 2 min and 1 % (v/v) acetic anhydride in 0.1 M triethanolamine pH 8.0 for 10 min at room temperature and dehydrated with a graded ethanol series. Hybridisation was carried out using DIG-labeled RNA probes. Sense and antisense probes were generated by *in vitro* transcription of linearised template DNAs in the presence of DIG-labeled dUTP as described by the manufacturer (Roche Molecular Biochemicals). The probe was diluted in hybridisation buffer (Sigma-Aldrich Chemie) to a final concentration of 200 ng.ml^{-1}. After overnight hybridisation and washing at 42 °C in 2xSSC 50 % formamide, the slides were treated with 1 % blocking reagent (Roche Molecular Biochemicals) in 100 mM Tris-HCl (pH 7.5) 150 mM NaCl for 1 h at room temperature and incubated for 1 h at room temperature with the antibody (Anti-DIG Fab fragments, Roche Molecular Biochemicals) diluted 1:3000 in the blocking solution. After washing the antibody was detected by incubating the slides in the dark with 150 μg.ml^{-1} Nitroblue tetrazolium, 75 μg.ml^{-1} 5-Bromo-4-chloro-3-indolylphosphate, 10 % polyvinyl alcohol (molecular weight 70 000 - 100 000 g.mol^{-1}), 100 mM Tris-HCl (pH 9.5) 100 mM NaCl, 50 mM MgCl$_2$ for up to 3 days. The slides were mounted and areas of hybridisation visualised using a Nikon Eclipse E400. Images were captured with a Nikon Coolpix 990.

5.3.2.6 Sequence analysis

Nucleotide sequences were obtained by using the Applied Biosystems PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit, following the manufacturers instructions. Samples were analysed by capillary electrophoresis in an Applied Biosystems automatic sequencer model ABI 3100.

Sequence homology searches amongst entries in the GenBank database were performed using the BLAST server at the National Center for Biological Information (NCBI) [32]. Multiple sequence

alignments were performed using the programs ClustalW 1.8, MAP and PIMA 1.4, provided by the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/). Software available at this same site was used to perform six frame translations. Amino acid composition, predictions of polypeptide molecular mass, charge and isoelectric points were obtained by using the program PEPSTATS: Protein statistics (EMBOSS), Biological Software Server, (http://bioweb.pasteur.fr). Other programs used included ProtScale for the analysis of hydrophobicity (Kyte-Doolittle prediction) [33] and Tmpred for the prediction of transmembrane spanning regions, provided by the ExPASy Molecular Biology Server (http://us.expasy.org) [34]. Sequence analysis software available from the Fruitfly Server (http://www.fruitfly.org) was used for prediction of the transcription initiation site.
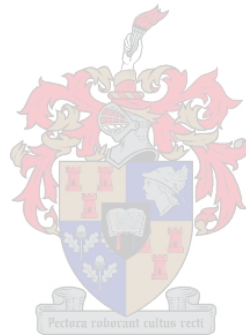
5.4 RESULTS

5.4.1 Isolation of *mrip*1 cDNA and genomic clones

The 128 bp ripening-related gene (clone C2-17) was identified by cDNA-AFLP analysis of 14- and 18-weeks post flowering (wpf) Clairette blanche berries and cloned following the implementation of additional steps to identify a specific ripening-related gene (Chapters 3 and 4). The gene was shown to be transcriptionally activated at the onset of Merlot grape berry ripening and was designated *mrip*1 (Merlot ripening-induced protein 1) (Chapter 4).

Full-length *mrip*1 cDNA was isolated by a combination of cDNA library screening and 5' RACE (Fig. 5.1A). The 128 bp cDNA fragment was used to screen 1152 clones of a Merlot 14-weeks post-flowering (wpf) cDNA library. Six clones hybridising to the 128 bp fragment were identified. Sequence analysis proved that all six cDNA sequences were identical and also contained the 128 bp fragment. Multiple alignment of these sequences with the *grip*15 cDNA sequence indicated that they were all partial sequences aligning to the 3' end of the *grip*15 sequence. Clone LH6 contained the longest fragment, 673 bp. Using 5' RACE the cDNA fragment from clone LH6 was extended to 800 bp. Sequence analysis confirmed that this fragment contained the ATG start codon.

Four fragments containing *mrip*1 5' and 3' untranslated region (UTR) and 5' UTR flanking sequences were amplified from genomic DNA digested with *Bgl*II, *Hind*III, *Nhe*I and *Xba*I (Fig. 5.1B). The size of fragments corresponding to the *mrip*1 5' UTR region, ranged from 4.2 kb to 5.5 kb (*Bgl*II, 4.5 kb; *Hind*III, 4.2 kb; *Nhe*I, 5.1 kb and *Xba*I, 5.5 kb). The transcription initiation site (TIS) was identified 28 bp upstream of the ATG start codon, confirming that the 5' RACE-product contained the 5' end of the *mrip*1 full-length cDNA (Fig. 1A). As observed in most of the plant genes analysed, the TIS was flanked by pyrimidine bases. The *mrip*1 AUG context, AAAG AUG TC, matched the plant consensus context, AA(C/A)A AUG GC, in seven of the nine positions [51]. The *mrip*1 cDNA sequence was deposited in Genbank, accession number AY341935.

5.4.2 Deduced amino acid sequence and primary structure of mrip1

The full-length *mrip*1 cDNA sequence (1 103 bp) contains a single open reading frame of 804 bp and a 5' and 3' UTR of 28 bp and 271 bp respectively (Fig. 5.2A, B and C). Evidently mrip1 is a proline-rich protein of 268 amino acids with a molecular mass of 28.77 kDa and an isoelectric point of 10.7.

Analysis of the deduced amino acid sequence revealed three clear regions (Fig. 5.2 and Fig. 5.3). The first is a N-terminal, hydrophobic region of 23 amino acids (amino acid residues 1 to 23) with features of a signal peptide as defined by von Heijne [35].

A

mrip1 full-length — 1076 bp (TIS *) — 673 bp — 276 bp (TGA) — 60 bp

Inverse PCR

5' RACE

cDNA library clone, LH6

128bp cDNA-AFLP fragment

B

XbaI, NheI, BglII, HindIII, PstI — PstI, BglII, XbaI, NheI — HindIII

+5 kb +4 kb +3 kb +2 kb +1 kb TIS Coding region -1 kb -2 kb -3 kb -4 kb -5 kb -6 kb
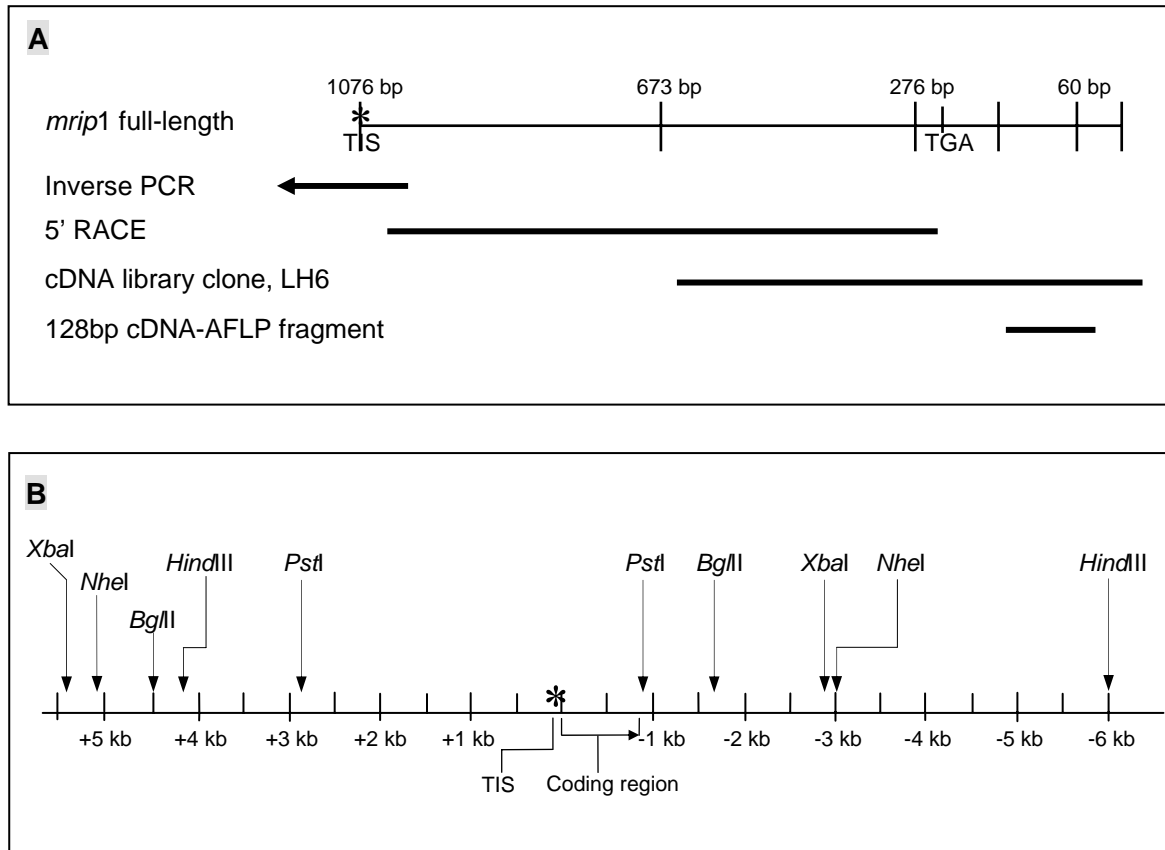
Figure 5.1 Schematic representation of the strategy to isolate the *mrip*1 full-length cDNA, and characterisation of *mrip*1 3' UTR and 5' UTR flanking regions by restriction endonuclease mapping. A: The *mrip*1 transcription initiation site (TIS) and position of the stop codon (TGA) are indicated. B: Restriction endonuclease mapping of the *mrip*1 3' UTR and 5' UTR flanking regions based on the size of fragments amplified from genomic DNA. Fragment sizes are indicated in basepairs (bp) or kilobase pairs (kb).

A: **Partial genomic sequence of *mrip*1 5' UTR. The putative TIS is in bold print.**
5'-TCCAGCCCTTTATAAAAGGCCACCCAATGCCTACAGTGTT**G**CGCCATCTTAGCTACAACTACTCAAAG-

B: ***Mrip*1 coding (804 bp) and deduced amino acid sequence (268 amino acids):**

```
        ATGTCTCCCACATGCTTGCTAGTATTGTTGCTTGGAATGGTGGTTCTCACCAGTACTCCA      60
         M   S   P   T   C   L   L   V   L   L   L   G   M   V   V   L   T   S   T   P
                        Region B
        TCTCTTGCTCATTACCCCAAGCCCCGCCATTTCAGAAGCCACCTCCGGAGCACAAGCCT     120
         S   L   A   H   Y   P   K   P   P   P   F   Q   K   P   P   P   E   H   K   P

        CCAGGGGAGAAGCCATCTCCAGAACACAAGCCACCAACAAAACCTCCCAAGGGAGAGAAG     180
         P   G   E   K   P   S   P   E   H   K   P   P   T   K   P   P   K   G   E   K

        CCACTCCCAGAACATAAGCCGCCGACTCCAATTGGCAAACCTCCCAAGGGAGAGAAGCCA     240
         P   L   P   E   H   K   P   P   T   P   I   G   K   P   P   K   G   E   K   P

        CCCCCAGAACATAAGCCGCCAACCCCAATTGGCAAACCTCCCAAGGGAAAAAAGCCACTT     300
         P   P   E   H   K   P   P   T   P   I   G   K   P   P   K   G   K   K   P   L

        CCAGAACATAAGCCGCCGACTCCAATTGGCAAACCTCCTAAAGGAGAGAAGCCACCCCCA     360
         P   E   H   K   P   P   T   P   I   G   K   P   P   K   G   E   K   P   P   P

        GAACATAAGCCGCCAACCCCAATTGGCAAGCCTCCCAAGGGAGAAAAGCCACTCCCAGAA     420
         E   H   K   P   P   T   P   I   G   K   P   P   K   G   E   K   P   L   P   E

        CATAAGCCGCCAACTCCAATTGGCAAACCTCCCAAGGGAGAGAAGCCGCCCCCAGAACAT     480
         H   K   P   P   T   P   I   G   K   P   P   K   G   E   K   P   P   P   E   H

        AAGCCGCCAACCCCAATTAGCAAACCTCCCAAGGGAGAGAAGCCACTCCCAGAACATAAG     540
         K   P   P   T   P   I   S   K   P   P   K   G   E   K   P   L   P   E   H   K

        CCGCCAACCCCAATTGGCAAACCTACCAAGGGAGAGAAACCACTCCCAGAACACAAGCCA     600
         P   P   T   P   I   G   K   P   T   K   G   E   K   P   L   P   E   H   K   P
                                      Region C
        CCAACCCCAATTGGTAAACCTCCTAAGGGAGAGAAGCCACCAAGCCCATTTGGCAAACCA     660
         P   T   P   I   G   K   P   P   K   G   E   K   P   S   P   F   G   K   P

        CCTACGGGAGAGAAGCCACCACATTATGGTCACACTCCCGGGCATCCTCCTGCACACTCA     720
         P   T   G   E   K   P   P   H   Y   G   H   T   P   G   H   P   P   A   H   S

        TACAAGCCGCCTCAGAAAATAAAGCCTCCTCCAACTCCTCCCCACAAGCCACCCCACAAA     780
         Y   K   P   P   Q   K   I   K   P   P   P   T   P   P   H   K   P   P   H   K

        CCACCATCTCCCACCTATCCCAACTGA                                     807
         P   P   S   P   T   Y   P   N   *
```


C: **M*rip*1 3'UTR cDNA sequence (271bp):**
TGAGTGAATTTGGTTATTACGAGTGGTGGTATACATGTGAAAAATAAGAG**CTGCAG**CGCACTGGTCTTGTCCTTT
CCTCTGGGTGGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTTATTCATGGAAG
TTCTTGTATGCTTGTAGGTGATCATGAACAATGTAACACTGCTTATTCTAATTCTATTTTATGGAGAATATTGGGT
GTCACCATTGCTGCTATCTACACTTTAGATTTGCTTTTATTAAA

Figure 5.2 Nucleotide and deduced amino acid sequence of *mrip*1. **A**: partial genomic sequence of the *mrip*1 5' UTR and 5' UTR flanking region, indicating the putative transcription initiation site (TIS) in bold print; **B**: nucleotide and deduced amino acid sequences of the *mrip*1 coding region. The putative cleavage site of the signal peptide sequence is indicated by the arrow. The ten repeats of the pentapeptide motif PEHKP are printed in bold and the extended motifs indicated in the shaded boxes. Sequence corresponding to the central (region B) and C-terminal region is indicated; **C**: 3' UTR cDNA sequence. A putative polyadenylation site, ATTAAA, is indicated in the box. Shown in shading, is the sequence corresponding to the 128 bp cDNA-AFLP fragment. The *Pst*I restriction site is indicated in bold print, and the annealing positions of the two primers used for iPCR, underlined.

The sequence downstream of the putative signal peptide is hydrophilic (Fig. 5.3) and consists of two distinct regions based on their respective amino acid composition and charge (Table 5.1). Both regions are rich in proline (38 % and 41 %) and have basic isoelectric points (10.78 and 10.6). When comparing the amino acid composition of the central region, (designated region B, amino acid residues 24 to 212) to that of the C-terminal region (amino acid residues 213 to 268), the central region is particularly rich in glutamate, isoleucine, leucine and lysine (Table 5.1). On the contrary, the C-terminal region is rich histidine, serine and tyrosine.
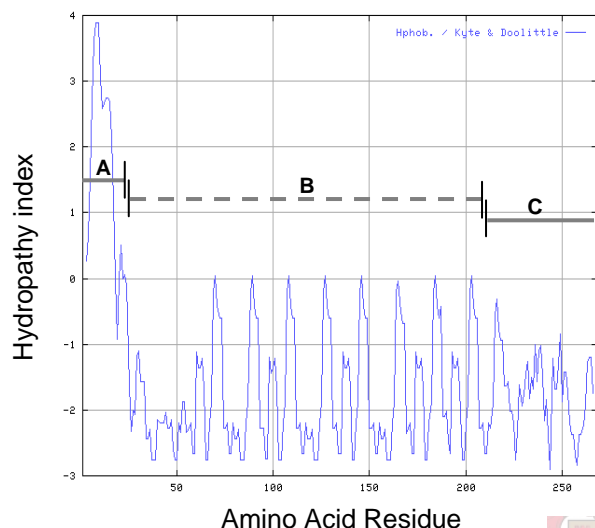
Figure 5.3 Hydropathy plot of *mrip*1. The hydropathy value of the amino acids was calculated by the algorithm of Kyte and Doolittile (1982) over a window of 5 amino acid residues and was plotted as a function of amino acid position. Values above the horizontal line indicate the hydrophobic regions. **A**: The putative signal peptide sequence; **B**: the central region containing the clustered pentapeptide motifs, and **C**: the C-terminal region are indicated.
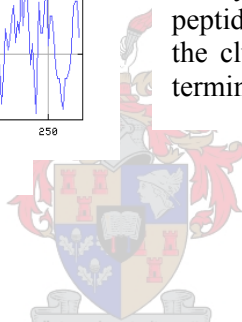
Table 5.1 Amino acid composition of the central (region B) and C-terminal regions identified in the deduced amino acid sequence of *mrip*1. The amino acid composition is indicated by the number of amino acid residues present in the respective regions and expressed as a percentage of the total number of residues present in the specific region. All amino acid residues present in the two regions are indicated.

Amino acid	Central region (region B) (amino acid residues 24 to 212)	C-terminal region (amino acid residues 213 to 268)
Alanine	0	1.786
Asparagine	0	1.786
Phenylalanine	0.529	1.786
Glutamate	10.053	1.786
Glutamine	0.529	1.786
Glycine	8.995	7.143
Histidine	5.82	10.714
Isoleucine	4.233	1.786
Leucine	2.646	0
Lysine	21.693	12.5
Proline	38.624	41.071
Serine	1.058	5.357
Threonine	5.291	7.143
Tyrosine	0.529	5.357

Collectively these data indicate that the three amino acids (proline, glutamate and lysine) accounts for 69 % of the amino acids of region B, while the amino acids proline, histidine and lysine accounts for 64.2 % of the C-terminal region.

The percentage of charged amino acids is significantly higher for region B than for the C-terminal region (37.8 % and 24.9 % respectively). This is reflected in the highly different charge values of the two regions. Region B is strongly positively charged (charge value of 27.5) while the C-terminal has a low positive charge value (charge value of 9.0).

The most obvious characteristic of region B is the presence of the pentapeptide motif PEHKP. The motif is repeated ten times in the region (Fig. 5.2). Eight of these repeats are contained in extended motifs of 19 amino acid residues *viz*. LPEHKPPTPIGKPPKGEKP (repeated 4 times) and one repeat of each of the motifs PPEHKPPTPIGKPPKGEKP, PPEHKPPTPIGKPPKG<u>K</u>KP, PPEHKPPTPI<u>S</u>KPPKGEKP and LPEHKPPTPIGKP<u>T</u>KGEKP. The underlined amino acid residues indicate single amino acid residue differences when compared to the first two extended motifs. Apart from the first 37 amino acid residues, the entire region is comprised of these consecutive extended motifs (amino acid residues 62 to 212). Interesting to note is how the extended motif LPEHKPPTPIGKPPKGEKP is alternated with the extended motifs starting with PPEHKPP.

5.4.3 *Mrip*1 homology search and analysis of sequence similarity

5.4.3.1 Deduced amino acid sequence analysis

Homology search using BLASTN revealed significant identity of the *mrip*1 cDNA sequence to eighty-two sequences in the GenBank database (BLASTN score $\geq$ 100). All of these were grapevine cDNA sequences isolated from ripening berries, seeds harvested at *véraison*, grape stems and from abiotic stressed leaves. Analysis of the deduced amino acid sequence of the cDNAs isolated from grape stems (PRP2, accession number AY046417) and abiotic stressed leaves (VVA001E07, accession number BM436276) revealed several repeats of the motif PEHKP and extended motifs, similar to those identified in *mrip*1. Homology analysis however, showed a low level of sequence identity to *mrip*1 (56 % and 65 % identity, respectively).

The 57 bp nucleotide sequence corresponding to the extended motif LPEHKPPTPIGKPPKGEKP, was submitted to a BLASTN homology search to identify cDNA sequences containing identical sequences in the GenBank EST and NR databases. Nineteen sequences were identified, all mRNAs from *véraison* and ripening berries from the cultivars Cabernet Sauvignon, Pinot Noir and Shiraz

(Table 5.2). Analysis of the deduced amino acid sequences revealed that each contained at least one repeat of the extended motif.

From the multiple sequence alignment data it is evident that the sequences share a high level of identity on the amino acid level (Fig. 5.4). The only exception is clone EST3815, which is only 72 % identical to mrip1. The highest level of sequence identity to mrip1 was revealed by the cDNA clones EST4872, EST2810 and G*rip*15 (Table 5.3). Variation in the deduced amino acid sequences is limited to a number of single amino acid differences in the central region containing the 19 amino acid extended motifs (mrip1 amino acid residues 62 to 213). Based on single amino acid differences, the cDNA sequences could be differentiated in two or three groups (refer mrip1 amino acid residues 68; 75; 91; 97; 110; 119; 157 and 167). However, no correlation could be found between the groups and phenotypic data like grape cultivar and ripening stage of the berries from which the mRNA was isolated. The deduced amino acid sequences corresponding to the C-terminal region of all the cDNAs, except for EST8207, were identical.

Table 5.2 Nineteen cDNA sequences in which deduced amino acid sequence at least one repeat of the extended motif LPEHKPPTPIGKPPKGEKP could be identified. The cDNA sequences were identified by submitting the 57 bp nucleotide sequence corresponding to the extended motif, to a BLASTN homology search of the GenBank EST and NR databases.

cDNA	GenBank database accession number	Tissue isolated from
EST0032	AW707985	Pinot Noir berries, *véraison* stage
EST2810	BQ793872	Shiraz berries, *véraison* stage
EST2891	BQ793953	Shiraz berries, *véraison* stage
EST3815	BQ794877	Shiraz berries, *véraison* stage
EST4284	BQ795346	Shiraz berries, ripening stage
EST4289	BQ795351	Shiraz berries, ripening stage
EST4387	BQ795449	Shiraz berries, ripening stage
EST4675	BQ795737	Shiraz berries, ripening stage
EST4737	BQ795799	Shiraz berries, ripening stage
EST4872	BQ795934	Shiraz berries, ripening stage
EST5768	BQ796830	Shiraz berries, ripening stage
EST5785	BQ796847	Shiraz berries, ripening stage
EST5968	BQ797030	Shiraz berries, ripening stage
EST6153	BQ797215	Shiraz berries, ripening stage
EST6162	BQ797224	Shiraz berries, ripening stage
EST8207	BQ792487	Cabernet Sauvignon 15,5-wpf berries
EST8322	BQ792602	Cabernet Sauvignon 15,5-wpf berries
EST8587	BQ792132	Cabernet Sauvignon berries, *véraison* stage
*Grip*15	AJ237984	Shiraz 10-wpf berries

```
                                                            One        Two
EST7923   1 ------------------------------------------------------------------------------------------- CS seeds, ver.
EST7822   1 ------------------------------------------------------------------------------------------- CS seeds, ver.
EST8322   1 MSPTCLLVLLLGMVVLTSTPSLAHYPKPPPFQKPPPEHKPPGEKPSPEHKPPTKPPKGEKPLPEHKPPTPIGKPPKGEKPPPEHKPPTPI CS seeds, 15,5-wpf
EST3815   1 -------------------------------------------------------PKGEKPPPEHKPPTPISKPPKGEKPLPEHKPPTPI Sh berries, ver.
EST4387   1 ------------------------------------------------------------------------------------------- Sh berries, rip.
EST4289   1 ------------------------------------------------------------------------------------------- Sh berries, rip.
EST8587   1 ------------------------------------------------------------------------------------------- CS berries, rip.
EST4284   1 ------------------------------------------------------------------------------------------- Sh berries, rip.
EST4675   1 --------------------------------------------------------------------------------------PTPI Sh berries, rip.
EST2891   1 ---------------------------------------------------------------------KGEKPPPEHKPPTPI Sh berries, ver.
EST4737   1 -----------------------------------------------------HEEHKPPTPIGKPPKGEKPPPEHKPPTPI Sh berries, rip.
EST0032   1 MSPTCLLVLLLGMVVLTSTPSLAHYPKPPPFQKPPPEHKPPGEKPSPEHKPPTKPPKGEKPLPEHKPPTPIGKPPKGEKPPPEHKPSTPI PN berries, ver.
EST5768   1 -----------------------------------------------------------EHKPPTPIGKPSKGEKPLPEHKPPTPI Sh berries, rip.
EST5968   1 ----------------------------STSLQGRSHLQNTSHQQNSQGEKPLSEHKPLTPIGKPPKGEKPPPEHKPPTPI Sh berries, rip.
EST6153   1 ---------------------------------------EHKPPTKPPKGEKPLPEHKPLTPIGKPPKGEKPPPEHKPPTPI Sh berries, rip.
EST8207   1 -------------------------------------------------------------PIGNPPKGEKPPPEHKPPTPI CS berries, 15,5-wpf
EST6162   1 -------------TSTPSLS-YPKPRHF-RSHLRSTSSREKPSPEHKPPTKTPKGEKPLPEHKPLTPIGKPPKGEKPPPEHKPPTPI Sh berries, rip.
EST5785   1 -----------------------LLIPKPRHSEAPP-EHKPPGEKPSPEHKPPTKPPKGEKPLPEHKPLTPIGKPPKGEKPPPEHKPPTPI Sh berries, rip.
EST4872   1 GLRTCLLVLLLGMVVLTSTPSLAHYPKPPPFQKPP----------------TKPPKGEKPLPEHKPPTPIGKPSKGEKPPPEHKPPTPI Sh berries, ver.
EST2810   1 ----------------------GTRPKPPPFQKPPPEHKPPGEKPSPEHKPPTKPPKGEKPLPEHKPPTPIGKPSKGEKPPPEHKPPTPI Sh berries, ver.
Mrip1     1 MSPTCLLVLLLGMVVLTSTPSLAHYPKPPPFQKPPPEHKPPGEKPSPEHKPPTKPPKGEKPLPEHKPPTPIGKPPKGEKPPPEHKPPTPI Mer berries, 12-wpf
Grip15    1 MSPTCLLVLLLGMVVLTSTPSLAHYPKPPPFQKPPPEHKPPGEKPSPEHKPPTKPPKGEKPLPEHKPLTPIGKPPKGEKPPPEHKPPTPI Sh berries, 10-wpf
                                                                                    68      75
            ->       Three       ->    Four      ->    Five      ->         Six          ->
EST7923   1 -----------KPPTPIGKPPKGEKPLPDLKLPTPIGKPPKGEKP----------------------------- CS seeds, ver.
EST7822   1 ---------PEHKPPTPIGNPPKGEKPLPEHKLPTPIGKPLKGEKP----------------------------- CS seeds, ver.
EST8322  91 GNPPKKKKKKKKKKH--------------------------------------------------------- CS berries, 15,5-wpf
EST3815  36 GKPPKGEKPLPEHKPPTPIGKPPKGEKPPPEYKPPTPV-KPP----PPPKHKTPT---LPPRVVRPPPTPKPPT- Sh berries, ver.
EST4387   1 -----PEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP----------- Sh berries, rip.
EST4289   1 -------EHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP----------- Sh berries, rip.
EST8587   1 ----KGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKLPTPIGKPPKGEKP------- CS berries, ver.
EST4284   1 ---PKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP--------------------- Sh berries, rip.
EST4675   5 SKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP------- Sh berries, rip.
EST2891  16 SKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP------- Sh berries, ver.
EST4737  30 SKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP------- Sh berries, rip.
EST0032  91 GKPXKGKKPLPEHKPPTPIGKPPKGEKPPSEHKPPTPIGKPPKGEKPLPEHKPSTPIGKPPKGEKPPPEHKPX--- PN berries, ver.
EST5768  28 GKPPKGEKPPPEHKPPTPISKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP----- Sh berries, rip.
EST5968  54 DKPPKGKKPLPEHKPPTPISKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP----- Sh berries, rip.
EST6153  44 DKPPKGKKPLPEHKPPTPISKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP----- Sh berries, rip.
EST8207  22 DKLPKGKKPLPEHKPPTPIGKPLKGEKPPPEHKPPTPIGKPSKGEKPLPEHKPPTPIGKPPKGEKPPPEHKPPTPISKPPKGEKPLPEHK CS berries, 15,5-wpf
EST6162  73 DKPPKGKKPLPEHKPPTPISKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP----- Sh berries, rip.
EST5785  68 DKPPKGKKPLPEHKPPTPISKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKP----- Sh berries, rip.
EST4872  74 GKPPKGKKPLPEHKPPTPIGKPPKGEKPPPEHKLPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPPPEHKPPTPISKPPKGEKPLPEHK Sh berries, rip.
EST2810  69 GKPPKGKKPLPEHKPPTPIGKPPKGEKPPPEHKLPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPPPEHKPPTPISKPPKGEKPLPEHK Sh berries, ver.
Mrip1    91 GKPPKGKKPLPEHKPPTPIGKPPKGEKPPPEHKLPTPIGKPPKGEKPLPEHKPPTPIGKPPIGKPPPEHKPPTPISKPPKGEKPLPEHK Mer berries, 12-wpf
Grip15   91 DKPPKGKKPLPEHKPPTPIGKPPKGEKPPPEHKPPTPIGKPSKGEKPLPEHKPPTPIGKLPKGEKPPPEHKPPTPISKPPKGEKPLPEHK Sh berries, 10-wpf
            91      97           110     119                         167       177
            ->   Seven    ->     Eight    ->      Additional    ->
EST7923  35 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP CS seeds, ver.
EST7822  38 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP CS seeds, ver.
EST8322     ------------------------------------------------------------------------------------- CS berries, 15,5-wpf
EST3815 102 ---------------------------------LPPIIVRPPPTKEPSEPHGHYPGHPPVET-PFSTPYKKPP Sh berries, ver.
EST4387  57 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST4289  57 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST8587  63 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP CS berries, ver.
EST4284  64 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST4675  71 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST2891  82 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, ver.
EST4737  96 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST0032     ------------------------------------------------------------------------------------- PN berries, ver.
EST5768 113 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST5968 139 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST6153 129 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST8207 112 PPTPIGKLPKGEKPFPEHKPPTPIGKPPKGERPFPEHKPPTPIGKPLKGEKPPSPFGKPITGEKPPHYGHTPGHPPAHSYKPPQKKKKK CS berries, 15,5-wpf
EST6162 158 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST5785 153 -----------------------------------------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, rip.
EST4872 164 PPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPPSPF------------------------------ Sh berries, rip.
EST2810 159 PPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPPSPFGKPPTGEKPPHYGHTPGHPPAH----------- Sh berries, ver.
Mrip1   181 PPTPIGKPTKGEKPLPEHKPPTPIGKPPKGEKP------------PSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Mer berries, 12-wpf
Grip15  181 PPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPLPEHKPPTPIGKPPKGEKPPSPFGKPPTGEKPPHYGHTPGHPPAHSYKPPQKIKPPP Sh berries, 10-wpf

EST7923  73 TPPP-PSHPTNHHL-PPIPTE--
EST7822  76 TPPHKPPPQN-----PPFPPYPN
EST8322     ----------------------
EST3815 141 TEEKKPWAPHHKHFKAPPPIHAN
EST4387  95 TPPHKP--PH----KPPSPTYPN
EST4289  95 TPPHKP--PH----KPPSPTYPN
EST8587 101 TPPHKP--PH----KPPSPTYPN
EST4284 102 TPPHKP--PH----KPPSPTYPN
EST4675 109 TPPHKP--PH----KPPSPTYPN
EST2891 120 TPPHKP--PH----KPPSPTYPN
EST4737 134 TPPHKP--PH----KPPSPTYPN
EST0032     ----------------------
EST5768 151 TPPHKP--PH----KPPSPTYPN
EST5968 177 TPPHKP--PH----KPPSPTYPN
EST6153 167 TPPHKP--PH----KPPSPTYPN
EST8207 202 KKKH------------------
EST6162 196 TPPHKP--PH----KPPSPTYPN
EST5785 191 TPPHKP--PH----KPPSPTYPN
EST4872     ----------------------
EST2810     ----------------------
Mrip1   252 TPPHKP--PH----KPPSPTYPN
Grip15  271 TPPHKP--PH----KPPSPTYPN
```
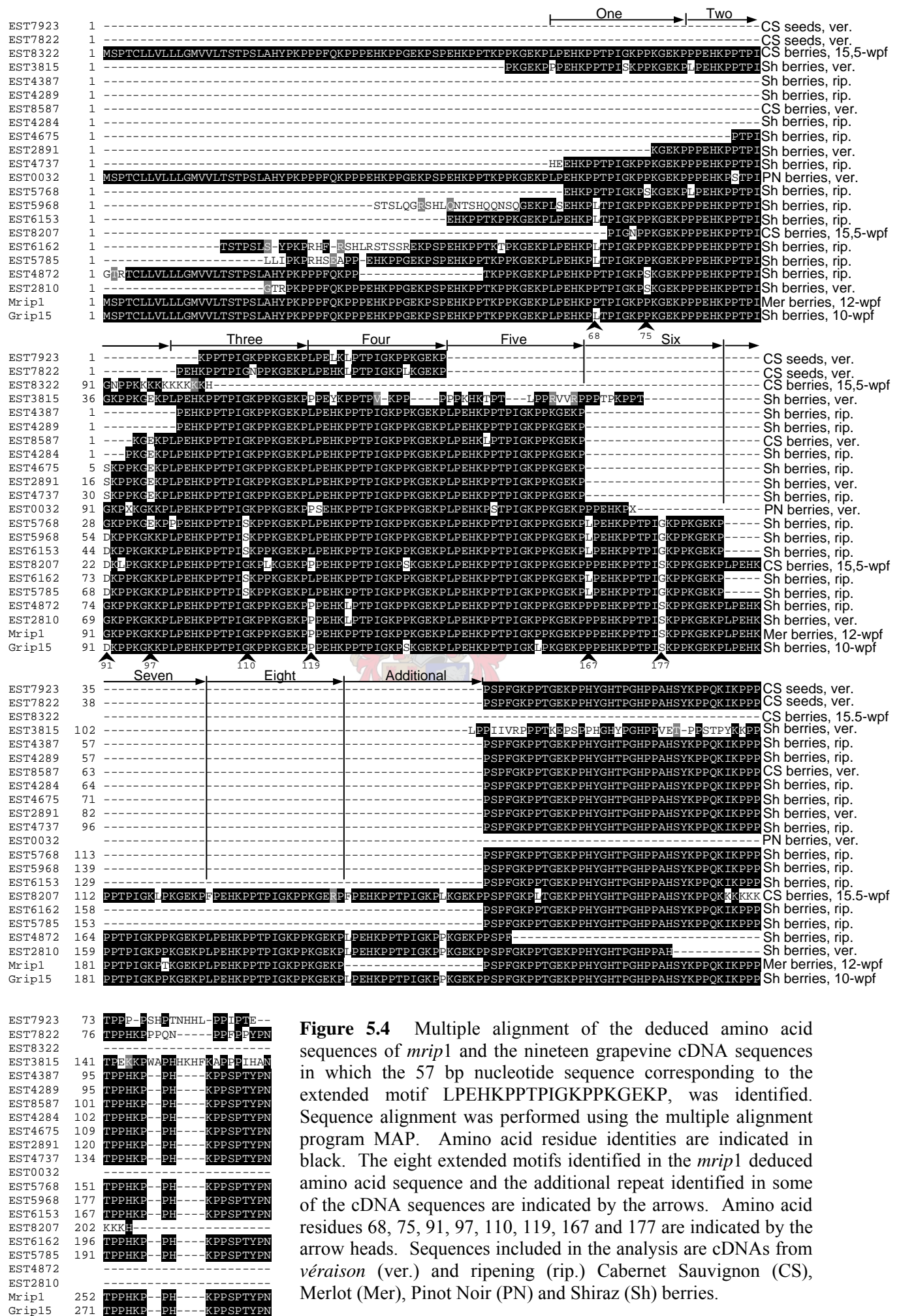
Figure 5.4 Multiple alignment of the deduced amino acid sequences of *mrip*1 and the nineteen grapevine cDNA sequences in which the 57 bp nucleotide sequence corresponding to the extended motif LPEHKPPTPIGKPPKGEKP, was identified. Sequence alignment was performed using the multiple alignment program MAP. Amino acid residue identities are indicated in black. The eight extended motifs identified in the *mrip*1 deduced amino acid sequence and the additional repeat identified in some of the cDNA sequences are indicated by the arrows. Amino acid residues 68, 75, 91, 97, 110, 119, 167 and 177 are indicated by the arrow heads. Sequences included in the analysis are cDNAs from *véraison* (ver.) and ripening (rip.) Cabernet Sauvignon (CS), Merlot (Mer), Pinot Noir (PN) and Shiraz (Sh) berries.

The most obvious difference between the sequences is the deletion of one of the extended motifs in mrip1 and the three, four and five-fold deletion of the motif in the other sequences (Fig. 5.4). Sequence homology analysis revealed that particular single amino acid differences correspond to the number of deletions identified, and that sequences could be categorised according to the level of sequence identity to *mrip*1. The cDNA sequences, in which no or four deletions of the extended motif could be identified, revealed the highest level of identity to *mrip*1 (Table 5.3). With the exception of EST8207, sequences with a lower level of identity revealed a three-fold deletion of the extended motif (Table 5.3). Complementary DNA sequences with no or a single deletion of the extended motif, are characterised by a proline residue in positions 119 and 167 of the mrip1 sequence. No correlation between the number of deletions identified in the deduced amino acid sequences and phenotypic data like grape cultivar or ripening stage of berries from which the mRNA was extracted was evident. Although the cDNAs from grape seeds did not contain the extended motif LPEHKPPTPIGKPPKGEKP, they revealed a high level of sequence identity to mrip1 (EST7822 - 91.9 %; EST7923 – 94 %) (accession numbers BQ792917 and BQ793018, respectively).

Table 5.3 Correlation found between the level of sequence identity, single amino acid differences and the number of deletions identified in the deduced amino acid sequences of ten cDNAs showing a high level of sequence identity to *mrip*1. Sequence identity is expressed as the number of identities in sequences corresponding to the central region of *mrip*1 (amino acids 62 to 213), excluding the deletions identified. Positions of single amino acid differences, differentiating cDNAs with none or one, and three or four of the extended motifs, are indicated in brackets.

cDNA	Sequence identity (%)	Number of deletions identified	Positions of amino acid residues (position of the amino acid residue in the *mrip*1 deduced amino acid sequence)
*mrip*1	-	One	Proline (119 and 167)
EST4872	98.68	None	
EST2810	98.02	None	
EST0032	97.03	Sequence to short to confirm absence or presence of deletions	
*Grip*15	96.7	None	
EST5768	93.8	Three	Serine (110)
EST5785	93	Three	Lysine (167)
EST5968	93	Three	Glycine (177)
EST6153	93	Three	
EST6162	93	Three	
EST8207	93.75	None	
EST2891	97.56	Four	Serine (91)
EST4675	97.18	Four	Glutamate (97)
EST4737	96.77	Four	
EST4387	Sequences much shorter than the rest. Sequence identity not calculated.	Four	
EST4289		Four	
EST8587		Four	
EST4284		Four	

Apart from the grapevine sequences, the pentapeptide motif PEHKP could only be identified in the deduced amino sequence of two plant cDNAs – a cDNA accumulating in carrots roots in response to wounding [23], and a cDNA from developing rose petals characterised by rapid petal elongation and accumulation of pigments (accession number BQ106587). Both sequences contained two repeats of the motif. Other sequences containing this motif included protein sequences from an insect diffusible secreted glycoprotein (accession number AAF98772) and an apical membrane antigen (accession number B44986). Homology analysis revealed that these sequences do not have any significant identity to mrip1.

5.4.3.2 Nucleotide sequence analysis

5.4.3.2.1 Codon usage in *mrip*1, *grip*15, EST2810 and EST4872

Although mrip1, grip15, EST2810 and EST4872 reveal high sequence identity, codon usage for glutamate, histidine, lysine and proline – which collectively account for 68,25 % of the total number of amino acid residues in the mrip1 sequence – revealed that *mrip*1 is closer related to *Grip* 15. For EST2810 and EST4872, the preferred codon for histidine is <u>CAT</u> (80 % and 100 %, respectively), followed by <u>CAC</u> (20 % and 0 %, respectively). On the contrary, codon preference for histidine in *mrip*1 and *grip*15 is not nearly as biased (58.82 %, <u>CAT</u> and 41.18 %, <u>CAC</u>).

5.4.3.2.2 Analysis of 3' UTR regions

Sequence analysis of the 3' UTR regions of *mrip*1, *grip*15 and the cDNAs in which three and four deletions of the extended motif were identified (Table 5.3), revealed that these sequences were almost identical (Fig. 5.5). Even the 3' UTRs of the two cDNAs from seed displayed this high level of sequence identity to mrip1. A single nucleotide difference 79 bp downstream of the stop codon, classified these cDNAs in two groups. Sequences with a guanidine in this position included *mrip*1, the three cDNAs in which four deletions of the extended motif were identified, and the two cDNAs from seed. Sequences with a thymidine in this position included *Grip*15 and the five cDNAs in which three deletions of the extended motif were identified.

```
        79
        ▼
Grip15   CCTTTCCTCTGGGTTGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST4737  CCTTTCCTCTGGGTGGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST2891  CCTTTCCTCTGGGTGGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST5768  CCTTTCCTCTGGGTTGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST5785  CCTTTCCTCTGGGTTGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST6162  CCTTTCCTCTGGGTTGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST6153  CCTTTCCTCTGGGTTGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST5968  CCTTTCCTCTGGGTTGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST4675  CCTTTCCTCTGGGTGGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST7822  CCTTTCCTCTGGGTGGATAGTGTTCTATGTATGAGTCTTTACGACGCCCTGTTGGCAATTGCTATGAGTT
EST7923  CCTTTCCTCTGGGTGGATAGTGTTCTATGTATGAGTCTTTACGACGCCCTGTTGGCAATTGCTATGAGTT
Mrip1    CCTTTCCTCTGGGTGGATAGTGTTCTATGTATGAGTCTCTACGACGCCCTGTTGGCAATTGCTATGAGTT
```

Figure 5.5 Partial 3' UTR sequence of grapevine proline-rich protein cDNA sequences indicating how the cDNAs are differentiated by the single nucleotide substitution 79 bp downstream of the stop codon. The substitution is indicated by the arrowhead.

5.4.4 Genomic analysis

Merlot genomic DNA was digested with eight different restriction endonucleases and probed with the 800 bp 5'-RACE fragment. Analysis performed under high-stringency conditions (0.1 x SSC, 65 °C) revealed a maximum of three fragments strongly hybridising to the probe (Fig. 5.6). As the RACE fragment used as probe do not contain restriction sites for any of the restriction endonucleases used, it is likely that these fragments represent the whole gene. The *Pst*I restriction site in the *mrip*1 3' UTR region (Fig. 5.1), implicates that the three fragments detected in the *Pst*I digest does not include sequences corresponding to *mrip*1 3' UTR sequences. The relative simple hybridisation pattern suggests that *mrip*1 is present in low copy number in the Merlot genome. The presence of other bands with a much lower hybridisation signal indicates the presence of genes with some level of sequence similarity to *mrip*1 in the Merlot genome.
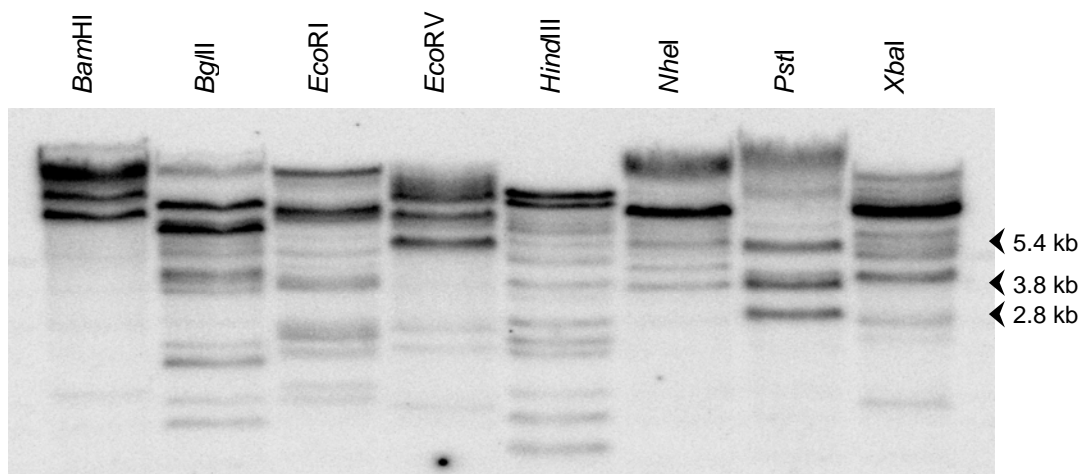
Figure 5.6 Southern blot analysis of *mrip*1 in the Merlot genome. Genomic DNA (8 µg) was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Nhe*I and *Xba*I, and probed with the 800 bp 5'-RACE fragment. Hybridisation washes were performed at high stringency conditions. The size of the fragments is indicated by the arrowheads.

5.4.5 *Mrip*1 transcription in ripening fruit and vegetative tissues

5.4.5.1 In grape berries and vegetative tissues

Transcription of *mrip*1 is ripening-related. In the Merlot grape berry, *mrip*1 transcription is initiated 10-wpf, shortly after *véraison* (characterised by the onset of glucose and fructose accumulation) and coinciding with the cessation of berry growth (Fig. 5.8A and B). The *mrip*1 transcript accumulates rapidly to the high levels visualised in 12- and 14-wpf berries, when glucose and fructose concentrations have reached a plateau. This is followed by a decrease in the level of *mrip*1 in fully matured 16-wpf berries. In Chardonnay, regulation and abundance of the *mrip*1 transcript in ripening berries was shown to be similar to that observed in Merlot.

*Mrip*1 mRNA is not expressed in flower or leaf tissue, but accumulates in maturing seeds 10- to 16-wpf and bunchstems of 10-wpf grape bunches (Fig. 5.8A and C). Although at a low level, *mrip*1 is expressed in immature 4- to 8-wpf seeds, which is different to the regulation of *mrip*1 transcription in ripening berries where no *mrip*1 mRNA could be detected in 2- to 8-wpf berries. The level of *mrip*1 transcription in bunchstems appears similar to that in fully matured 16-wpf berries. Two poorly-labelled fragments were detected in total RNA from roots.

Compared to *grip*15, the regulation of *mrip*1 during Merlot and Chardonnay berry ripening is distinctly different (Table 5.4).

5.4.5.2 In strawberry and tomato fruit

No *mrip*1 mRNA or *mrip*1-related mRNAs could be detected in ripening strawberry or tomato fruit (Fig. 5.7 and 5.8C). A single poorly-labelled fragment was visualised in total RNA from leaf material, which suggests the transcription of a gene with some sequence similarity to *mrip*1 (Fig. 5.8C). Northern blot analysis performed at lower stringency did not provide any evidence of *mrip*1-related genes in strawberry or tomato (Fig. 5.7).
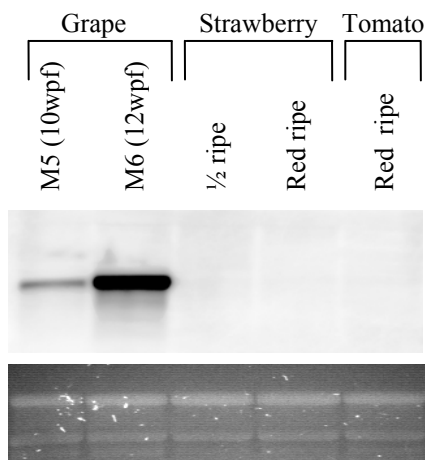
Figure 5.7. *Mrip*1 transcription in ripening grape, strawberry and tomato fruit. *Mrip*1 transcription was visualised by using the 800bp 5'-RACE as probe. The membrane was washed at low stringency conditions. Bottom panel: Total RNA visualised in ethidium-stained-gels to demonstrate the intactness and relative loadings of the RNA samples used in Northern blot analysis.
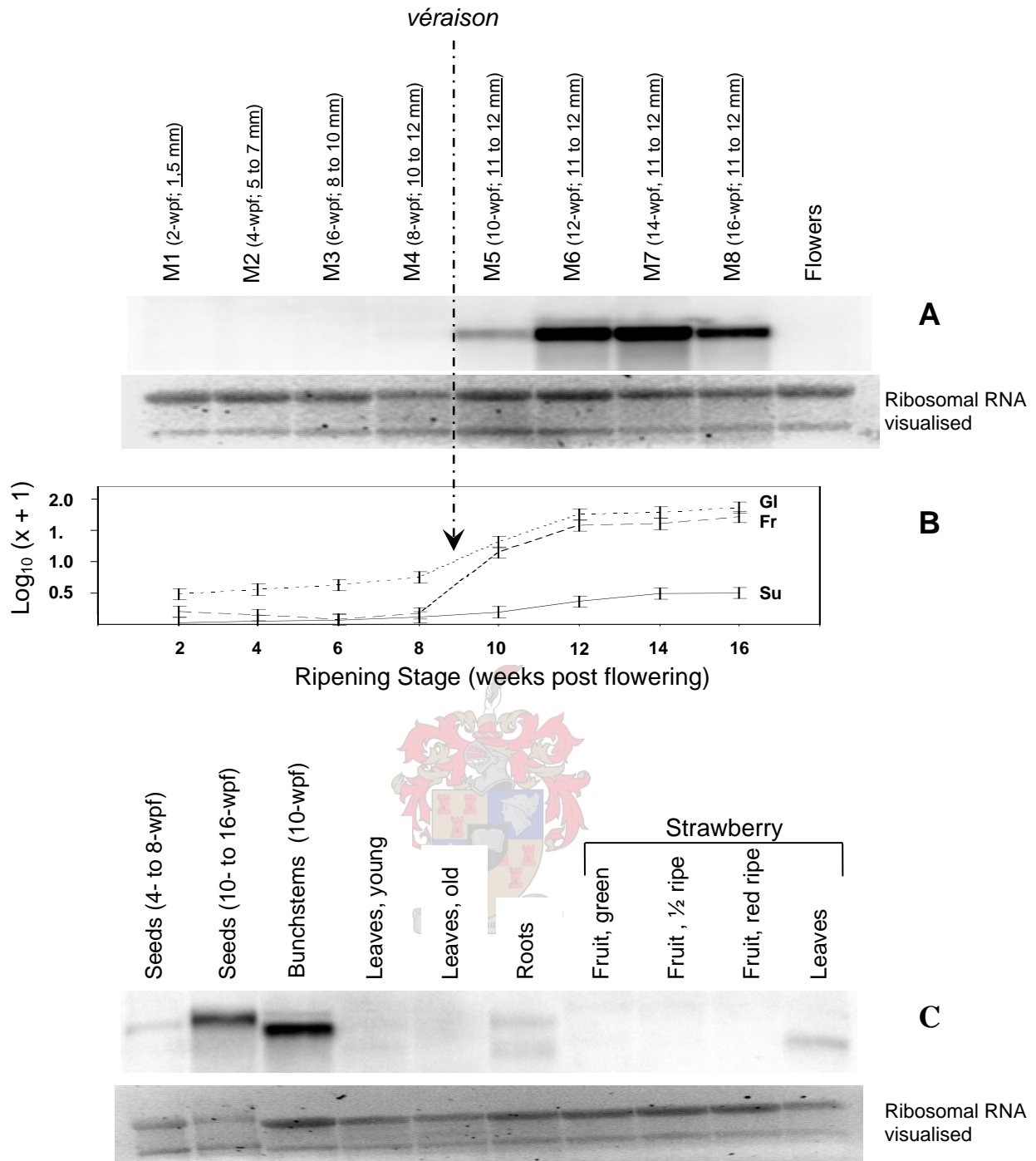
Figure 5.8 M*rip*1 transcription in ripening berries and vegetative tissue of grapevine, cv. Merlot and strawberry. *Mrip*1 transcription was visualised by using the 800 bp 5'-RACE fragment as probe. **A:** Total RNA from grape berries collected at two-week intervals, starting at 2-weeks post flowering (wpf) and continued until berries reached full maturity, 16-wpf (M1 – M8). The berry size (diameter) is underlined; **B:** Fructose (Fr), Glucose (Gl) and Sucrose (Su) concentration of ripening, 2- to 16-wpf grape berries as determined by HPLC analysis. The scale of measurement of raw data ($\mu mol.g^{-1}$ fresh weight) has been transformed to $\log_{10}(x+1)$, as is appropriate for growth stage data of this nature which include fractions smaller than 1; **C:** Total RNA from young (collected from immature, 4- to 8-wpf berries) and mature grape seeds (collected from maturing, 10- to 16-wpf berries), bunchstems of 10-wpf grape bunches, young and old grape leaves, and roots of hydroponically grown grape plants. Total RNA from strawberry includes that from green, half ripe (half green, half red) and full red ripe fruit, and young leaves. **Bottom panels**: Ribosomal RNA visualised in ethidium-bromide stained gels to demonstrate the intactness and relative loadings of the RNA samples used in Northern blot analysis.

Table 5.4 A comparison of the transcription levels of *mrip*1 and *grip*15 in various grape tissues. Detectable levels of the transcript, as measured by Northern blot analysis, is indicated by the (+). Differences between the transcription levels of *mrip*1 and *grip*15 are highlighted by the shaded boxes.

Tissue type	*Mrip*1[1]	*Grip*15[2]
Flower	-	-
Berry:		
2 to 8-wpf	-	-
10-wpf	+, Low level of the transcript	+, Abundant levels of the transcript
12-wpf	+, Abundant levels of the transcript	-
14-wpf	+, Abundant levels of the transcript	-
16-wpf	+, Lower level of the transcript	-
Seeds:		
4 to 8-wpf	+, Very low level of the transcript	-, 4-wpf
10 to 16-wpf	+, High level of the transcript	Not shown
Leaf	-	-
Root	Two poorly-labelled fragments detected.	-

[1]As shown in both Merlot and Chardonnay.
[2]From Davies and Robinson [15].

5.4.6 Localisation of *mrip*1

In situ hybridisation analysis confirmed that the transcription of *mrip*1 is ripening-related. No *mrip*1 mRNA could be detected in berries 2-wpf (Fig. 5.9A), whilst 8-wpf berries were characterised by low levels of *mrip*1 (Fig. 5.9B and I). In 14-wpf berries the *mrip*1 transcript was abundant (Fig. 5.9C). As shown by the arrows in figure 5.9B, C and I, the *mrip*1 antisense probe hybridised to the transcript in cells only throughout the mesocarp and exocarp of 8-wpf (Fig. 5.9B and I) and 14-wpf berries (Fig. 5.9C).

No hybridisation of the antisense strand probe to cross-sections of grape root and leaf tissue could be detected (Fig. 5.9G and J).

Control experiments using the sense strand probe showed very low levels of background hybridisation (Fig. 5.9D, E, F, J and K).
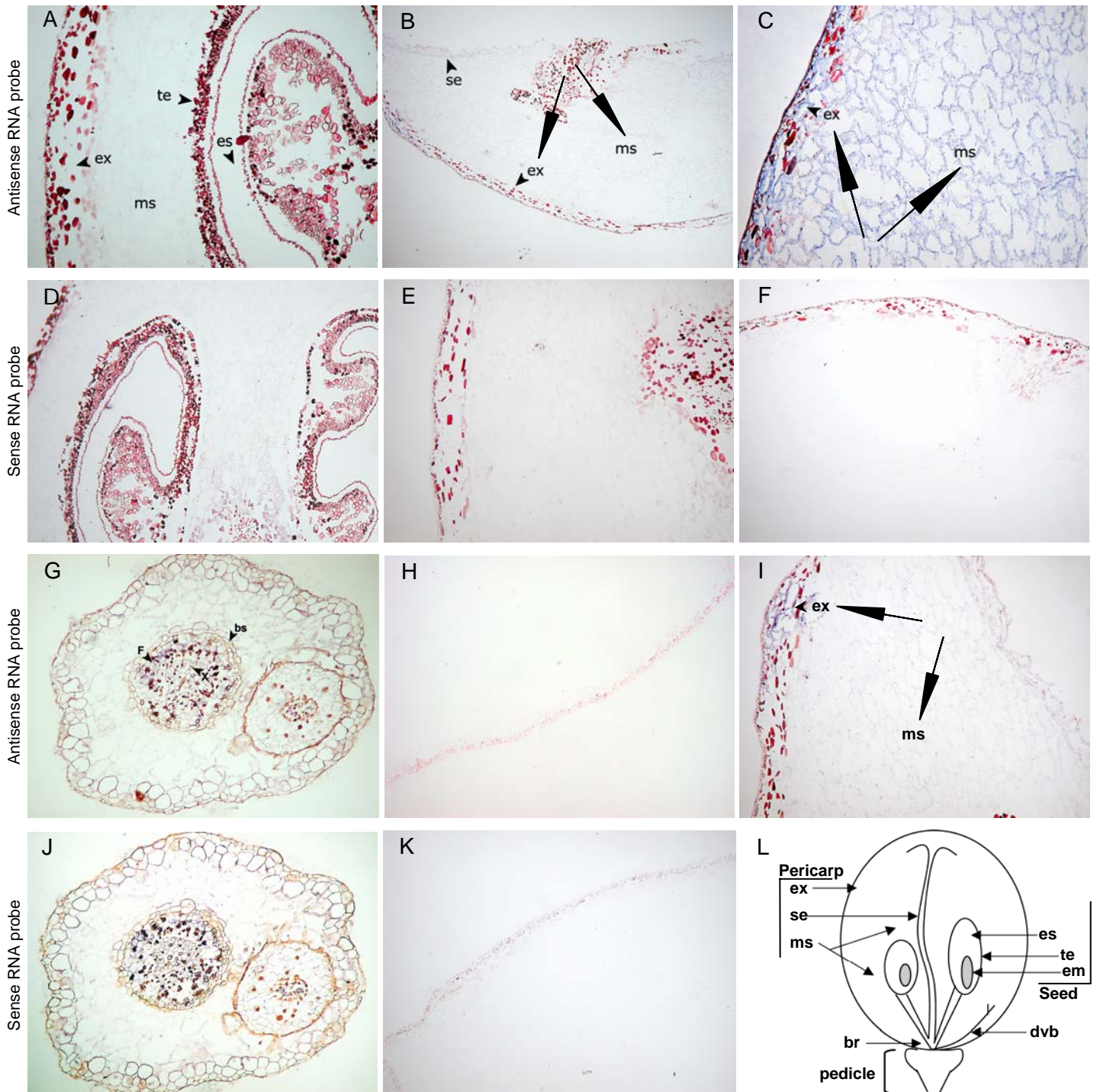
Figure 5.9 Localisation of *mrip*1 mRNA in Merlot berry, leaf and root tissue. Hybridisation of the single-stranded *mrip*1 RNA probe was visualised by immunocytochemistry using alkaline phosphatase-conjugated anti-DIG Fab fragments. Hybridisation was observed as blue areas and is indicated by the arrows. **A**, **B**, **C** and **I:** Cross-sections of berries 2-wpf (**A**), 8-wpf (**B and I**) and 14-wpf (**C**) hybridised with the antisense probe; **I:** Magnification of a cross-section of a 8-wpf berry hybridised with the antisense probe; **D**, **E** and **F:** Cross-sections of berries 2-wpf (**D**), 8-wpf (**E**) and 14-wpf (**F**) hybridised with the sense probe; **G and J:** Cross-section of root tissue hybridised with antisense (**G**) and sense probe (**J**) **H and K:** Cross-sections of leaf tissue hybridised with antisense (**H**) and sense probe (**K**); **L:** a diagrammatic longitudinal cross-section of a grape berry showing the location of the different cell types (*Courtesy M. Venter)*; bs = bundle sheath, br = brush, dvb, dorsal vascular bundle, em = embryo, es = endosperm, ex = exocarp, F = phloem, ms = mesocarp, se = septum, te = testa, x = xylem.

5.5 DISCUSSION

Mrip1 is a distinct member of the plant PRP family

Despite having features common to proline-rich proteins, such as a hydrophobic putative leader sequence, a high proline content and the repetitive nature of the pentapeptide motifs, the Merlot PRP is clearly different from the other reported plant PRPs. Also, mrip1 does not fit any of the three groups of PRPs described by Josè and Puigdomènech [27]. Although mrip1 contains a bipartite structure, the proline-rich repeats are limited to the central region, and the C-terminus region is hydrophilic and not cysteine-rich. The only PRP with similar features, is the potato guard cell PRP, StGCPRP [21]. Like mrip1, StGCPRP did not fit any of the three groups of PRPs and was classified a hybrid PRP. Unlike StGCPRP, the N-terminal region of mrip1 is not proline-poor. It can therefore be concluded that mrip1 represents a member of a group of PRPs undoubtedly different from the groups described by Josè and Puigdomènech, but similar to the hybrid PRP, StGCPRP.

The mrip1 is characterised by a high level of proline, glutamate, histidine and lysine. This is unlike most of the PRPs which are characterised by high levels of valine, tyrosine and the absence (or low levels) of glutamate and histidine [6]. The only two exceptions include ENOD2, a cDNA for nodulin-75 of soybean root nodules [24] and ENOD2, a cDNA clone from nodules of *Maackia amurensis* [26]. Mrip1 is however distinguished from both the soybean and *M. amurensis* proteins by a strongly positive charged proline and lysine-rich region, pI 10.7.

The most outstanding characteristic of mrip1 is the tandem nature of the eight extended proline-rich motifs identified in the hydrophilic central region of the putative protein. Although the function of the repetitive motifs is not clear, the clustered nature of the glycine–proline–proline motifs could indicate that the motifs have a structural function [6]. Physical properties of the glycine-proline sequences will allow the polypeptide backbone to fold into a tight U-shaped structure, whilst molecular modelling has shown that tandem repeats of proline-rich repeats frequently form β-turn helices. These multiple repeats of glycine-proline are similar to the glycine-proline-hydroxyproline repeats of collagen and elastin. Considering the characteristic nature of this region - the presence of the proline-rich repeats, the strongly positively charged nature, all single amino acid differences and 19 amino acid residue deletions or insertions, limited to this region - it is proposed that this region is involved in aspects of cell wall composition and structure different from the C-terminal region.

The hydrophilic nature of both the central and the C-terminal region implies that the mrip1 is not "locked" into the membrane, but suggests an extended structure that may interact with itself and/or with other cell wall components through ionic interactions. Based on the relatively high histidine

content of the mrip1 C-terminal region, and a hypothesis by Baldwin and co-workers that the basic histidine-rich region of the carrot arabinogalactan protein confers pectin-binding properties [13], is possible that mrip1 is involved in pectin "gelling" and intercellular adhesion. This possibility is further supported by the basic isoelectric point predicted for mrip1 (pI 10.7), which implies that it may interact with the acidic pectin network within the cell wall [13]. An interesting observation is the sequence consensus observed within the C-terminal region of most the grapevine cDNAs analysed in this study.

Mrip1 constitutes part of a grapevine PRP family

The large number of grapevine cDNAs with high sequence identity to *mrip*1, indicates that *mrip*1 constitutes a member of a multigenic PRP family in grapevine. Based on single amino acid differences, deletions of one or more of the 19 amino acid residues motifs and single nucleotide differences in the 3' UTR regions, these cDNA sequences could be differentiated in two or three groups. As these differences do not simply represent varietal differences within the same gene, further characterisation may prove that they constitute different members of the grapevine PFP family. It is suggested that the relative simple pattern of genomic fragments, showing strong hybridisation to the *mrip*1 probe, is representative of these closely related members of the Merlot PRP gene family. The existence of a multigenic PRP family in the Merlot genome is in accordance with evidence that dicotyledonous plant PRPs are encoded by multi-gene families, each gene characterised by a distinct pattern of regulation [38, 39].
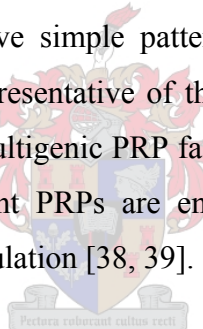
In addition to the sequence variation between *mrip*1 and *grip*15 (five amino acid differences and the deletion of one of the extended motifs), the regulation of *mrip*1 in Merlot and Chardonnay is distinctly different from the regulation of *grip*15 in Shiraz. Studies in grapevine has shown that despite high identity within the translated regions of the stilbene synthase and alcohol dehydrogenase genes, these genes are differently regulated (*Vst1* and *Vst2* – 93 % identity; Vv*Adh*1, Vv*Adh*2, Vv*Adh*3 – 77.9 to 80 % identity). They were confirmed isogenes of the *Vst* and *Adh* gene families, based on substantial differences in the 5'- and 3'-UTRs of these genes [36, 37]. It is therefore proposed that *mrip*1 constitutes a novel member of the grapevine PRP family. To clarify this issue, further studies should be aimed at the isolation and characterisation of the *grip*15 5' UTR flanking region. The high sequence identity of the two sequences and the repetitive nature of the extended motifs, would make differentiation by the use of sequence-specific probes or primers, extremely difficult.

Transcription of mrip1 is ripening-related, and associated with cessation of cell expansion

Although *mrip*1 constitutes a member of a closely related Merlot PRP family, and the observed transcript levels could reflect transcription of more than one gene, the data allow for some definite conclusions. If the data do reflect transcription of more than one gene, no transcription of any one occurs prior to its initiation in 8-wpf berries. The *mrip*1 transcript only accumulates once the berries have developed to *véraison* and thereafter. Evidently, the onset of *mrip*1 transcription follows shortly after the onset of fructose and glucose accumulation and the change in the fructose:glucose ratio observed in 8- to 10-wpf berries, and coincides with the cessation of berry growth. The observation that the change in the fructose:glucose ratio coincides with the cessation of berry growth, corresponds well with the observation by Winkler *et al*. [40] that the change in the fructose:glucose ratio serves as indication of cessation of berry growth (cell expansion). Post-*véraison* berry ripening is associated with many physiological changes [41]. However, most noticeably is the accumulation of reducing sugars, decrease in total acidity [40, 42], cessation of growth and accumulation of water [40]. Consequently there is an increase in osmotic pressure [43]. Considering the proposed involvement of PRPs in cell wall rigidification, and the accumulation of *mrip*1 in the mesocarp and exocarp cells of the ripening berry which is initiated at the onset of berry ripening, it is proposed that mrip1 could either be involved in the "locking" of the primary cell wall once cell elongation is completed, or in architectural changes to reinforce the thinning cell walls of the mesocarp and pericarp cells as the principal growth of the berries is by cell enlargement, which results in the thinning of the cell walls.
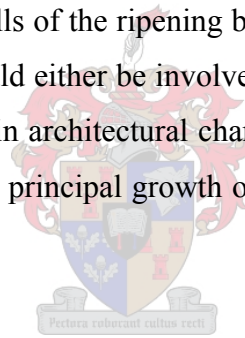
The strong accumulation of *mrip*1 in maturing seeds coincides with the cessation of seed growth and the initiation of secondary cell wall metabolism in seeds [44]. Since the mature embryo occupies only a small part of the seed, the abundance of *mrip*1 in maturing seeds is most probably representative of the presence of the *mrip*1 transcript in the endosperm or integument tissue of the seed. Considering the proposed involvement of PRPs in cell wall rigidification, it is especially in the integument with its lignified cells, and cells with spiral thickenings and irregular thickenings on the radial walls, that mrip1 could play a role. Accumulation of *mrip*1 in seeds and berries, suggests that *mrip*1 is transcriptionally activated by elements common to berries and seeds.

Mrip1 is specific to particular cell types

Neither transcription analysis in ripening strawberry and tomato fruit, nor sequence homology searches, revealed any sequences other than grapevine cDNAs, with significant homology to *mrip*1. This, despite the large number of cDNAs identified in ripening strawberry and tomato fruit [45-49]. These results indicate either a low level of sequence identity between grapevine, strawberry and

tomato *mrip*1-like PRPs, or that *mrip*1 is specific to particular fruit kinds or cell types. It is suggested that *mrip*1 is not common to ripening in fruit, but probably specific to particular fruit kinds with cell types similar to the mesocarp and exocarp cells of the ripening grape berry.

Referring to the proposed function of PRPs in the rigidification of the plant cell wall, the presence of the *mrip*1 transcript in ripening grape berries, and the absence of sequences with significant homology to *mrip*1 in strawberry or tomato, attention was consequently drawn to the classification of these fruits based on the swelling properties of their cell walls during fruit ripening. In strawberry and tomato - which develop a soft melting texture during ripening - swelling and softening of the cell wall (due to the dissociation of the pectin matrix) is evident [50]. In grapes, and other fruits such as apple, pear and watermelon - which ripen to a crisp, fracturable texture - no cell wall swelling is observed [2, 50]. The presence of *mrip*1 in grape berries, where pectin 'gelling' and intercellular adhesion is evident, but not in strawberry and tomato, in which ripening is characterised by the dissociation of the pectin matrix, suggests a potential role for mrip1 in this aspect of cell wall architecture.

Further studies in which *mrip*1 will be down regulated, should shed light on the role of *mrip*1, and could open potential applications for the use of this gene in the control of cell wall architecture in fruits. However, as manipulation of fruit properties in grape berries will be most important in the later stages of ripening, *mrip*1 is an ideal candidate gene for the isolation of a fruit- and ripening-specific promoter to regulate transgene transcription in genetically modified grapevine.
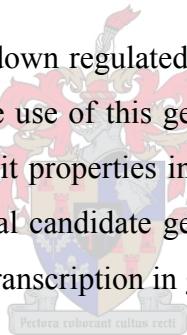
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CHAPTER 6

Grapevine promoter directs gene expression

in the nectaries of transgenic tobacco.

Research Results

Grapevine promoter directs gene expression in the nectaries of transgenic tobacco

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6.1 ABSTRACT

A major obstacle in the genetic manipulation of fruit ripening in grapes, remains the lack of regulatory elements (promoters) to regulate transgene transcription in the ripening berry. In this study, the gene encoding the grapevine ripening-induced proline-rich protein, *mrip*1, was used to isolate and characterise a tissue-specific, ripening-related promoter from grapevine. Sequence analysis of a 5.5 kb sequence corresponding to *mrip*1 5' untranscribed flanking region (UTR), revealed the presence of a proline-rich protein directly upstream of *mrip*1. In the 2.8 kb upstream of the *mrip*1 transcription initiation site, several putative *cis*-acting regulatory elements were identified. These include a spectrum of hormone-, light-, phytochrome-, sugar-and stress-responsive elements, as well as elements involved in tissue-specific transcription. A 2.2 kb fragment of the *mrip*1 5' UTR flanking region was selected for functional analysis of the *mrip*1 promoter. Functionality of *mrip*1:*sgfp*S65T fusion gene was confirmed by transient expression in green pepper pericarp tissue, before introduction into tobacco by *Agrobacterium*-mediated transformation. In transgenic tobacco, transcription of the mri*p*1:*sgfp*S65T fusion was developmentally-regulated and specific to the ovary and nectary-tissue-specific of the developing flower. Whilst low in immature flowers, GFP rapidly accumulated to the high level of expression visualised in the flower in full-bloom, followed by a decrease in the final stages of ovary development. These observations suggest that the 2.2 kb *mrip*1 promoter is functional and that this promoter region harbours *cis*-elements necessary for tissue- and developmental-specific regulation of GFP expression. It furthermore suggests that the transcriptional activation of *mrip*1 is mediated by developmental signals present in both grapevine berries and tobacco flowers.

Key words: Grapevine, ripening-related promoter, ripening-related proline-rich protein, fruit ripening, transgenic tobacco, green fluorescent protein (GFP)

6.2 INTRODUCTION

Fresh or processed fruits form an essential part of the human diet, providing essential nutrients and health promoting compounds (Tucker, 1993). As such, there is an ever-increasing demand for improved quality, including aesthetic attributes like flavour, colour and texture. As the molecular era opened new avenues for plant improvement by genetic engineering, such improvements can be achieved by a thorough understanding of the biochemical and molecular basis of fruit ripening, and mechanisms to manipulate or regulate gene transcription during fruit development.

Commercially, trade is dominated by a relatively small number of fruit – with grape, banana, citrus, pome (apples and pears) and tomato being of greatest importance. However, with nine million hectares of vineyards producing about 60 million tonnes of fruit in the early 1990's, the grape is considered the world's most important fruit crop. Its uses include wine-making, distilled liquors, fresh consumption (table grapes), dried fruit (raisins), juice and concentrate, rectified must, and limited industrial products. Wine-making is however the most important use, with a total of 250 to 300 million hectolitre of wine produced in the world annually (Kanellis and Roubelakis-Angelakis, 1993; Robinson, 1994). In recent years, the value and competitive nature of the grape and wine industry led to an increasing emphasis on producing quality fruit for the production of quality wines.

As such, the grape berry is one of the main targets for the genetic manipulation of fruit quality traits. In recent years, several cDNA clones responding to ripening-related genes, have been isolated from grape (Ablett *et al*., 2000; Davies and Robinson, 2000; Terrier *et al*., 2001; Venter *et al*., 2001). A major obstacle in the genetic manipulation of fruit ripening in grapes, however, remains the lack of regulatory elements (promoters) to regulate transgene transcription in the ripening berry (Botha, 1999; Boss and Davies, 2001). As the most dramatic changes in the characteristics that determine the quality of the final product (wine) occur as the fruit enters into the ripening phase, ideally transgene transcription should be regulated to the post-*véraison* stages of berry ripening ("*véraison*" refers to the onset of berry ripening). To date, the isolation and characterisation of only two fruit-specific, late ripening-related promoters have been reported, *viz*. tomato *E4* and *E8* (Deikman *et al*., 1992; Montgomery *et al*., 1993; Coupe and Deikman, 1997). Although a number of genes that are transcriptionally activated at the onset of berry ripening have been reported (Lincoln and Fischer, 1988; Deikman *et al*., 1992; Rose *et al*., 1997; Tattersall *et al*., 1997; Civello *et al*., 1999; Davies and Robinson, 2000; Harrison *et al*., 2001), the isolation of their promoter sequences has not been described.

In this study, the gene encoding the grapevine ripening-induced proline-rich protein, *mrip*1, was used to isolate and characterise a tissue-specific, ripening-related promoter from grapevine. Although characterisation of proline-rich proteins expressed during plant development is well documented (Josè and Puigdomènech, 1993; Showalter, 1993; Sommer-Knudsen, 1997), very little is known about their regulation; particularly, during fruit ripening. Factors known to affect PRP expression during plant development include abscisic acid (Josè-Estanyol *et al*., 1992; Josè and Puigdomènech, 1993; Guo *et al*., 1994; Deutch and Winicov, 1995; Anderson, 2001), auxin (Satoh and Fujii, 1988; Davis *et al*., 1997), sucrose (Banfalvi *et al*., 1996) and environmental factors such as wounding, elicitors and infection (Josè and Puigdomènech, 1993; Milioni *et al*., 2001). Interestingly, most of these factors have also been implicated in fruit ripening (Kanellis and Roubelakis-Angelakis, 1993).

Here, a 5.5 kb sequence corresponding to 5' untranslated flanking region (UTR) of *mrip*1 was characterised by nucleotide sequence analysis, restriction endonulease mapping and homology analysis. For functional analysis of the *mrip*1 promoter, a 2.2 kb fragment directly upstream of the *mrip*1 transcription initiation site was translationally fused to the green fluorescent protein (GFP) reporter gene. Functional analysis of the fusion gene was performed by transient expression analysis by particle bombardment of intact green pepper pericarp tissue. For functional analysis of the fusion gene in stably transformed plant, the gene was introduced into tobacco. Compared to the low genetic transformation efficiency, slow regeneration and long reproductive life cycle of grapevine (Torregrosa *et al*., 2001; Kikkert *et al*., 2001), genetic transformation of tobacco is highly efficient, transgenic shoots are rapidly regenerated (about 4 months) and the reproductive cycle is short. Observations that the genetic mechanisms effecting developmental and tissue-specific control are evolutionarily conserved between many dicotyledon species (Lin *et al*., 1993), and that fruit developmental regulation of the kiwifruit actinidin promoter is reserved in transgenic petunia, prompted us to evaluate tobacco as an alternative system for the functional analysis of a ripening-promoter from grapevine.

6.3 RESULTS

6.3.1 Characterisation of the 5.5 kb *mrip*1 5' UTR flanking region

Sequences of the first 700 bp upstream of the *mrip*1 ATG translation start codon of the four clones HindIII[4.2], BglII[4.5], NheI[5.1] and XbaI[5.5], are identical. The putative *mrip*1 transcription initiation site (TIS) is 28 bp upstream of the ATG translation start codon. A putative TATA box sequence (TATAAAA) was identified 24 bp upstream of the TIS, and the promoter element CAAT, 96 bp further upstream from the putative TATA-box. Relative to the *mrip*1 TIS, the position of both the putative TATA box sequence and promoter element CAAT is within the conserved range identified for plant promoters (Joshi, 1987).

Analysis of the A/T content of the 5.5 kb sequence revealed two A/T-rich regions (Fig. 6.1C, areas A - B, and E - I). A region with an A/T content 30 % lower than that of the first two, was identified 3.58 to 4.5 kb upstream of the *mrip*1 TIS (Fig. 6.1C, regions C - D). Sequence analysis revealed a 99 % identity of this sequence with a cDNA clone from pre-*véraison* Cabernet Sauvignon berries, and 96 % sequence identity with *grip*13 from Shiraz (accession numbers CB979133 and VVI237983, respectively). No other sequences with significant sequence homology to the 5.5 kb sequence were revealed. The 5.5 kb *mrip*1 5' UTR flanking region sequence was deposited in the NCBI genetic database, accession number AY542687. The 2.8 kb *mrip*1 5' UTR flanking region, that is the region up to the *Pst*I restriction site, was selected for further characterisation of the *mrip*1 promoter region.
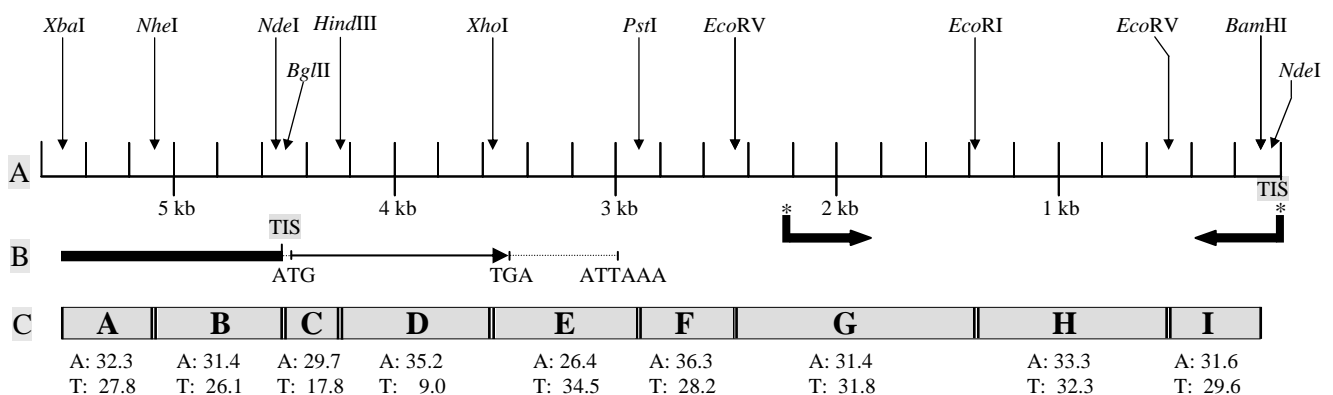

Figure 6.1. Characterisation of the 5.5 kb *mrip*1 5' UTR flanking region by restriction endonuclease mapping and nucleotide sequence analysis. **A, C**: Based on the restriction endonuclease sites *Bam*HI, *Bgl*II, *EcoR*I, *EcoR*V, *Hind*III, *Nhe*I, *Pst*I and *Xho*I, the 5.5 kb sequence were divided into nine areas (A – I). The A/T content of each of these areas is indicated as a percentage of the total number of nucleotides in the specific area. The solid arrows indicate the 2.2 kb fragment which was used to construct the *mrip*1:GFP fusion gene. **B**: Position of the *mprp2* coding region and 0.97 kb of its 5' UTR flanking region. The ATG start codon, TGA stop codon and a putative 3' polyadenylation signal are indicated. Transcription initiation sites (TIS) are indicated in the shaded boxes. The map is to scale, with the size indicated in kilobase pairs (kb).

6.3.2 Characterisation of the proline-rich protein located directly upstream of *mrip*1

The sequence revealed a single open reading frame of 1024 bp, encoding a proline-rich protein. The ATG translational start codon of this gene was 21 bp downstream of the *Bgl*II restriction site (Fig. 6.1A and B). A putative 3' polyadenylation signal, ATTAAA, was located 471 bp downstream of the stop codon (Fig. 6.1B). Sequence identity of *mprp*2 to the *mrip*1 coding sequence was 88 %. This gene was designated Merlot proline-rich protein 2, *mprp*2.

The 1 kb sequence upstream of the *mprp*2 ATG translational start codon, revealed a putative TIS 26 bp from the ATG start codon, and a TATA-like sequence (TATAAAA) and several putative *cis*-acting regulatory elements in the 974 bp 5' UTR flanking region.

6.3.3 Characterisation of the 2.8 kb *mrip*1 promoter region

A total of 23 promoters of genes transcribed in fruit tissue, and five promoters of genes encoding PRPs, were retrieved from the Genbank genetic database (Table 6.1). None of these showed significant sequence identity to the 2.8 kb *mrip*1 promoter sequence.

Two sequences, located respectively 751 bp and 1417 bp upstream of the *mrip*1 TIS, were present in several sequences in the NCBI non-redundant database. The first, a 26 bp sequence ATTTATGTATGTATGTGCATATAAAA, was present in twenty-eight nucleotide sequences from *Arabidopsis,* fruitfly, human and mouse genomic DNA sequences. The second, a 23 bp sequence TCTGTTTTTCATTTTTGTTTTGT, was present in seventeen sequences including DNA sequences from human and mouse chromosomal clones. BLAST sequence analysis did not provide any information on the identity or possible function of the two sequences. Nor could any putative *cis*-acting regulatory elements be identified in any of the two sequences.

Several putative *cis*-acting regulatory elements were identified in the 2.8 kb *mrip*1 promoter region. Elements identified included a spectrum of hormone-, light-, phytochrome-, sugar-and stress-responsive elements, as well as elements involved in tissue-specific transcription (Fig. 6.2). All the putative hormone-responsive elements are located in the 1.4 kb region flanking the 5' UTR. Two consensus sequences, BOXIINTPATPB, conserved in several NCII promoters of plastid genes, and MYBPLANT, a sequence related to box P in promoters of phenylpropanoid biosynthetic genes, were identified. Sequence homology analysis revealed the presence of the sugar-responsive element SURE1STPT21 in only six of the promoters listed in Table 6.2, *viz. Vvht*1, melon ACC-01, strawberry *pdc*1, *E8* and *MsPRP2*.

Table 6.1 Promoter sequences of genes transcribed in ripening fruit, and genes encoding proline-rich proteins, retrieved from the NCBI genetic database. The sizes of the sequences are indicated in base pairs (bp) and include 5' UTR and 5' UTR flanking region. Only promoter sequences longer than 250 bp were included in this list. Promoters of genes encoding proline-rich proteins are indicated in the shaded box.

| Crop	Promoter	Size (bp)	Genbank accession number
Grapevine	Alcoholdehydrogenase (GV-*Adh*1)	306	U36586
	Alcoholdehydrogenase (GV-*Adh*7)	390	AF195867
	Chitinase	403	AJ430782
	Dihydroflavonol reductase (*dfr)*	2278	AF 280768
	β-1,3-glucanase (not detected in grape berries)	922	AJ430781
	Hexose transporter 1 (Vv-*ht*1)	2441	AJ001062
	Leucoanthocyanidin dioxygenase (ldox)	2429	AF290432
	Inward rectifying potassium channel (SIRK)	2916	AF359522
	Stilbene synthase (*vst*-1)	1013	Y18532
	Sucrose transporter 2 (*VvSUT2*)	2184	AF439321
	UDP glucose:flavonoid 3-O-Glucosyltransferase (F1UFGT1)	458	AB047098
	Vacuolar pyrophosphatase (*vpp*)	1567	AJ544719
Apple	ACC oxidase	2056	AF030859
	Polygalacturonase (*MdPG*)	2629	AF031233
Avocado	Cellulase (*Cel*1)	1630	X59944
Kiwi	Actinidin	590	X57551
	Polygalacturonase (PG)	3200	L12019
Melon	ACC oxidase (*CM-ACO*1*)*	826	X95551
	ACC oxidase (*CM-ACO*3*)*	2300	X95553
Strawberry	Pyruvate decarboxylase (*pdc*1)	780	AF333772
Tomato	2A11	4752	M37631
	E4	1441	S44898
	E8	2160	AF515784
Alfalfa (*Medicago sativa*)	MsPRP2	1552	AF028841
Barrel medic (*Medicago truncatula*)	MtPRP4	380	L23504
Giant dodder (*Cuscuta reflexa*)	HyPRP (*hyprp*)	424	L20755
Pea	PsENOD12A	2210	X81366
Pea	PsENOD12B	2060	X57232
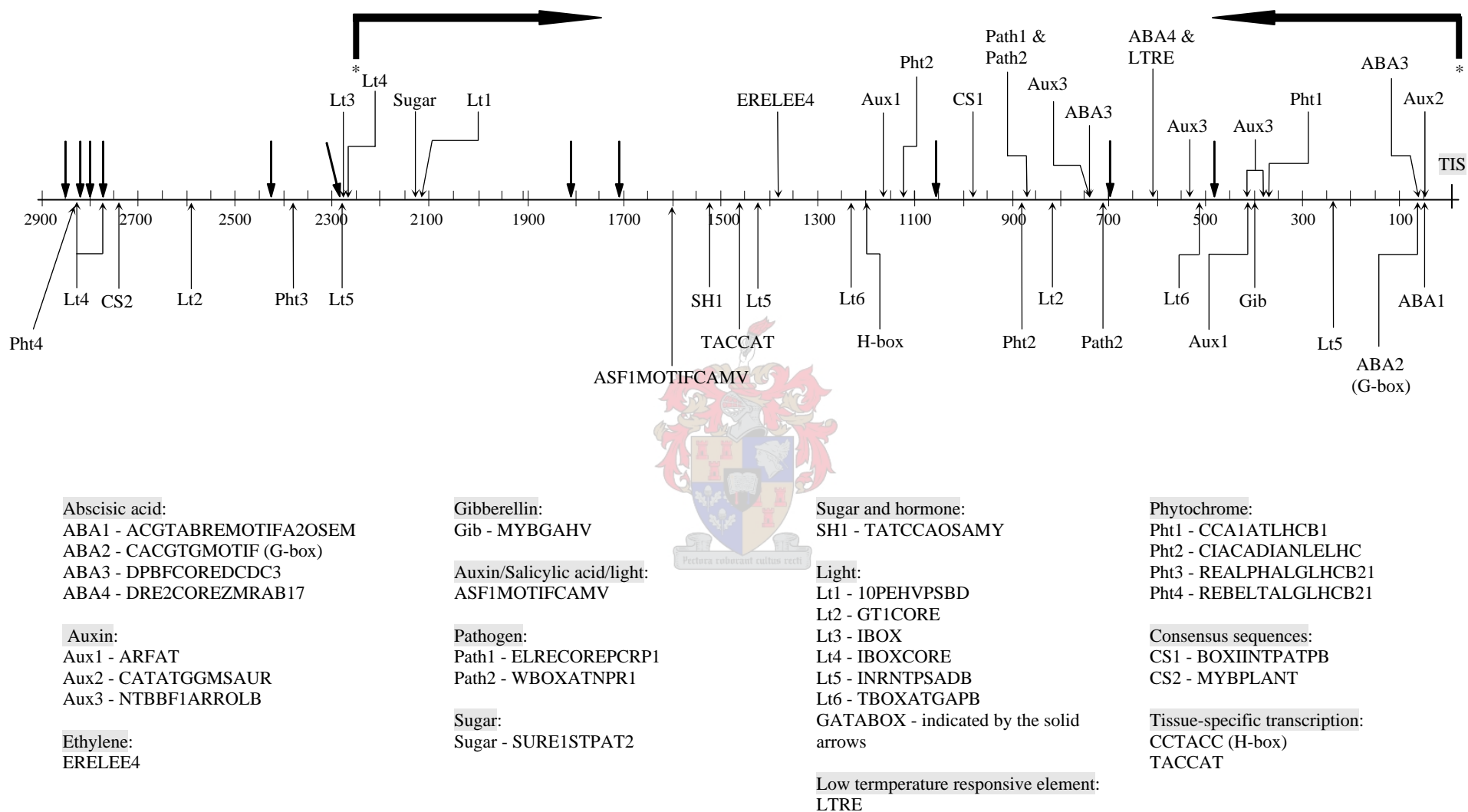
Figure 6.2. Schematic representation of the positions of the putative hormone-, light-, sugar-, pathogen- and phytochrome-responsive elements identified in 2.8 kb *mrip*1 5' UTR flanking region. Also indicated are two elements implicated in tissue-specific expression (H-box and TACCAT), and two consensus sequences (BOXIITPATPB and MYBPLANT). The large solid arrows indicate the 2.25 kb fragment used to construct the *mrip*1:GFP fusion gene. The *mrip*1 transcription initiation site (TIS) is indicated in the shaded box. The map is to scale, and the position of the elements indicated in base pairs (bp).

Based on the putative sugar-responsive element 2.1 kb upstream of the *mrip*1 TIS, a 2.2 kb fragment of the *mrip*1 5' flanking region was selected for functional analysis of the *mrip*1 promoter. The in-frame fusion of the 2.2 kb *mrip*1 promoter and the reporter gene, *sgfp*S65T, was confirmed by sequence analysis (not shown).

6.3.4 Genomic analysis of *mrip*1

Merlot genomic DNA was digested with eight different restriction endonucleases and probed with the *Bam*HI digested, 2.2 kb *mrip*1 promoter fragment. As this fragment corresponded only to the *mrip*1 5' UTR flanking region (refer Materials and Methods, and the *Bam*H I restriction site 87 bp upstream of the *mrip*1 TIS in Fig. 6.2), no genomic fragments <u>only</u> corresponding to the *mrip*1 coding region would be visualised.

A maximum of two fragments were visualised by hybridisation, suggesting that the *mrip*1 5' UTR flanking region was present in low copy number in the Merlot genome (Fig. 6.3). By using a combination of results obtained by endonuclease restriction mapping (Fig. 5.1B and Fig. 6.1) and nucleotide sequence analyses, genomic fragments corresponding to *mrip*1 could be identified (Table 6.2 and Fig. 6.3). Hybridisation of the probe to a second fragment, suggests the presence of a second gene with high homology to the *mrip*1 5' UTR flanking region.

The single fragment visualised in the *Eco*RV digest, was found to be consistent with the 1.94 kb *Eco*RV-*Eco*RV fragment visualised in the *mrip*1 5' UTR flanking region (Fig. 6.1).
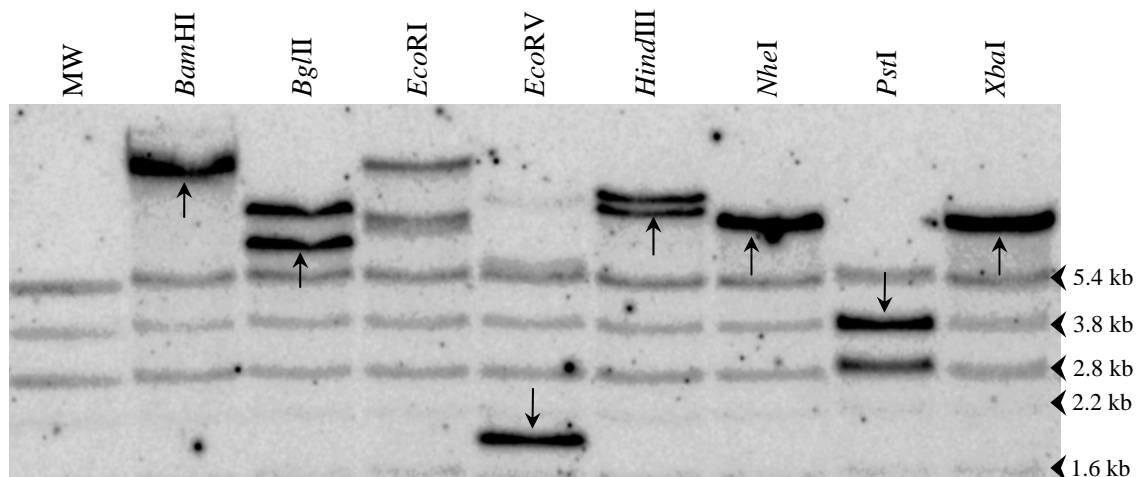

Figure 6.3 Southern blot analysis of *mrip*1 in the Merlot genome. Genomic DNA (8 µg) was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hind*III, *Pst*I, *Nhe*I and *Xba*I and probed with a 2.2 kb sequence corresponding to the *mrip*1 5' UTR flanking region. Hybridisation washes were performed at high stringency conditions. Fragments corresponding to *mrip*1 are indicated by the arrows. Fragments corresponding to the molecular weight marker are shown in lane 1 (MW). The size of the fragments is indicated by the arrowheads.

Table 6.2 Sizes of the sequences corresponding to the *mrip*1 5' UTR flanking, 5' UTR, coding and 3' UTR regions. Fragment sizes are indicated in base pairs (bp) and kilobase pairs (kb).

Digest	5' UTR flanking region[1]	5' UTR and coding region[2]	3' UTR[2] region	Total
*Bam*HI		None (Refer to the *Bam*HI site 87 bp upstream of *mrip*1 transcription inititation site)	-	>5.5 kb
*Bgl*II	4.5	0.84 kb (28 bp 5' UTR + 807 bp coding region)	1.7	7.03
*Hind*III	4.25	0.84 kb	6.0	11.09
*Pst*I	2.84	0.84 kb	0.05	3.73
*Nhe*1	5.1	0.84 kb	3	8.93
*Xba*I	5.4	0.84 kb	2.8	9.03

[1]Refer Figure 6.1
[2]Refer Figure 5.1

6.3.5 Characterisation of *mrip*1 promoter activity in transient expression assays

Transient expression of the *mrip*1:sgfpS65T fusion gene was visualised in green pepper pericarp and leaf tissue 48 h after particle bombardment (Fig. 6.4). No difference in the level of GFP expression in pericarp discs of maturing (small, medium and large) peppers could be observed. GFP expression was also visualised in pericarp tissue bombarded with plasmid DNA of the binary plasmids, pBIN.*mrip*1:sgfpS65T and pBIN.35S:*sgfp*S65T, which were used for plant genetic transformation (not shown).

6.3.6 Characterisation of *mrip*1 promoter activity in stably transformed tobacco plants

Fifteen primary transgenic plants expressing the *mrip*1:*sgfp*S65T fusion gene, were analysed. In five of these plants, GFP was abundantly expressed in the nectary tissue of maturing flowers. These plants were selected for further analysis. GFP expression in these tobacco plants indicated that *mrip*1:*sgfp*S65T transcription is tissue-specific and developmentally-regulated (Fig. 6.5C). Immature tobacco flowers (stage 2) was characterised by a low level of GFP expression, specific to the nectary tissue. The level of GFP expression increased during flower development, and flowers in full-bloom were characterised by abundant GFP expression (Fig. 6.5C: S5). GFP expression was abundant in the nectary tissue, but also accumulated in the base of the ovary wall, in tissue adjacent to the septum and in the fused lateral bundles (Fig. 6.5C: Cs2-4 and Fig. 6.5E). No GFP expression could be visualised in the placental tissue or the developing ovules. The level of GFP expression decreased during the final stages of ovary development (stages 7 – 8). In vegetative tissues of these tobacco plants, no GFP expression could be observed (not shown).

Six primary transgenic plants expressing the CaMV 35S:*sgfp*S65T fusion gene, were analysed. The level of GFP expression in flowers of these plants was low, compared to the high levels of GFP

visualised in flowers expressing the *mrip*1:*sgfp*S65T fusion gene (Fig. 6.5F). Due to the low level of GFP expression, and the increasing level of autofluorescence during flower development, GFP expression was best visualised in immature flowers (Fig. 6.5A, stage 2).

No GFP expression could be visualised in the flowers of untransformed plants (Fig. 6.5D) or in their vegetative tissue (not shown).
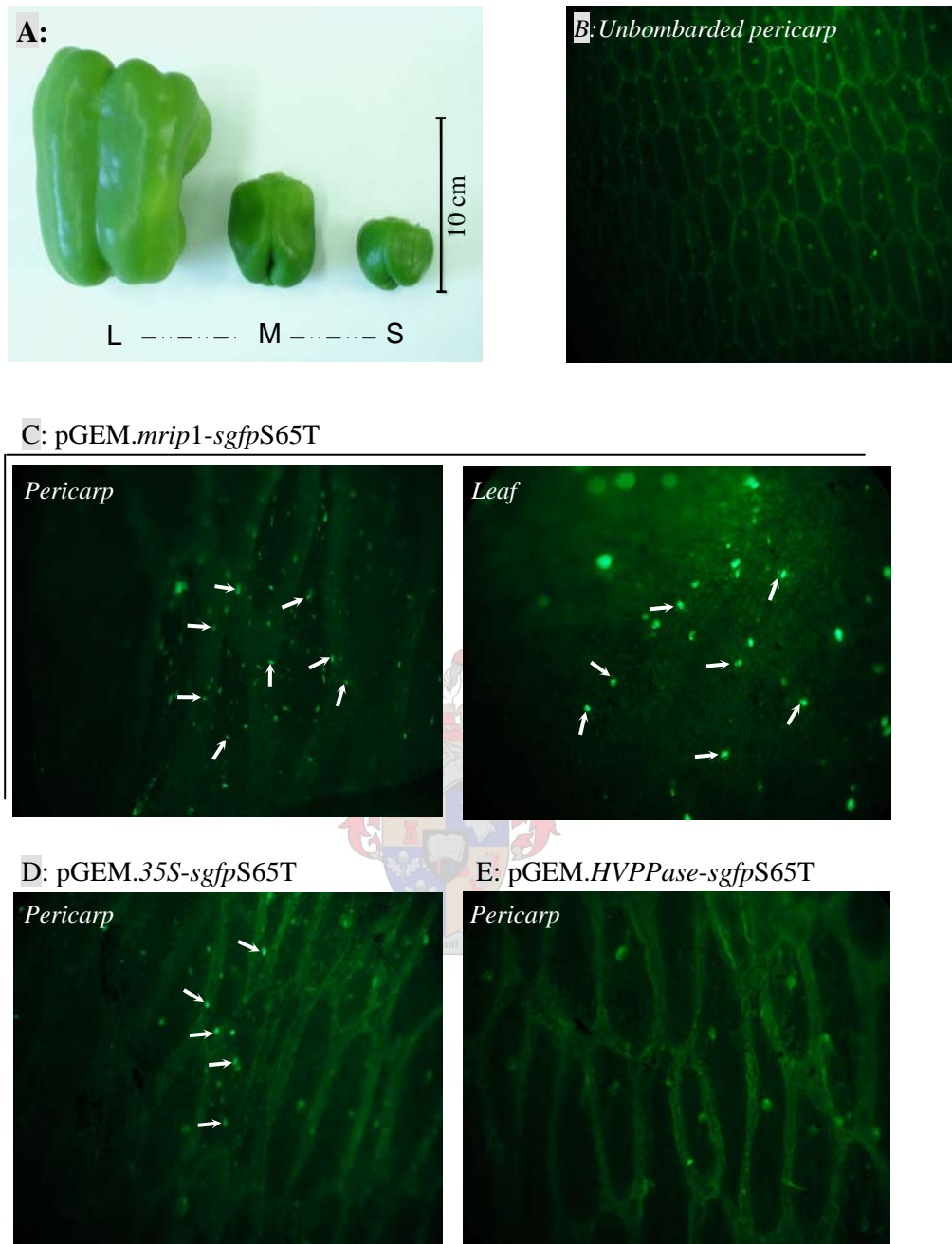
Figure 6.4 Characterisation of *mrip*1 promoter activity by transient expression in green pepper pericarp tissue. A: Immature (S), mid-size (M) and mature (L) green peppers were subjected to the analyses. B: Image of unbombarded pericarp tissue. C: Visualisation of GFP expression in the pericarp tissue of mature pepper, and leaf tissue 48 h after particle bombardment using the construct *mrip*1:*sgfp*S65T. Arrows indicate some of the nuclei visualised by GFP fluorescence. D: GFP expression in the pericarp tissue of mature pepper bombarded with the construct pGEM.35S:*sgfp*S65T, which was used as positive control. D: No GFP expression could be visualised in the pericarp tissue of mature pepper bombarded with the promoter-less construct, pGEM.*HVPPASE*:*sgfp*S65T.
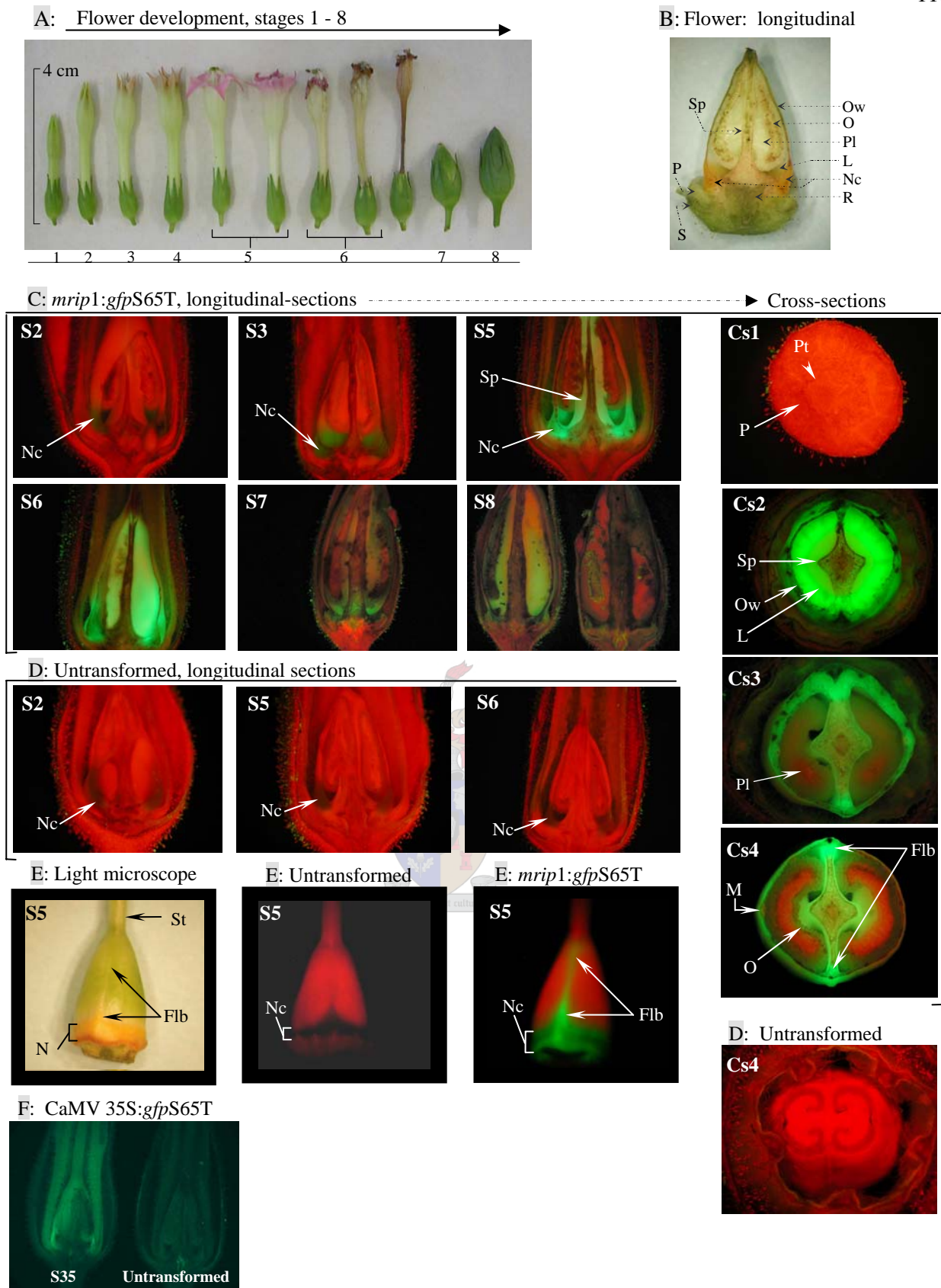
A: Flower development, stages 1 - 8

4 cm

1 2 3 4 5 6 7 8

B: Flower: longitudinal

Sp
P
S
Ow
O
Pl
L
Nc
R

C: *mrip*1:*gfp*S65T, longitudinal-sections ------------► Cross-sections

S2 — Nc
S3 — Nc
S5 — Sp, Nc
S6
S7
S8

Cs1 — Pt, P
Cs2 — Sp, Ow, L
Cs3 — Pl
Cs4 — Flb, M, O

D: Untransformed, longitudinal sections

S2 — Nc
S5 — Nc
S6 — Nc

E: Light microscope — S5 — St, Flb, N
E: Untransformed — S5 — Nc
E: *mrip*1:*gfp*S65T — S5 — Nc, Flb

D: Untransformed — Cs4

F: CaMV 35S:*gfp*S65T — S35 Untransformed

Figure 6.5 Characterisation of *mrip*1 promoter activity in stably transformed tobacco plants expressing the *mrip*1:*sgfp*S65T fusion gene. With the excitation filters used, GFP fluoresces green, while chlorophyll fluoresces red. **A**: Stages of flower development, classified as stages 1 – 8. **B**: Longitudinal section of a tobacco flower, stage 5, indicating the different tissue types identified. **C**: Longitudinal- and cross-sections of developing flowers expressing *mrip*1:*sgfp*S65T - stages of development are indicated as S2 to S8. The four cross-sections were prepared by cutting four discs of 1 mm each, starting at the bottom of the ovule (Cs1) and then moving up the through the ovule (Cs2 – 4). **D**: Longitudinal-sections of developing flowers of an untransformed plant. A cross-section is also shown. **E**: Side-view of an ovary indicating the different tissues types; an ovary of a flower of an untransformed plant; an ovary expressing *mrip*1:*sgfp*S65T. Flowers in full-bloom (stage 5) were used for side-view and cross-section images. **F**: Longitudinal section of a flower expressing S35:sgfpS65T. **Flb**: fused lateral bundles, **L**: locule, **Mb**: median bundle, **Nc**: nectaries, **O**: ovules, **Ow**: Ovary wall, **P**: petal, **Pl**: placenta, **Pt**: petiole attachment, **R**: receptacle, **S**: sepal, **St**: style, **Sp**: septum or partitioning.

6.4 DISCUSSION

Characterisation of the 5.5 kb *mrip*1 5' UTR flanking region

The 5.5 kb *mrip*1 5' UTR flanking region presents the longest 5' UTR flanking region of a ripening-related gene characterised to date. As such, the characterisation of this sequence was the first to reveal the presence of another member of the grapevine proline-rich protein family directly upstream of the *mrip*1 promoter region. Apart from providing an insight into the chromosomal organisation of grapevine PRPs, and clues to its evolutionary history, the 5.5 kb clone provides a tool to isolate and characterise more members of the grapevine PRP family by chromosome walking. Gene families organised in tandem arrays have been reported in *Arabidopsis* (The Arabidopsis Genome Initiative, 2000), where up to 17 % of all genes are arranged in this manner.

Characterisation of the 2.8 kb *mrip*1 promoter region

As has been implicated in fruit ripening and PRP expression, putative *cis*-regulatory elements for abscisic acid-, auxin-, ethylene, sugar- and pathogen-responsive elements were identified in the 2.8 kb *mrip*1 promoter region. The 2.8 kb *mrip*1 promoter region did however not reveal any significant sequence identity to any of the promoters of genes transcribed in ripening fruit, or to the promoter of PRPs, currently present in Genbank. Considering that a high level of sequence similarity has been indicated for promoters that are similarly regulated (Fillion *et al*., 1999), and that no other ripening-related promoter from grapevine is currently available in Genbank, this observation is not surprising. The only ripening-related promoters present in the genetic database were the tomato *E4* and *E8* genes. Despite observations made in green pepper and tomato suggesting that climacteric (like tomato) and non-climacteric fruits (like green pepper and grapevine) share common regulatory mechanisms (Kuntz *et al*., 1998), this study could not show significant sequence identity between the *mrip*1 and the *E4* and *E8* promoters.

Ethylene is known that play an important role in climacteric fruit ripening (Lincoln *et al*., 1987; Deikman *et al*., 1992; Hobson and Grierson, 1993; Montgomery *et al*., 1993). However, ethylene is only produced in very small amounts during the ripening of non-climcacteric fruit like grape, and evidence for the regulation of grape berry ripening by ethylene is fragmented (Hobson and Grierson, 1993; Kanellis and Roubelakis-Angelakis, 1993). The putative ethylene-responsive element, ERELEE4, identified in the *mrip*1 promoter, is an indication of the involvement of ethylene in the regulation of ripening-related gene transcription in non-climacteric fruit. Identification of putative pathogen-responsive elements identified in the *mrip*1 promoter, and the known involvement of ethylene in the regulation of some pathogen- and stress-related genes, supports the indication that ethylene is involved in *mrip*1 regulation. Robinson and co-workers (1997) stated that fruits, being

rich in sugars and other nutrients, provide ideal targets for pathogens. In this respect, it is thus not surprising to find pathogen-related elements, and the associated ethylene-responsive elements, in the regulatory sequences (promoters) of ripening-related genes.

Apart from the grapevine *mrip*1 and *Vvht*1, melon ACC-01, strawberry *pdc*1, tomato *E8* and alfalfa *MsPRP2* promoters – the sugar-responsive element, SURE1STPT21, was also identified in the promoter of the tobacco developmentally-related, nectary-specific nectarin I precursor (*NEC1*) gene (Carter *et al*., 1999). Although the element has been identified in the promoter region of genes transcriptionally activated at the onset of sugar accumulation *viz. Vvht*1 (Fillion *et al*., 1999) and *Nec*1 (Esau, 1977; Carter *et al*., 1999), the element does not seem necessary for the regulation of ripening-related (the stage associated with higher sugar content) gene transcription. With the exception of the tomato *E8* promoter, the element was not identified in any of the ripening-related promoters of climacteric fruit. Identification of the element in the promoter of the root-specific PRP, *MsPRP2*, suggests that the element is not characteristic of fruit-specific genes only (Deutch and Winicov, 1995). Although sugar has been implicated in the regulation of PRP expression, the element SURE1STPT21 was not identified in any of the two other PRP promoters. If the element proves to be associated with increased sugar concentration, the relationship between sugar content and the induction of *MsPRP2* transcription in roots, remains to be resolved.

Genomic analysis of *mrip*1

Southern blot analysis, with using the *mrip*1 promoter fragment as probe revealed a hybridisation pattern different from that obtained when the *mrip*1 coding region was used as probe (Chapter 5, Section 5.4.4). In this study, only one other gene with high sequence homology to the *mrip*1 5' UTR flanking region was found. These results suggest that the genomic copy of the *mrip*1 5' UTR flanking region is less complex than that of the coding region, implying that they are probably a number of related PRP genes with similar coding regions (and repeated motifs) but more variable 5' UTR flanking regions. In addition, based on the size of the genomic fragments hybridising to the *mrip*1 promoter fragment, and the size of the *mrip*1 5' UTR flanking region, coding and 3' UTR regions, it is suggested that the *mrip*1 coding region, like that of *mprp*2, does not contain any introns. Although not a typical feature of PRPs, similar observations have been reported (Govers *et al*., 1991; Jose-Estanyol *et al*., 1992; Deutch and Winicov, 1995). No relationship between *mrip*1 and these PRPs could be established.

Functional analysis of the 2.2 kb *mrip*1 promoter region

Transient expression by particle bombardment of intact green pepper pericarp tissue proved to be a rapid and effective system for the verification of the functionality of the constructs. Ultimately, it demonstrated the functionality of the 2.2 kb *mrip*1 promoter fragment. Transient assays by particle bombardment did however not provide any evidence regarding the tissue- and developmentally-regulated specificity of the *mrip*1 promoter fragment.

Although tobacco has been widely used for the functional analysis of promoters of ripening-related genes (Lin *et al*., 1993; Blume and Grierson, 1997; Lasserre *et al*., 1997; Schubert *et al*., 1997; Püringer *et al*., 2000), this is the first report on a promoter from a non-climacteric fruit, driving tissue- and developmental-specific transcription in tobacco. Considering that anatomically, fruits are swollen ovaries that may also contain associated flower parts, the tissue-specific expression of the *mrip*1:GFP fusion in transgenic tobacco corresponds well with the presence of the *mrip*1 transcript in the grape berry meso- and exocarp-tissue. Furthermore, developmental regulation of the GFP accumulation in the nectary tissue of transgenic tobacco flowers is very similar to the ripening-related regulation of *mrip*1 transcription in grape berries (Chapter 5, Figure 5.7). These observations suggest that the *mrip*1 2.2 kb promoter region harbours *cis*-elements necessary for tissue- and developmental-specific regulation of GFP accumulation, and that the transcriptional activation of *mrip*1 is mediated by developmental signals present in both grapevine berries and tobacco flowers. Results presented here suggest that tobacco can be more generally applied as heterologous system for the functional analysis of ripening-related promoters.

In conclusion, characterisation of the 5.5 kb *mrip*1 5' UTR flanking region contributes towards the characterisation of the genes encoding fruit-specific proline-rich proteins, and towards a better understanding of the regulatory mechanisms involved in non-climacteric fruit ripening. Characterisation of *mrip*1 promoter region forms a basis for future experiments to define the key *cis*-acting elements necessary for tissue- and cell-specific gene regulation in fruit, more specifically in grapevine. In addition, the *mrip*1 promoter is an ideal candidate for ripening-related regulation of transgene transcription in the berries of genetically modified grapevine. Ultimately, functional analysis of the promoter during fruit ripening in transgenic grapes will verify the usefulness of this promoter in the genetic manipulation of fruit metabolism in grapevine.

6.5 MATERIALS AND METHODS

6.5.1 Plant material:

Freshly picked sweet bell peppers *Capsicum annuum* (green pepper) cv. King Arthur were obtained from a local vegetable grower. Small (3.5 cm), medium (7 cm) and large peppers (13 cm) peppers were used. The size refers to the length of the pepper. *In vitro* propagated tobacco plants (*Nicotiana tabacum* cv. Petit Havana, SR1) were used for plant genetic transformation. Tobacco plants were propagated on rooting and proliferation medium[1] and maintained at 25 °C under a 16 h photoperiod.

6.5.2 Methods:

6.5.2.1 Sequence analysis of the *mrip*1 5' UTR flanking region

Four clones containing 4.2 kb, 4.5 kb, 5.1 kb and 5.5 kb of *mrip*1 5' UTR and 5' UTR flanking region, was obtained (Chapter 5, Section 5.4.1). These will be referred to as clones HindIII[4.2], BglII[4.5], NheI[5.1] and XbaI[5.5], respectively. The four clones were subjected to sequence homology analysis. Clone NheI[5.1] was used for characterisation of the *mrip*1 5' UTR flanking region.

An endonuclease restriction map of the NheI[5.1] was compiled. The compiled map was used to identify shorter fragments, which were excised and re-ligated into pBlueScript (Stratagene), and used as templates for nucleotide sequence analysis. Plasmid DNA was sequenced using primers T3 and T7 and the Applied Biosystems PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit, following the manufacturer's instructions. Sequencing reactions were analysed by capillary electrophoresis in an Applied Biosystems automatic sequencer model 3100.

Promoter Prediction software available from the Berkeley Drosophila Genome Project: Analysis Tools (http://www.fruitfly.org/seq_tools/other.html) was used for prediction of the *mrip*1 putative transcription initiation site. Putative *cis*-acting regulatory elements in the *mrip*1 5' UTR flanking region and in eighteen other plant promoters, were identified by submitting the sequences to the PLACE Signal Scan Search (http://www.dna.affrc.go.jp/htdocs/PLACE) (Higo *et al*., 1999). Sequence homology searches were performed using the BLAST server at the National Center for Biological Information (NCBI) (Altshul *et al*., 1990).

6.5.2.2 Southern blot analysis

Southern blot analysis of Merlot genomic DNA was performed according to procedures described in Chapter 5, section 5.3.2.4. To specifically probe sequences corresponding to the 5' UTR flanking region of *mrip*1, the fragment corresponding to the 2.2 kb 5' UTR flanking region of *mrip*1 was

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[1]MS salts and vitamins (Murashige and Skoog, 1962) containing 30 % (m/v) sucrose and 0.2 µgml[-1] kinetin.

amplified using oligonucleotide primers mrip1promfrw and mrip1promrev (Table 6.1), subjected to *Bam*HI endonuclease treatment, and purified by agarose gel electrophoresis. The radio-labeled probe was synthesised by random prime labelling of 75 ng of the purified product. Hybridisation was visualised by phosphor-imaging using the Cyclone™ Phosphorimager (Packard Instrument Co.) and an AlphaImager™2000 documentation and analysis system (Alpha Innotech Corporation).

6.5.2.3 Preparation of constructs for transient expression studies

The plasmid pGEM.Ubi1-*sgfp*S65T (Elliott *et al.*, 1999), from which the maize polyubiquitin 1 promoter and intron were removed, was used for plasmid construction (Fig. 6.6). Three plasmids were constructed for transient expression studies. These included 1) pGEM.*mrip*1-*sgfp*S65T, containing 2.2 kb of the *mrip*1 5' UTR flanking region, 2) pGEM.35S-*sgfp*S65T, a positive control containing the CaMV 35S promoter, and 3) pGEM.*HVPPase-sgfp*S65T, a promoter-less construct containing a 2.3 kb *Hordeum vulgare VPPase* cDNA fragment (Fig. 6.6).
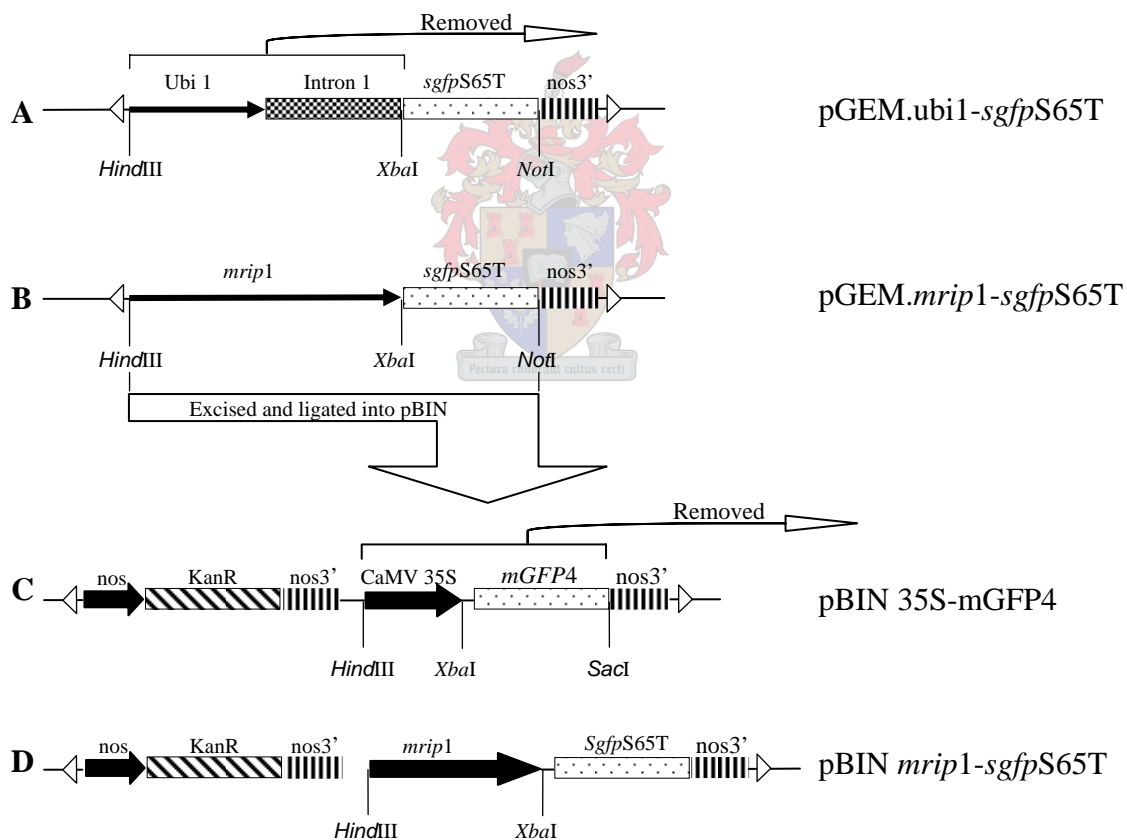

Figure 6.6 Schematic representation of two of the plasmids constructed for transient expression assays and plant genetic transformation (not to scale). **A**: Plasmids for transient expression assays were constructed using the plasmid pGEM.Ubi1-*sgfp*S65T from which the maize polyubiquitin 1 promoter and intron was removed. **B**: pGEM.*mrip1-sgfp*S65T was constructed by ligating the 2.2 kb *Hind*III-*Xba*I fragment of *mrip*1 5' UTR flanking sequence into pGEM. **C**: pBIN.35S-mGFP4 was used to construct plasmids for plant genetic transformation. **D**: The plasmid pBIN *mrip*1-*sgfp*S65T was constructed by replacing the CaMV 35S promoter and mGFP4 of pBIN.35S-mGFP4, by the *mrip1-sgfp*S65T fusion. Plasmids used as positive and negative controls were constructed similarly. Restriction sites relevant to the construction of the plasmids are indicated.

Amplification of the 2.2 kb of *mrip*1 5' UTR flanking region was performed using the clone NheI[5.1] as template and oligonucleotide primers mrip1promfrw and mrip1promrev (Table 6.3). The amplification product was digested with *Xba*I and *Hind*III and ligated into the promoter-less pGEM.Ubi1-*sgfp*S65T. Introduction of the *Xba*I restriction site immediately downstream of the *mrip*1 ATG start codon, facilitated the in-frame ligation of the *mrip*1 ATG start codon with the *sgfp*S65T gene. The recombinant plasmid was designated pGEM.*mrip*1-*sgfp*S65T (6.379 kb).

The positive control, pGEM.35S-*sgfp*S65T (5.037kb), was constructed by ligating the 865 bp CaMV 35S promoter fragment from pBIN 35S-mGFP4 (Haselhoff *et al*., 1997) into the promoter-less pGEM.ubi1-*sgfp*S65T plasmid. The plasmid pBIN 35S-mGFP4 was kindly provided by Jim Haselhoff, MRC, Cambridge, United Kingdom.

The negative control, the promoter-less pGEM.HPPase-*sgfp*S65T (6.529 kb) was constructed by ligating a 2.357kb fragment of *Hordeum vulgare VPPase* cDNA into the promoter-less pGEM.ubi1-*sgfp*S65T construct. The 2.357 kb *Hind*III-*Xba*I *VPPase* cDNA fragment was excised from a clone supplied by Yoshiyuki Tanaka, National Institute of Agrobiological Resources, Tsukuba Ibaraki, Japan (Tanaka *et al*., 1993).

All chimeric fusions were verified by sequence analysis, using *sgfp*S65T-rev1 primer (Table 6.3).

Table 6.3 Oligonucleotide primers used for nucleotide sequence analysis and amplification. Restriction endonuclease sites to be introduced by amplification, are underlined.

Primer name	Primer sequence, in the 5' to 3' convention	Annealing position
sgfpS65T-rev1	ATT CTT GTA CAG CTC GTC CAT G	nt 696 to 717 downstream of the *sgfp*S65T ATG start codon
sgfpS65T-rev2	GAT GAA CTT CAG GGT CAG CTT GC	nt 119 to 141 downstream of the *sgfp*S65T ATG start codon
mrip1prom-frw	CTC <u>AAG CTT</u> ATG AAC TGC ATG CAA AG	nt +2176 to +2159 upstream of the *mrip*1 putative transcription initiation site. The *Hind*III restriction endonuclease site to be introduced, is underlined.
mrip1prom-rev	TCC C<u>TC TAG A</u>CA **T**CT TTG AGT AGT TGT AGC	Includes the *mrip*1 ATG start codon and the 17bp upstream of the start codon. The *mrip*1 start codon is indicated in boldface and the *Xba*I restriction endonuclease site to be introduced, is underlined.
CaMV 35S-frw	TCC ACT GAC GTA AGG GAT GAC,.	nt +99 to +79 upstream of the CaMV 35S fragment 3' end
VPPase-frw	CAG ACA GCC ATG GCG ATC C	nt +2274 to +2293 upstream of the *Xba* I site, which is where the gene was fused to the *sgfp*S65T gene

6.5.2.4 Preparation of constructs for plant genetic transformation

The plasmid pBIN 35S mGFP4, from which the CaMV 35S and mGFP4 containing *Hind*III-*Sac*I fragment was removed, was used for plasmid construction (Fig. 6.6).

Three plasmids were constructed *viz.* 1) pBIN.*mrip*1-*sgpf*S65T, 2) pBIN.35S-*sgfp*S65T, the positive control and 3) pBIN.*VPPase- sgfp*S65T, the negative control. The plasmids were constructed by subcloning the *Hind*III-*Not*I fragment of the plasmids pGEM.*mrip*1-*sgfp*S65T, pGEM.35S-*sgfp*S65T and pGEM.*VPPase-sgfp*S65T into pBIN 35S mGFP4. For subcloning, the *Not*I- and *Sac*I- generated ends from the inserts and pBIN 35S mGFP4 were polished T4 DNA polymerase, prior to *Hind*III digestion. The size of the constructed plasmids ranged was 14.956 kb (pBIN.*mrip*1-*sgpf*S65T) and 13.615 kb (pBIN.35S-*sgfp*S65T).

Presence and orientation of the fusion genes in pBIN were confirmed by restriction analysis and PCR. Amplification by PCR was performed using the sgfpS65T reverse2 primer and a forward primer specific to either the *mrip*1 promoter or the CaMV 35S promoter (Table 6.1).

6.5.2.5 Transient expression studies by particle bombardment

Transient expression assays were performed by particle bombardment of sweet bell pepper fruit pericarp tissue. Bombardment was performed using a particle bombardment device, constructed according to the Finer Particle Inflow Gun design (Finer *et al*., 1992) and tungsten particles (0.7 µm, Grade M10, Bio-Rad) coated with the plasmid DNA.

For preparation of the target tissue, peppers were rinsed in sterile water before tissue segments (2 x 3 cm) were cut from the pericarp. Pericarp segments were placed skin side down, in 9 cm petri dishes containing a single sheet of sterile filter paper and 4 ml sterile distilled water. Petri dishes contained one tissue segment each. The surface of the tissue segments was partially dried by leaving the open petri dishes in the laminar flow for 10 min.

Plasmid DNA was precipitated unto sterile tungsten particles by mixing 10µl of plasmid DNA (10 µg), 50 µl of tungsten (5 mg), 50 µl of 2.5 M $CaCl_2$ and 20 µl of 100 mM spermidine, free base. The mixture was incubated on ice for 5 min, before 100 µl of the supernatant was removed and discarded.

For bombardment, 5 µl of the dispersed tungsten-DNA preparation was pipetted onto the centre of the support screen of the disassembled 13 mm stainless steel syringe filter unit. Petri dishes containing the target tissue was placed 12.5 cm from the support screen in the syringe filter unit.

The tissue was covered with a baffle made of 2 mm stainless steel screen, mounted 9 cm above the tissue (Finer *et al*., 1992). Particle bombardment was performed with a chamber vacuum of 85 kPa and a helium delivery pressure of 1000 kPa. The helium solenoid valve relay was set at 50 msec. Tissue discs were bombarded only once. For negative control experiments tungsten particles unto which no plasmid DNA was precipitated, were used. Procedures were performed in triplicate for each of the three constructs and the negative control. After bombardment, petri dishes were sealed and incubated at 28 °C for 48 h.

Visualisation of GFP fluorescence in plant tissues was achieved using a Leica MZFLIII stereomicroscope with a fluorescence GFP Plus filter module (Leica Microscopy and Scientific Instruments) (Elliot *et al*., 1991). Images were captured using a Nikon Coolpix 990.

6.5.2.6 Plant genetic transformation: *Agrobacterium*-mediated transformation of *Nicotiana tabacum*

Plasmids for plant genetic transformation were mobilised into the *Agrobacterium tumefaciens* strain EHA 105 (Hood *et al*., 1993) using the freeze-thaw method (An *et al*., 1988). The presence of the introduced plasmids in *Agrobacterium* was confirmed by amplification of the fusion genes using 1 µl of *Agrobacterium* overnight culture and the *sgfp*S65T reverse primer in combination with forward primers specific to each of the fusion genes (Table 6.1).

Agrobacterium-mediated transformation was performed using a 2 % (m/v) sucrose- *Agrobacterium* suspension. Bacterial cells from an overnight culture (a single colony of transformed *Agrobacterium* grown in 50 ml YEP[2] containing 50 µg.ml⁻¹ kanamycin sulphate and 50 µg.ml⁻¹ rifampicin for overnight at 28 °C and 250 rpm) were collected by centrifugation at 5500 g for 15 min at 10 °C. The pelleted cells were resuspended and diluted to a density of 0.3 OD_{550} using 2 % (m/v) sucrose.

Four-week old, *in vitro* propagated tobacco plants were used for leaf disc transformation. The three upper leaves of the plants were collected, segmented (8 – 12 mm) and wounded by making three incisions across the veins. Wounded leaf segments were dipped into the sucrose-*Agrobacterium* suspension, blotted dry and transferred to shoot regeneration medium[3] for a co-cultivation period of 48 h at 23 °C in the light (1500 lux). Inoculated leaf segments were transferred to shoot regeneration medium supplemented with antibiotics[4] to eliminate *Agrobacterium* and to select

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[2] 10 g.l⁻¹ Peptone, 10 g.l⁻¹ yeast extract, 5 g.l⁻¹ NaCl, pH 7.2
[3] MS salts and vitamins containing 30 % (m/v) sucrose, 0.1 µg.ml⁻¹ NAA and 1 µg.ml⁻¹ 6-BAP (Murashige and Skoog, 1962).
[4] 200 µg.ml⁻¹ cefotaxime and 100 µg.ml⁻¹ kanamycin sulphate.

genetically transformed shoots regenerating from the leaf segments. These shoots were excised and transferred to rooting and proliferation medium supplemented with cefotaxime and kanamycin sulphate. Primary transgenic tobacco plantlets were hardened off and grown in a containment glasshouse under standard glasshouse conditions at 25 °C.

6.5.2.7 Visualisation of GFP fluorescence in plant tissues

To minimise the level of red fluorescence produced by the high chlorophyll content of the tobacco vegetative tissues, untransformed and tobacco plants expressing the fusion genes *mrip*1:*sgfp*S65T and S35:*sgfp*S65T, were grown in the dark for three weeks. Vegetative tissues were screened for GFP fluorescence by using a Spectroline high-intensity ultraviolet lamp (365 nm) (Spectronics Corporation).

6.6 ACKNOWLEDGEMENTS

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C H A P T E R   7

Concluding Remarks and Future Prospects.
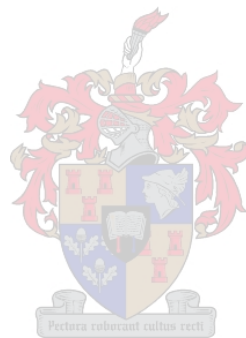
Concluding Remarks and Future Prospects

🍇 7.1 CONCLUDING REMARKS

In recent years, the value and competitive nature of the grape and wine industry led to an increasing emphasis on producing quality fruit for the production of quality wines. As such, the grape berry is one of the main targets for the genetic manipulation of fruit quality traits. However, two of the main obstacles in the successful application of this technology remain the lack of a thorough understanding of the biochemistry and molecular biology of the fruit ripening process, and mechanisms to regulate plant gene transcription during berry ripening.

To address some aspects of these two issues, this study embarked on the analysis of gene transcription during Clairette blanche and Cabernet Sauvignon berry ripening. The analyses led to the identification of 175 ripening-related cDNAs (Chapter 3) and to the cloning of a specific ripening-related gene, clone C2-17, which was shown to be transcriptionally activated at the onset of berry ripening (Chapter 4). This gene was designated Merlot ripening induced protein 1 (*mrip*1). Characterisation of *mrip*1 provided evidence that the gene is a novel ripening-related gene, and the putative protein is a distinct member of the plant proline-rich protein family (Chapter 5). Functional analysis of the *mrip*1 promoter (Chapter 6) showed that the promoter is tissue- and developmentally-regulated; and therefore a good candidate to regulate transgene transcription in during berry ripening in genetically modified grapevine. Additionally, the study provided valuable insight into the suitability of certain techniques in the identification, isolation and characterisation of ripening-related genes, and the functional analysis of promoter elements in transient assays and in stably transformed plants.

Results suggested that gene transcription in the ripening berries of the two cultivars, which differ largely phenotypically and in berry- and wine-making qualities, is remarkably similar (Chapter 3). Obvious differences in ripening-related gene transcription were, however, identified. These included 86 ripening-related, cultivar-specific cDNAs and 37 cDNAs which were differently regulated in the two cultivars. These cDNAs were considered candidates for further characterisation and the isolation of other ripening-related genes, but moreover, for the identification of genes possibly involved in berry and wine-making qualities. Considering that, although gene transcription in the ripening berries of the two cultivars are remarkably similar, most of the ripening-related cDNAs are cultivar-specific / or differently regulated in the two cultivars, these cultivar-specific cDNAs could correspond to genes involved in specific berry qualities. These qualities could include

aspects such as anthocyanin accumulation, sugar and acid concentration changes, compounds involved in improved aroma, and cell wall architecture and changes thereof.

Further characterisation of one of the 175 ripening-related cDNAs led to the isolation of a novel ripening-related gene from Merlot, *mrip*1 (Merlot ripening-induced protein 1) (Chapter 5). This gene was specifically transcribed in the fruit tissue, seed and bunchstems of grapes, from 10-weeks post flowering (wpf) (*véraison*) to the final stages of berry ripening. Sequence analysis revealed that the gene encodes a distinct member of the plant proline-rich protein (PRP) family. Although PRPs expressed during plant development has been extensively studied, the characterisation of this gene is the first to reveal the novel properties of a ripening-related PRP. The most outstanding characteristic of mrip1 is the tandem nature of the eight extended proline-glycine motifs identified in the central region of the putative protein. The function of the repetitive motifs is not clear, but the clustered nature of the glycine–proline–proline motifs could indicate that the motifs have a structural function. Physical properties of the glycine-proline sequences will allow the polypeptide backbone to fold into a tight U-shaped structure, whilst molecular modelling has shown that tandem repeats of proline-rich repeats frequently form β-turn helices. The multiple repeats of glycine-proline are similar to the glycine-proline-hydroxyproline repeats of collagen and elastin. These properties, as well as the initiation of *mrip*1 transcription 10-wpf, which coincides with the cessation of berry and seed growth, and the initiation of secondary cell wall metabolism in seeds, fits well with the proposed role of PRPs in cell wall rigidification associated with the cessation of cell expansion.
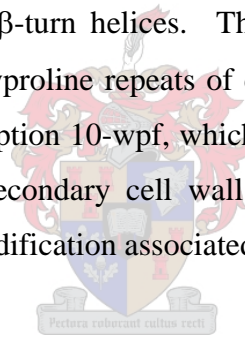
Very little is known about the sub-cellular localisation of PRPs. As one of the major sub-groups of hydroxyproline-rich glycoproteins, PRPs are considered extracellular, structural components of the plant cell wall. The identification of the signal peptide in the *mrip*1 deduced amino acid sequence corresponded with this proposed extracellular localisation. The hydrophilic nature of both the central and the C-terminal region implies that the mrip1 is not "locked" into the membrane, but suggests an extended structure that may interact with itself and/or with other cell wall components through ionic interactions. Based on the basic predicted isoelectric point of mrip1 (pI 10.7) it was suggested that mrip1 may interact with the acidic pectin network within the cell wall, possibly involved in pectin "gelling" and intercellular adhesion. A potential role for mrip1 in controlling aspects of cell wall architecture, and a potential application of this gene in the control of cell wall architecture (texture) in ripening fruits, needs further investigation.

Characterisation of the 5.5 kb sequence directly upstream of the *mrip*1 transcription initiation site (Chapter 6), led to the identification of a proline-rich protein encoding gene, *mprp*2, directly upstream of the *mrip*1 transcription initiation site; but more importantly, to the isolation of the first

reported tissue-specific, ripening-related promoter element from grape berries. This promoter element contains several putative *cis*-acting regulatory elements, which provides the first insight into the factors possibly involved in the regulation of ripening-related gene transcription in grape berries. These included hormone-, light-, phytochrome-, sugar-and stress-responsive elements, as well as elements involved in tissue-specific transcription (Chapter 6). The identification of the ethylene-responsive element is the first indication of the involvement of ethylene in ripening-related gene transcription in a non-climacteric fruit. The isolation and characterisation of the *mrip*1 promoter presents a significant contribution to the pool of tissue- and ripening-related promoter sequences available for comparative analysis, since the only other fruit-specific, ripening-related promoters reported to date is that of the tomato *E4* and *E8* genes. As manipulation of fruit properties in grape berries is most important in the later stages of ripening, the *mrip*1 promoter is considered a good candidate for the regulation of the transgene in the genetic manipulation of fruit metabolism in grapevine.

The study provided valuable insight into the suitability of certain techniques used in the identification, isolation and characterisation of ripening-related genes, and the functional analysis of the *mrip*1 promoter element. (Chapters 3, 4, 5 and 6, respectively). Concluding remarks on the most important contributions are included in the following section. **Firstly,** particle bombardment and transient expression in green pepper pericarp discs proved to be a rapid and effective system for the functional analysis of the *mrip*1 promoter element (Chapter 6). **Secondly**, the data clearly demonstrated the suitability of tobacco, as a heterologous system, for the functional analysis of a fruit-specific, ripening-related promoter element from a non-climacteric fruit (Chapter 6). As such, it was suggested that functional analysis of ripening-related promoters in tobacco, could be more generally applied. **In addition**, the green fluorescent protein (GFP) reporter gene *sgfp*S65T was shown ideal for studying promoter activity in developing tobacco flowers (Chapter 6). A primary advantage of GFP as reporter gene, compared to alternatives such *uid*A (Jefferson *et al*., 1986; Jefferson, 1987; Jefferson *et al*., 1987) and luciferase (the other commonly used *in vivo* reporter) (Riggs and Chrispeels, 1987; Van Leeuwen *et al*., 2000; Mankin and Thompson, 2001) is that the GFP assay *in vivo* requires neither sample preparation nor uptake of exogenous substrate. It is especially this non-destructive nature of GFP expression analysis that was found of particular advantage in this study. Difficulties experienced with the visualisation of GFP expression under control of the CaMV S35 promoter, corresponds with reports on difficulties to distinguish between autofluorescence and GFP expression in some plant tissues (Garabagi and Strommer, 2000). Moreover, these difficulties emphasized the need for appropriate controls in GFP-reporter-based experiments.

7.2 FUTURE PROSPECTS

The availability of the *mrip*1 promoter presents a significant step towards the understanding and the regulation of ripening-related gene transcription in transgenic grapevine. As such, one of the first objectives now would be to verify the tissue-specificity and ripening-related regulation of this promoter in ripening grape berries. With the focus on fruit quality and the genetic manipulation of grape berry metabolism, equally important would be the identification of genes to manipulate fruit- and wine-making qualities of the ripening berry. Since the South African wine industry's most challenging task is to counteract low total acidity, high pH and excessive accumulation of sugars generally associated with hot grape producing regions - strategies should include the identification of genes to manipulate organic acid metabolism and the accumulation of hexoses. Additionally, identification of genes to manipulate the accumulation of anthocyanins, which is essential for the production of fine red wine, and the development of compounds involved in improved aroma, would be of major importance. In this study, several ripening-related, cultivar-specific cDNAs were identified by the analysis of ripening-related gene transcription in Cabernet Sauvignon and Clairette blanche. Although only one of these was taken through comprehensive further analysis, the remainder of this pool of cDNAs could be exploited for the identification of genes involved in berry- and wine-making qualities.

The isolation and characterisation of *mrip*1, together with other initiatives on the isolation of more ripening-related genes and promoter elements, may lead to an excellent model system to study the molecular basis of the processes involved in grape berry ripening. Further analysis of ripening-related promoter elements, either by comparative analysis, deletion analysis or induction studies, may lead to the identification of key regulatory elements involved in tissue-, ripening-related specificity. Since tobacco has been shown an effective heterologous system for functional analysis of the *mrip*1 promoter, it provides a suitable system for further promoter characterisation by deletion analysis, or possibly induction studies. Ultimately, information gained from promoter analyses, will contribute to questions on the universal nature of promoter recognition factors, and to a better understanding of gene regulation at the core promoter level. In turn, this information could be exploited for strategic modifications to promoter elements, tailoring transgene transcription for specific applications.

7.3 LITERATURE CITED

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