Kalliopi A. Roubelakis-Angelakis
Editor

Grapevine Molecular Physiology & Biotechnology

Springer

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Second Edition

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Edited by

Kalliopi A. Roubelakis-Angelakis
University of Crete, Heraklion, Greece

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Editor
Prof. Dr. Kalliopi A. Roubelakis-Angelakis
University of Crete
Dept. Biology
714 09 Heraklion, Crete
Greece
poproube@biology.uoc.gr

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to Andreas,
the partner of my life

PROLEGOMENA

With nearly the first decade of the 21st century behind us, in the era of post-'omics' (genomics, proteomics, metabolomics, etc), we are amazed by the storm of new scientific knowledge. However, we have been convinced that we still are far from fully understood how the extrinsic and intrinsic cues signal and regulate plant growth, development, metabolism and responses to biotic and abiotic stresses.

With the global world climatic and environmental changes and the threat of food crisis ahead, the plants are becoming the most important organisms on earth, which, if properly used, can help to successfully overcome most of these problems.

Grapevine is one of the most widely cultivated plant species worldwide. The publication of the grapevine genome sequence in 2007 has opened new horizons in grapevine research. The previous Edition 'Molecular Biology & Biotechnology of the Grapevine' published by Kluwer Publishers in 2001, is out of print. Thus, we felt that a new Edition could fill the gap in the literature. In this Edition 10 Chapters refer to new aspects of Grapevine Molecular Physiology and Biotechnology and 11 Chapters were revised and updated. To achieve this goal, 73 scientists from 15 countries worldwide have collaborated. We all hope that the outcome will reflect the time and efforts invested.

The Book is intended to become the reference book for all researchers, scientists and biotechnological companies, who want to be updated in viticultural research, but also it can be used as textbook for graduate and undergraduate students.

Sincere thanks are due to all worldwide-leading scientists in the field, who have contributed, and especially for their impeccable collaboration during the preparation of this Book. Also, special thanks are due to Dr Anastasia Papadakis, who undertook the formatting of the Book and the preparation of the ready-to-camera material. Finally, Dr Meran Owen and Mrs Tanja van Gaans, in Springer Publishers, are cordially thanked for their continuous interest and assistance.

Heraklion, December 2008

Kalliopi A. Roubelakis-Angelakis
University of Crete, Greece

CONTENTS

Chapter 11

CONTRIBUTING AUTHORS

Dr Philippe Abbal
INRA, UMR SPO
2 Place Viala, F-34060 Montpellier Cedex 1
France
abbal@supagro.inra.fr

Dr. Alice Agasse
Centro de Fisiologia Molecular e Biotecnologia de Plantas
Departamento de Biologia, Universidade do Minho, 4710-057 Braga
Portugal
aliceagasse@yahoo.fr

Prof. Sara Amâncio
DBEB/CBAA, Instituto Superior de Agronomia
Universidade Técnica de Lisboa, 1349-017 Lisboa
Portugal
samport@isa.utl.pt

Prof. Ryszard Amarowicz
Department of Biochemistry, Faculty of Biology
University of Warmia and Mazury in Olsztyn
Oczapowskiego Street 1A, Pl-10-957 Olsztyn
Poland
amaro@pan.olsztyn.pl

Prof. Atanas Atanassov
AgroBio Institute, 8 Dragan Tsankov Blvd, Sofia 1164
Bulgaria
atanas_atanassov@abi.bg

Prof. Aziz Aziz
Dept. Biologie-Biochimie, Université de Reims Champagne-Ardenne
Campus Moulin de la Housse, B.P. 1039, 51687 Reims Cedex 2
France
aziz.aziz@univ-reims.fr

Prof. Luigi Bavaresco
Istituto di Frutti-Viticoltura, Università Cattolica del Sacro Cuore
Via Emilia Parmense 84 29100 Piacenza
Italy
luigi.bavaresco@unicatt.it

Dr. Paul K. Boss
Commonwealth Scientific and Industrial Research Organisation
Plant Industry, P.O. Box 350
Glen Osmond, South Australia 5064
Australia
paul.boss@csiro.au

Dr. Christine Böttcher
Commonwealth Scientific and Industrial Research Organisation
Plant Industry, P.O. Box 350
Glen Osmond, South Australia 5064
Australia
christine.bottcher@csiro.au

Dr. Günther Buchholz
RLP-AgroScience GmbH, AlPlanta
Institute for Plant Research, Breitenweg 71
D-67435 Neustadt / W
Germany
gunter.buchholz@agroscience.rpl.de

Dr. Véronique Cheynier
Equipe Polyphénols et Interaction
INRA- UMR Sciences pour l'Œnologie
2 Place Viala, 34 060 Montpellier Cedex 01
France
cheynier@supagro.inra.fr

Prof. Violetka Colova-Tsolova
Center for Viticulture & Small Fruit Research
College of Engineering Sciences, Florida Agric. and Mechanical University
Tallahassee, FL 32317
USA
violetka.colova@famu.edu

Dr. Carlos Conde
Centro de Fisiologia Molecular e Biotecnologia de Plantas
Departamento de Biologia, Universidade do Minho, 4710-057 Braga
Portugal
conde@bio.uminho.pt

Dr. Laura Costantini
Edmund Mach Foundation Research Centre - IASMA
Genetics and Molecular Biology Department
Via E. Mach 1, 38010 San Michele all'Adige (TN)
Italy
laura.costantini@iasma.it

Prof. Pierre Coutos-Thevenot
Université de Poitiers, Laboratoire de Physiologie et Biochimie Végétale
UMR CNRS 6161, 40 Avenue du Recteur, Pineau, 86022 Poitiers
France
pierre.coutos.thevenot@univ-poitiers.fr

Prof. Alain Deloire
INRA, UMR 1083 Sciences for the Enology
2 Place P. Viala, F-34060 Montpellier
France
deloire@supagro.inra.fr
After 01/01/2009
University of Stellenbosch
Department of Viticulture and Oenology
Private Bag XI, Matieland, 7602 Stellenbosch, South Africa
deloire@sun.ac.za

Prof. Serge Delrot
Université de Bordeaux 2
UMR Ecophysiologie et Génomique Fonctionnelle de la Vigne
Domaine de la Grande Ferrade, INRA, 33883 Villenave d'Ornon
France
serge.delrot@bordeaux.inra.fr

Dr. Christopher Davies
Commonwealth Scientific and Industrial Research Organisation
Plant Industry, P.O. Box 350
Glen Osmond, South Australia 5064
Australia
christopher.davies@csiro.au

Mr. Jake D. Dunlevy
School of Biological Sciences, Flinders University
GPO Box 2100, Adelaide, South Australia 5001
Australia
jake.dunlevy@csiro.au

Dr. Flor Etchebarne
INRA, UMR 1083 Sciences for the Enology
2 Place P. Viala, F-34060 Montpellier
France
etchebar@supagro.inra.fr

Dr. João Carlos Fernandes
DBEB/CBAA, Instituto Superior de Agronomia
Universidade Técnica de Lisboa, 1349-017 Lisboa
Portugal
joao.h.fernades@clix.pt

Ms Natacha Fontes
Centro de Fisiologia Molecular e Biotecnologia de Plantas
Departamento de Biologia, Universidade do Minho
4710-057 Braga
Portugal
nfontes@bio.uminho.pt

Dr Costanza Fregoni
Istituto di Frutti-Viticoltura, Università Cattolica del Sacro Cuore
Via Emilia Parmense 84, 29100 Piacenza
Italy
costanza.fregoni@ovisnigra.it

Prof. Hernãni Gerós
Centro de Fisiologia Molecular e Biotecnologia de Plantas
Departamento de Biologia, Universidade do Minho
4710-057 Braga
Portugal
geros@bio.uminho.pt

Dr. Matthew Gilliham
School of Agriculture Food and Wine, University of Adelaide
Waite Campus, PMB 1 Glen Osmond, South Australia 5064
Australia
matthew.gilliham@adelaide.edu.au

Prof. Eric Gomès
Institut des Sciences de la Vigne et du Vin
UMR INA/Univ. Bordeaux, Domaine de la Grande Ferrade, INRA
33883 Villenave d'Ornon
France
eric.gomes@bordeaux.inra.fr

Dr. Maria Stella Grando
Edmund Mach Foundation Research Centre, IASMA
Genetics and Molecular Biology Department
Via E. Mach 1, 38010 San Michele all'Adige (TN)
Italy
stella.grando@iasma.it

Dr. Ivana Gribaudo
Plant Virology Institute IVV-CNR, Grugliasco Unit
Via Leonardo da Vinci 44, 10095 Grugliasco (TO)
Italy
i.gribaudo@ivv.cnr.it

Prof. Bertrand Hirel
Unité de Nutrition Azotée des Plantes, UR 511, INRA de Versailles
R.D. 10, F-78026 Versailles Cedex
France
hirel@versailles.inra.fr

Dr. Curtis Kalua
Commonwealth Scientific and Industrial Research Organisation, Food Futures Flagship and Plant Industry, PO Box 350, Glen Osmond, South Australia 5064, Australia
curtis.kalua@csiro.au

Dr. Christian Kappel
Laboratoire de Physiologie Moléculaire du Transport des Sucres chez les Végétaux FRE 3091, 40, Avenue du Recteur Pineau, bât GON,
86 022 Poitiers Cedex
France
christian.kappel@bordeaux.inra.fr

Dr. Brent N. Kaiser
School of Agriculture Food and Wine, University of Adelaide
Waite Campus, PMB 1 Glen Osmond, South Australia 5064
Australia
brent.kaiser@adelaide.edu.au

Dr. Robert A. Keyzers
Commonwealth Scientific and Industrial Research Organisation
Food Futures Flagship and Plant Industry, PO Box 350
Glen Osmond, South Australia 5064
Australia
rob.keyzers@csiro.au

Dr. Stoyanka Krastanova
Department of Plant Pathology, Cornell University
New York State Agricultural Experimental Station, 14456 Geneva, NY
USA
tania1949@hotmail.com

Prof. Francois Lefort
Plants and Pathogens Group, Research Institute Earth Nature and Landscape School of Engineering of Lullier, University of Applied Sciences of W. Switzerland 150 Route de Presinge, 1254 Jussy
Switzerland
francois.lefort@hesge.ch

Prof. Konstantinos A. Loulakakis
Department of Horticulture, Technological Educational Institution of Crete
Estavromenos, 71 500 Heraklion, Crete
Greece
loulakak@steg.teiher.gr

Mr. Edi Maletic
Faculty of Agriculture, University of Zagreb
Svetošimunska 25, HR-10000 Zagreb
Croatia
emaletic@agr.hr

Dr. Lucia Martinelli
Cellular and Molecular Biology Research Unit
Fondazione Edmund Mach (IASMA) Research Center
Via E. Mach 1, 38010 San Michele all'Adige (TN)
Italy
lucia.martinelli@iasma.it

Prof. José M. Martínez-Zapater
Departamento de Genética Molecular de Plantas
Centro Nacional de Biotecnología, CSIC
Campus de la Universidad Autónoma de Madrid
Cantoblanco, 28049 Madrid
Spain
zapater@cnb.csic.es

Dr. Gwenda Mayo
Australian Centre for Plant Functional Genomics, School of Agriculture
Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA 5064
Australia
gwenda.mayo@acpfg.com.au

Dr. Flavia M. Moreira
Edmund Mach Foundation Research Centre, IASMA
Genetics and Molecular Biology Department
Via E. Mach 1, 38010 San Michele all'Adige (TN)
Italy
flavia.moreira@iasma.it

Dr. Jean-Frangois Morot-Gaudry
Unité de Nutrition Azotée des Plantes, UR 511, INRA de Versailles
R.D. 10, F-78026 Versailles Cedex
France
morot@versailles.inra.fr

Mr Panagiotis N. Moschou, Grad. student
Department of Biology, University of Crete
P.O. Box 2208, 71 409 Heraklion, Crete
Greece
panagis1982@yahoo.gr

Dr. Hernan Ojeda
INRA, UE 0999 Pech Rouge, F-11430 Gruissan
France
ojeda@supagro.inra.fr

Dr. Dider Ollé
Equipe Polyphénols et Interaction
INRA- UMR Sciences pour l Œnologie
2, Place Viala, 34 060 Montpellier Cedex 01
France
olle@supagro.inra.fr

Dr. Etti Or
Department of Fruit Tree Sciences, Institute of Horticulture
Agricultural Research Organization, the Volcani Center, Bet Dagan 50250
Israel
vhettior@volcani.agri.gov.il

Dr. Anastasia K. Papadakis
Department of Biology, University of Crete
P.O. Box 2208, 71 409 Heraklion, Crete
Greece
papadaki@biology.uoc.gr

Dr. Konstantinos G. Paschalidis
Department of Biology, University of Crete
P.O. Box 2208, 71 409 Heraklion, Crete
Greece
kpaschal@biology.uoc.gr

Pror. Ivan Pejić
Faculty of Agriculture, University of Zagreb
Trg marsala Tita 14, HR-10000 Zagreb
Croatia
ipejic@agr.hr

Prof. Avihai Perl
Department of Fruit Tree Breeding and Molecular Genetics
Institute of Horticulture Agricultural Research Organization
The Volcani Center P.O. Box 6, 0250 Bet-Dagan
Israel
perlx@int.gov.il

Dr. Goetz M. Reustle
RLP-AgroScience GmbH, AlPlanta, Institute for Plant Research Breitenweg 71,
D-67435 Neustadt
Germany
goetz.reustle@agroscience.rlp.de

Prof. Kalliopi A. Roubelakis-Angelakis
Department of Biology, University of Crete, P.O. Box 2208
71 409 Heraklion, Crete
Greece
poproube@biology.uoc.gr

Dr. Suren Samuelian
Center for Viticulture and Small Fruit Research , College of Engineering
Sciences Technology and Agriculture, Florida Agricultural and Mechanical
University Tallahassee, FL 32317
USA
suren.samuelian@famu.edu

Dr. Kristina Sefc
Institut für Zoologie, Karl Franzens Universität Graz
Universitätsplatz 2, 8010 Graz
Austria
kristina.sefc@uni-graz.at

Dr. Megan S. Shelden
Australian Centre for Plant Functional Genomics, School of Botany, University of Melbourne, Parkville, Victoria, Australia 3010 Australia
megan.shelden@anu.edu.au

Dr. Damianos V. Skopelitis
Department of Biology, University of Crete
P.O. Box 2208, 71 409 Heraklion, Crete
Greece
skopelit@cshl.edu

Dr. Cátia Sousa
DBEB/CBAA, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1349-017 Lisboa, Portugal
miss_catiasousa@hotmail.com

Dr. Silvia Tavares
DBEB/CBAA, Instituto Superior de Agronomia
Universidade Técnica de Lisboa ,1349-017 Lisboa
Portugal
satavares@isa.utl.pt

Dr. Nancy Terrier
Equipe Polyphénols et Interaction
INRA- UMR Sciences pour l'Œnologie
2, Place Viala, 34 060 Montpellier Cedex 01
France
terrier@supagro.inra.fr

Dr. Catherine Tesniere
INRA, UMR SPO
2 Place Viala, F-34060 Montpellier Cedex 1
France
tesniere@supagro.inra.fr

Prof. Mark R. Thomas
Commonwealth Sci. and Industrial Research Organization
Plant Industry, PO Box 350, Glen Osmond, South Australia 5064
Australia
mark.r.thomas@csiro.au

Ms. Joanne Tilbrook
School of Agriculture Food and Wine, University of Adelaide
Waite Campus, PMB 1 Glen Osmond, South Australia 5064
Australia
jtilbrook@ozemail.com.au

Ms Imene Toumi, Grad. student
Laboratory of Grapevine Molecular Physiology
Route Touristique de Soliman 95, Hammam-Lif 2050
Tunisia
toumine@yahoo.fr

Prof. Steve Tyerman
School of Agriculture Food and Wine, University of Adelaide, Waite Campus
PMB 1 Glen Osmond, South Australia 5064
Australia
steve.tyerman@adelaide.edu.au

Dr. Rebecca K. Vandeleur
School of Agriculture Food and Wine, University of Adelaide, Waite Campus
PMB 1 Glen Osmond, South Australia 5064
Australia
rebecca.coleman@adelaide.edu.au

Dr. Maria Isabel Van Zeller De Basto Macedo Gonzalves
Istituto di Frutti-Viticoltura, Università Cattolica del Sacro Cuore,
Via Emilia Parmense 84, 29100 Piacenza
Italy
maria.vanzeller@unicatt.it

Mr Christos N. Velanis, Grad. Student
Department of Biology, University of Crete
P.O. Box 2208, 71 409 Heraklion, Crete
Greece
xvelanis@edu.biology.uoc.gr

Dr. Celine Vignault
Laboratoire de Physiologie Moléculaire FRE 3091
40, Avenue du Recteur Pineau, bât GON,
86 022 Poitiers Cedex
France
celine.vignault@gmail.com

Dr. Silvia Vezzulli
IASMA Research Center, Via E. Mach 1 38010
San Michele all'Adige (TN)
Italy
silvia.vezzulli@iasma.it

Dr. Clotilde Verriès
Equipe Polyphénols et Interaction
INRA, UMR Sciences pour l'Œnologie, 2 place Viala
34 060 Montpellier Cedex 01
France
verries@supagro.inra.fr

Prof. Stanislaw M. Weidner
Department of Biochemistry, Faculty of Biology
University of Warmia and Mazury in Olsztyn
Oczapowskiego Street 1A, Pl-10-957 Olsztyn
Poland
weidner@uwm.edu.pl

Dr. Eva Zyprian
Julius Kühn-Institut (JKI) Bundesforschungsinstitut für Kulturpflanzen
Institut für Rebenzüchtung Geilweilerhof 76833, Siebeldingen
Germany
e.zyprian@bafz.de

1

GRAPE BUD DORMANCY RELEASE – THE MOLECULAR ASPECT

E. Or

Department of Fruit Tree Sciences, Institute of Horticulture,
Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, ISRAEL

1. THE GROWTH CYCLE OF GRAPEVINE

Grapevine is a woody temperate-zone perennial. As such, it presents a period of active growth from spring to fall, followed by a rest period in the winter. Soon after bud burst in the spring, a complex bud is formed within the axil of each leaf on the young shoot. In the prophyll of the prompt bud, which may burst within the same growing season and develop into a lateral shoot, three latent buds are formed, known as the primary, secondary and tertiary buds. During the spring and early summer, about 10 leaf primordia develop in each of these buds, while inflorescence primordia develop mainly in the primary bud (Boss et al. 2003 and references therein). In mid-summer, the latent buds enter a phase of paradormancy, in which bud burst is repressed by factors originating in other plant organs, such as auxin from the apical meristem (Lang 1987, Lavee and May 1997).

Decreasing photoperiod and temperatures in the fall induce bud endodormancy (ED), a stage in which bud burst is repressed by factors within the bud (Lang 1987, Lavee and May 1997). Further progression of the bud through the dormancy cycle requires exposure to adequate chilling temperatures, which ultimately lead to ED release (Lavee and May 1997, Dokoozlian 1999). Although low, compared to other temperate woody perennials, chilling requirements of the grape bud must be fulfilled to allow proper bud break, and maximum bud break improves with increased chilling exposure (Dokoozlian and Williams 1995, Lavee and May 1997, Dokoozlian 1999). Once the chilling requirements are met, buds will quickly and efficiently burst under controlled forcing conditions. However, in the vineyard, they may go through an additional stage, termed ecodormancy (ECD), during which bud burst may still be inhibited if environmental conditions are inadequate to support growth (Lang 1987, Or et al. 1999).

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The dormancy cycle is commonly represented by a dormancy curve, where the percentage of bud break is assessed at weekly intervals, under forcing conditions, from fall to spring. Data are presented as percent bud break (Fig. 1) or 50% of bud break (BR_{50}; Perez et al. 2007).

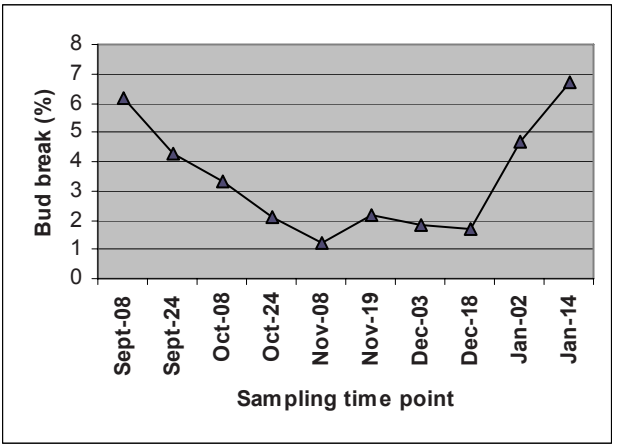

Fig. 1. Dormancy status of grape buds during the natural dormancy cycle in the Jordan valley. Ten groups of 10 single-node cuttings were prepared from cans collected in the vineyard at 2-week intervals from September to January, and incubated in a growth chamber at 23°C under 12/12 light/dark. Dormancy status is expressed as the average percentage of bud break after 18 days (E. Or, unpublished).

2. THE IMPORTANCE OF DORMANCY RELEASE FOR COMMERCIAL GRAPE PRODUCTION

Control of dormancy is very important for the commercial production of grape and other temperate crops (Saure 1985). In cold winter regions, early release from ED may contribute to low-temperature injury: crop losses are common and frost-protection measures represent a major production cost (Cattivelli and Bartlet 1992, Kadir and Proebsting 1994). Therefore, varieties need to be identified or bred for these regions that resume growth only under favorable conditions, to avoid plant damage and reduction in yield.

In warm winter regions, lack of sufficient chilling during the winter is a major obstacle for the commercial production of table grapes. This is due to the symptoms of prolonged dormancy that often appear, including delayed and low percentages of bud burst, delayed and protracted flowering season, irregular ripening, decreased yields, quick decline in growth vigor and early senescence of the vines (Dokoozlian and Williams 1995, Lavee and May 1997, Dokoozlian

1999). For successful production in these regions, it is mandatory to chemically or physically overcome the dormancy cycle (Erez 1987, Erez and Lavee 1974). Among the currently available dormancy-breaking compounds, hydrogen cyanamide (H_2CN_2; HC) is the most useful for controlled induction of grape bud break (Dokoozlian 1999 and references therein; Shulman et al. 1983). This compound successfully compensates for the lack of chilling and is in wide use in the table grape industry in warm winter regions (Dokoozlian and Williams 1995 and references therein).

One major factor complicating the commercial application of HC is the difficulty in deciding when to apply the treatment, as its efficacy and phytotoxicity depend on the dormancy status of the bud, and the outcome is dependent on both dose and timing (Fig. 2). Early application reduces the number and the size of the clusters, and is correlated with low yields. Late application may be harmful, as resistance to the chemical's toxicity declines rapidly upon release from ED (Or et al. 1999 and references therein).

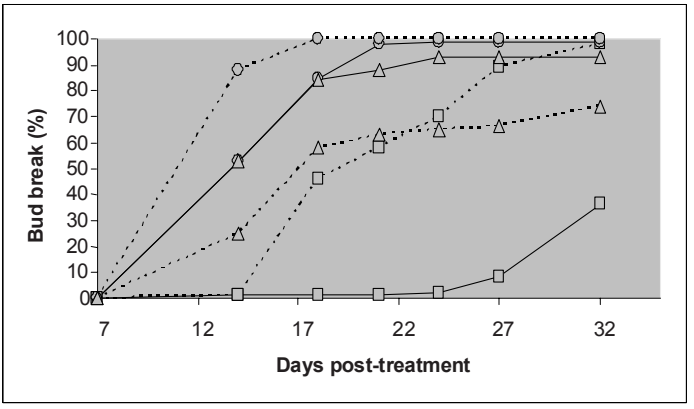

Fig. 2. The effect of dose and timing of HC application on dormancy release of grape buds. Cans were collected in the vineyard on November 5th (solid line) and December 5th (dashed line). Groups of single-node cuttings were collected at each sampling point as described in Fig. 1 and treated with 3% HC (circles), 5% HC (triangles) or water (squares). See legend to Fig. 1 for incubation details (E. Or, unpublished).

3. THE NEED FOR A MECHANISTIC UNDERSTANDING OF DORMANCY RELEASE

Due to the absence of visual changes during the bud dormancy cycle, there is no means of monitoring dormancy status of the bud in real time. The number of chilling hours has been used to estimate the bud's progress through

the dormancy cycle (Erez et al. 1990 and references therein) but it does not appear to be a reliable means of accurately determining the optimal timing of HC application (Dokoozlian and Williams 1995). This entails the risk of serious bud damage, which unfortunately often occurs following commercial application of HC. A truly reliable method for determining the dormancy status of a bud, based on endogenous changes, would enable better timing of the application of dormancy-breaking chemicals. Alternatively, development of new effective dormancy breaking strategies that are free from phytotoxic effects is required to control dormancy in warm winter region.

A prerequisite for such practical developments is the establishment of a comprehensive understanding of the biochemical mechanisms responsible for dormancy release of grape buds. However, while extensive studies have been performed on the physiological and horticultural aspects of grape-bud dormancy release (Lavee and May 1997 and references therein), only limited information on the network of biochemical pathways involved in this process in grape buds is available. This chapter describes the knowledge generated in this direction in the last decade with the aid of molecular tools.

4. THE EXPERIMENTAL SYSTEMS USED TO STUDY DORMANCY RELEASE OF GRAPE BUDS

To date, two main approaches have been used to follow molecular changes in the bud during dormancy release. One involves sampling bud material from the vineyards at 1-week to 1-month intervals from fall to bud burst (Or et al. 2002, Pacey-Miller et al. 2003, Perez et al. 2007), and study of the molecular changes occurring within the buds during the natural course of the dormancy cycle. With this approach, it is of the utmost importance to analyze the dormancy status of the sampled buds at each sampling point, so as to properly correlate the observed molecular changes with changes in dormancy status during the long sampling period. Unfortunately, some studies are lacking in such characterizations. The other approach is to carry out controlled induction of dormancy release by either natural (i.e controlled chilling) or artificial [HC, heat shock (HS), sodium azide] means, applied to either potted vines (Mathiason et al. 2008) or single-node cuttings (Or et al. 2000a, b, 2002, Perez and Lira 2005, Keilin et al. 2007, Pang et al. 2007, Halaly et al. 2008, Perez et al. 2008).

The bud-break response in single-node cuttings appears to be well-correlated with bud behavior on the vine, and it has therefore been used as a common and reliable indicator to describe dormancy depth of grapevines under forcing conditions (Shulman et al. 1983, Koussa et al.1994, Lavee and May 1997, Or et al. 1999). The use of this system enables the study of ED-related is-

sues, without the interference of paradormant and ecodormant effects. Another advantage is the possibility of working with a large number of buds, allowing proper representation of the dormancy status of a given bud population at a selected time point during the dormancy cycle.

While natural means for inducing dormancy release, such as chilling, may be instinctively considered better for tracking changes during dormancy release, such an experimental system presents limitations that do not exist when artificial means are used. The artificial stimuli supply controlled, evident, synchronized and relatively rapid induction of dormancy release within a well-characterized time frame (Halaly et al. 2008, Keilin et al. 2007, Or et al. 2000b, 2002, Pang et al. 2007), thereby creating a traceable system that facilitates the sampling of buds within the range of the actual induction period. On the other hand, the long period of chilling treatment (800 to 1500 chilling hours), combined with the absence of diagnostic means to accurately define the actual induction timing of ED release, limits the practical ability to sample chilled buds within the effective range (Dokoozlian 1999, Mathiason et al. 2008) and to follow the transient changes occurring during dormancy release (Halaly et al. 2008).

Artificial means such as HC, HS and sodium azide allow a comparison to controls that are placed under identical environmental conditions for identical periods of time. Such controls are needed to separate changes caused by the treatment from others, caused by time-dependent developmental processes. On the other hand, the only appropriate comparison in the controlled chilling system is to buds that have not been exposed to chilling, or that have been exposed to a limited chilling period which does not lead to bud break. However, such a control does not allow differentiating changes caused by chilling from time-dependent developmental changes. A comparison of biochemical changes during dormancy release in chilled buds with those in non-chilled buds incubated for an identical period of time is unacceptable, since buds exposed to different temperatures will develop at different rates during such a long period. Thus, differences in the endogenous metabolism at a given point may be reflecting differences in developmental status that are not related to dormancy release.

There is some concern regarding the detection of changes that are caused by the treatment, natural or artificial, but are not necessarily related to the process of dormancy. Neither use of the above-described controls, nor use of non-dormant material, can satisfy this concern, for reasons already discussed. Thus, it appears that the best approach currently available for the identification of dormancy-related pathways is a parallel analysis of biochemical changes following the application of various signals (natural and artificial) that induce bud break. Identification of similar changes may become a potent tool for the identification of processes and pathways that could potentially play a central role in

dormancy release. Preliminary work in this area has been recently published (Halaly et al. 2008).

5. THE USE OF MOLECULAR TOOLS TO STUDY DORMANCY RELEASE OF GRAPE BUDS

5.1. Identification of alterations in the expression of selected genes in response to artificially induced dormancy release

The first attempt to identify alterations in gene expression during early stages of dormancy release in grape buds was reported by Or and co-workers in 2000 (Or et al. 2000b). A comparison of RNA populations from HC-treated and control buds by differential display revealed a transcript for a Sucrose Non Fermenting (SNF)-like protein kinase that was upregulated following induction of dormancy release by HC. Evidence at the time from other systems suggested that SNF-like protein kinases may function as stress pseudoreceptors in yeast and mammals (Trewavas and Malho 1997). Accordingly, the parallel transcriptional regulation of a few plant SNF-like kinases by stress-related stimuli was recorded (Anderberg and Walker-Simmons 1992, Hardie 1994, Trewavas and Mahlo 1997). Therefore, it was suggested that the grape dormancy breaking-related protein kinase (GDBRPK) might be involved in the perception of a stress signal induced by HC (Or et al. 2000b).

The metabolism of HC and the mechanism by which it induces dormancy release in plants is not well understood. However, it has been previously shown (Nir et al. 1986), and recently confirmed (Perez and Lira 2005, Perez et al. 2008), that HC and other dormancy-breaking agents inhibit catalase (CAT) activity in grape buds shortly after its application. A sharp decline in the level of a CAT transcript suggested that HC exerts its effect through regulation of CAT gene expression (Or et al. 2002). However, the relationship between the HC-induced inactivation of CAT expression/activity and the effect of HC on dormancy release remains unclear.

There are indications that signals activating plant defense, such as HS and salicylic acid (SA), lead to a reduction in CAT activity and a significant increase in H_2O_2 (Foyer et al. 1997, Dat et al. 1998). It has also been shown that some of the effects of low temperature stress in plant systems are mediated by reactive oxygen species (ROS; Prasad 1996). Therefore, it was assumed that accumulation of H_2O_2 during stress serves as part of the stress-induced signalling cascade (Conrath et al. 1997, Foyer et al. 1997, Anderson et al. 1998). Or et al. (2000b) proposed that GDBRPK may be involved in the perception of an oxidative stress-induced signal, stimulated by the temporary HC-driven accumulation

of H_2O_2 in the buds. Analysis of H_2O_2 level in chilling and HC-challenged buds was then carried out and clearly confirmed that a transient induction of H_2O_2 level precedes the release of ED (Perez and Lira 2005, Perez et al. 2007, 2008). Additionally, exogenous application of H_2O_2 advanced bud break, supporting the involvement of H_2O_2 in dormancy release (Perez et al. 2008). However, its efficiency was 50% lower when compared to HC treatment. While this result may reflect differences in penetration or stability, it may also indicate that application of HC induces an effect that cannot be fully reproduced by external application of H_2O_2. Hence, the proposed signal's identity is not yet clear and it may be either H_2O_2 itself or another small molecule, generated during stress.

A transient but significant induction of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) genes, both involved in anaerobic respiration and induced in response to respiratory stress, was recorded in grape buds by Or and co-workers (2000b), following the application of HC. These data suggest the induction of transient respiratory stress by HC, which may result in a temporary increase in the AMP:ATP ratio. Hence, it was hypothesized that the SNF-like GDBRPK could serve as the sensor for a change in AMP:ATP ratio, which is the signal sensed by mammalian SNF kinases (Hardie 1994, Or et al. 2000b). The fact that both sodium azide and cyanide, which inhibit oxidative phosphorylation, are effective inducers of grape bud break provided supportive evidence that respiratory interference and possibly a change in the AMP:ATP ratio are involved in dormancy release (Or et al. 2000b).

5.2. The use of bud expressed sequence tags (EST) collections to identify changes in the bud trancriptome during dormancy release

The above-described experimental system led to the conclusion that gene expression is affected by the induction of dormancy release, and exposed changes in the expression of several genes that appear to be relevant to dormancy release, based on the existing horticultural and physiological knowledge (Or et al. 2000b). However, to gain a broad perspective of the complex biochemical network responsible for the regulation and execution of the dormancy-release process, more detailed insight into the coordinated induction/repression of metabolic cassettes that act together during dormancy release is required. High-throughput analysis of the expression profiles of large subsets of genes may be valuable in this endeavor.

5.2.1. Detection of changes in the trancriptome during a natural dormancy cycle

The first attempt to carry out a high-throughput study was reported by Pacey-Miller et al. (2003). Buds were collected from a vineyard at weekly intervals, beginning 9 weeks prior to bud burst. One cDNA library representing all sampling points was generated, from which 4270 clones were randomly selected, sequenced and printed on nylon membranes. RNA from each sampling point was P^{33}-labeled and used to probe the membranes.

While the scope of the study was wide, the dormancy status of the buds at the different sampling points was not analyzed (i.e. a dormancy curve was not established), making the interpretation of the results somewhat questionable. From the indicators supplied in that study—bud swell at time point 6 and bud burst at time point 9—only partial and indirect information could be obtained. Since bud swelling represents the beginning of active growth, which starts only after ED is released, and bud burst commonly occurs no earlier than 3 weeks from the time of ED release under winter field conditions, a valid hypothesis could be that ED was released at no later than time point 5. However, if an ECD effect was involved, the situation would be more complicated: ED release could occur even earlier, and bud swelling could be delayed until the environmental conditions become satisfactory. Hence, it is difficult to properly differentiate between changes related to ED, ECD and meristem growth in the analyzed population. Accordingly, only expression data obtained from buds sampled at time points 1-5 will be considered here, as they most likely represent ED buds during their transition towards dormancy release.

The ESTs with identified functions (65%) were divided into subcategories according to their cellular role, and the outcome led the authors to conclude that because the whole metabolism of the bud is being assayed, it is difficult to clearly distinguish between the different stages of the developmental process. The diffuculty to reach a conclusion from these data may partially stem from the mixed analysis of ED, ECD and active growth stages. As active growth may represent at least 40% of the biomass used for the library pool, it can account for the high percentage of genes related to cell structure and plant growth. A comparison to the size and content of similar sub-divisions in non-dormant buds or actively growing organs would likely be instrumental in revealing potentially unique features of the mature bud during late stages of ED. Nevertheless, there are indications of a high percentage of defense/stress-related genes, which support the results of other studies (Or et al. 2000b, 2002, Keilin et al. 2007, Mazzitelli et al. 2007, Halaly et al. 2008, Mathiason et al. 2008, Perez et al. 2008).

The authors presented the array-based expression patterns of a few candi-

date genes that were either associated to the dormancy-release process in previous studies, or represented related functions. An increase in *ADH* at time point 2 was followed by induction of members of the antioxidative machinery, such as ascorbate peroxidase (*APX*), glutathione reductase (*GR*), glutathione peroxidase (*GPX*) and glutathione S-transferase (*GST*), at the time points 3 and 4, which may reflect the timing of dormancy release. These findings support the aforementioned hypothesis regarding the development of respiratory and oxidative stress during dormancy release (Or et al. 2000b) and suggest that the antioxidant machinery is induced to cope with the increased H_2O_2 level in the bud during natural dormancy release. Significant induction of phospholipid hydroperoxide glutathione peroxidase and the stress-induced gene *OSMOTIN* at time point 3 is in line with this suggestion. A clear and stable decrease in the transcript level of pathogenesis-related genes and *DEHYDRIN* was also reported, which will be discussed further on.

While this study provided some valuable insight, the high-throughput data could not be further exploited; the number of unigenes in the EST collection was not reported, the ESTs were not made available to the public for further analyses, and expression profiles of the entire collection were not supplied. In addition, the lack of validations for the array-based profiles made it difficult to assess the reliability of the arrays and the quality of the profiles, particularly those that fluctuate redundantly between time points, as in the case of *CAT* and *SNF*-related kinase ESTs.

5.2.2. Expression profiling of grape-bud EST collections from dormant and active buds

The next attempt at taking a high-throughput approach was presented by Keilin and co-workers (2007), who generated a publicly available large-scale grape-bud EST collection. Clones for this collection were selected from libraries from buds at various developmental stages: (i) mature woody buds sampled in the vineyard during the dormancy cycle; (ii) young green buds sampled during shoot development; (iii) mature buds sampled at different time points after ED release by HC; and (iv) untreated buds sampled in parallel to the HC-treated ED buds. The collection included 5516 consensus sequences, of which 59% were not included in the *Vitis* TIGR collection at the time of analysis. About 22% of these transcripts showed no similarity to any known plant transcript.

When mature and young bud libraries were compared, it appeared that the functional distance between the young and mature buds was reflected by both quantitative and qualitative differences within the clone populations in each of the functional categories analyzed. The young bud library presented a much larger variety of functions which were related to active differentiation and growth. Overall, the data indicated that mature buds are biochemically active,

but exhibit a different repertoire of biochemical activities. On the other hand, when the HC-treated and control libraries were compared, changes in several functional categories reflected mainly changes in the expressions of single genes. This indicates a significant effect of the treatment on the expression of certain potentially key genes. Among other findings, a comparison between the HC-treated library and its control revealed significant differences in ubiquitin ligase complex, ribonucleoprotein complex, plasma membrane, cell division, cell cycle and biopolymer metabolism functional categories. The categories of response to stress and response to biotic stimuli appeared to be significantly more highly represented in the mature bud library, confirming data presented previously (Pacey-Miller et al. 2003). On the other hand, signal transduction, carbon utilization, photosynthesis, regulation of metabolism, secondary metabolism, cell cycle and cell division were more intensively represented in the library prepared from young buds.

The abundance of a particular transcript in an EST collection from a non-normalized cDNA library can be used to estimate its expression level in the tissue from which the library originated (Fei et al. 2004). Following this rationale, Keilin and co-workers (2007) detected significant differences in the number of clones between HC and control libraries for a collection of unigenes. Detailed expression analysis was reported for seven selected genes with increased expression and two genes with decreased expression at 6, 12, 24, 48 and 96 h post-HC treatment, validating the 'virtual' blot and tracking changes at the early stages of the bud response.

DEHYDRIN expression was shut down following application of HC, in agreement with the profile detected during the natural dormancy cycle (Pacey-Miller et al. 2003). Dehydrins are normally synthesized during cellular dehydration; they are present in mature seeds and buds exposed to chilling and they disappear once growth resumes (Lang. 1994, Muthalif and Rowland 1994, Salzman et al. 1996, Faust et al. 1997, Rinne et al. 1999, Welling et al. 2002). The observed reduction in transcription level supports the putative effect of HC on growth resumption as early as 48 h after its application, and suggests that *DEHYDRIN* transcript level is controlled by dormancy-status regulators that might be induced by stimuli other than chilling temperature (Keilin et al. 2007).

Two oxidative-stress-related genes, thioredoxin h (*TRXH*) and *GST*, were markedly induced following HC application, supporting the hypothesis that HC application leads to the development of oxidative stress. Thioredoxins play a role in redox regulation of target enzymes and transcription factors. They also serve as hydrogen donors to peroxiredoxins which reduce hydroperoxides to water (Jacquot et al. 2002, Sweetlove et al. 2002, Marchand et al. 2004, Serrato et al. 2004). Accordingly, induction of *TRXH* expression has been reported in response to oxidative stress (Laloi et al. 2004, Serrato et al. 2004, Rey et al.

2005) and *TRXH* has been shown to localize to the mitochondria and regulate the activity of the alternative oxidase (AOX; Gelhaye et al. 2004).

Analysis of the expression profile of *GST* supplied solid and controlled confirmation of data reported by Pacey-Miller et al. (2003). Among other functions, GSTs serve as GPXs, reducing the organic hydroperoxides produced during oxidative stress. They also function as dehydroascorbate reductases (DHARs), reducing dehydroascorbate to ascorbic acid to allow proper function of the ascorbate-glutathione cycle, a major player in the antioxidant defense system (Edwards and Dixon 2005). Accordingly, expression of *GST1* transcript in *Arabidopsis* is induced by oxidative stress (Reuber et al. 1998, Kliebenstein et al. 1999), and import of the major cytosolic GST into mitochondria was demonstrated in mouse cells following oxidative stress (Raza et al. 2002). Evidence from mammalian systems strongly suggests that GSTs play an important physiological role in the regulation of cellular signalling processes (Awasthi et al. 2005).

Among the other genes that were significantly induced were those encoding for Rab GTP-binding protein, actin depolymerization factor (ADF), ADP-ribosylation factor (ARF), calmodulin-binding protein (CBP) and polyubiquitin (Keilin et al. 2007). Interestingly, analysis of the impact of oxidative stress on the proteome of plant mitochondria revealed a similar combination of induced genes, including those encoding for disulfide isomerase, peroxiredoxin, GST, ADF and the endoplasmic reticulum-based Ca^{2+}-sequestering protein calreticulin (Sweetlove et al. 2002). Differential expression of *ADF* has also been associated with cold hardiness in wheat (Ouellet et al. 2001). The combined induction of ADF, ARF, and small GTPases led Keilin and coworkers (2007) to raise the possibility that the intracellular vesicle trafficking system plays a role in dormancy release. Interestingly, involvement of small GTPase in the regulation of actin dynamics and in the modulation of hydroperoxide production has been previously reported during polar cell growth and in response to abiotic stress (Mazel et al. 2004, Molendijk et al. 2004).

Another gene that was clearly induced in HC-treated buds was annotated as sucrose synthase (*SuSy*), catalyzing sucrose cleavage to fructose and UDP-glucose. The functional significance of SuSy is particularly important under low oxygen conditions and its requirement for survival under such conditions was shown using *SuSy*-deficient mutants (Subbaiah and Sachs 2003, Koch 2004). *SuSy* up-regulation is also evident in response to cold shock, water deprivation, anoxia and salt stress (Marana et al. 1990, Baud et al. 2004, Subbaiah and Sachs 2003, Gu et al. 2004). A potential role for SuSy in dormancy release may be inferred from findings connecting sugar-sensing systems and SuSy to regulation of plant-hormone biosynthesis and sensing, and to meristem development. In this context, it is interesting to note that the *SuSy* gene is one of the first genes

induced as leaf primordia differentiate from the apical meristem (Pien et al. 2001, Koch 2004).

5.2.3. The potential of transcriptome profiling to reveal factors potentially involved in dormancy release: possible role of calcium signalling in bud dormancy release

Under stress conditions upregulation of *SuSy* is accompanied by calmodulin upregulation and potentially mediated by Ca^{2+} (Subbaiah and Sachs 2003, Gu et al. 2004). Interestingly, a Ca^{2+}-binding protein (CBP) that interacts with calmodulin in a Ca^{2+}-dependent manner was markedly induced following HC application (Keilin et al. 2007). Thus, the possibility that Ca^{2+}-signalling is involved in grape-bud dormancy release and that cytosolic Ca^{2+} might be a transducer of the HC signal was proposed. This assumption may be supported by the findings that oxidative stress induces Ca^{2+} signalling in plant cells (Price et al. 1994, Pei et al. 2000, Foreman et al. 2003, Rentel and Knight 2004), and that cytosolic Ca^{2+} is a transducer of low oxygen signals in maize seedlings, leading to changes in the expression of various genes including *SuSy* (Subbaiah and Sachs 2003).

The involvement of Ca^{2+}/calmodulin-dependent NAD^{+} kinase (Gallais et al. 2000, 2001), calcineurin B-like protein kinase (Kim et al. 2003, Pandey et al. 2004) and Ca^{2+}-dependent protein kinase (CDPK) (Anil et al. 2000) in seed germination has been documented, suggesting a mechanistic role for Ca^{2+}-signalling in seed germination/dormancy release. However, evidence for the involvement of Ca^{2+} signalling in bud dormancy release was not reported until recently. In their report, Pang and co-workers (2007) showed HC-induced expression of Ca^{2+}-ATPase, which in turn could be an indication that HC treatment evokes an increase in Ca^{2+}_{cyt}. Similar induction was confirmed for calmodulin, CBP and CDPK. Both the Ca^{2+}-channel blocker $LaCl_3$ and the calcium chelator EGTA blocked the inductive effect of HC, and their inhibitory effect was removed by supplying exogenous Ca^{2+}. Calcium-dependent histone phosphorylation was up to 70% higher in HC-treated buds. Endogenous protein-phosphorylation assays detected a 47-kD protein that presented strong and Ca^{2+}-dependent phosphorylation only in HC-treated buds. The potential role of a CDPK in the phosphorylation of this protein was supported by an immunoprecipitation assay. Based on these data, the authors suggested that calcium signalling is involved in the mechanism of bud dormancy release (Pang et al. 2007).

5.3. Comparative analysis of the effect of different artificial stimuli of dormancy release on changes in gene expression

A recent study of changes in the transcriptome of raspberry (*Rubus idaeus* L.) lateral buds during ED release stimulated by controlled chilling was carried out using custom-made cDNA microarrays (Mazzitelli et al 2007). The recorded induction of *APX*, *GST* and *GR* genes and enhanced import of sugars were in line with findings presented above for grape buds. Reduction in the level of *DEHYDRIN* transcript and induction of members of the ubiquitination machinery, calmodulin-regulated ion channel, ATPase, GTP-binding proteins and SNF-related kinase also resembled the changes detected in grape buds. This similarity may reinforce the relevance of the changes detected in grape buds for dormancy release and raise the hypothesis that similar mechanisms of dormancy release are employed by buds from different species in response to various stimuli. The stress-related characteristics shared by natural and artificial stimuli of dormancy release such as chilling, HS, HC and sodium azide may further support this hypothesis. The similar changes described above for grape buds in response to natural chilling and HC provide preliminary support for this assumption.

A combined study of changes in gene expression induced by different dormancy-breaking stimuli might be instructive in determining whether different or similar mechanisms are activated by these dormancy-release stimuli. If analogous mechanisms are activated, the identification of genes showing similar changes in expression pattern in response to different stimuli could provide a potent tool for the discovery of pathways that are essential for dormancy release, independent of the primary artificial signal inducing the process. Two independent studies that followed this rationale have recently been reported (Halaly et al. 2008, Perez et al 2008).

5.3.1. Comparative analysis of the effects of HC and HS on the expression patterns of selected genes

Halaly and co-workers (2008) presented a comparative study of the effects of HC and HS on the expression patterns of selected genes (Fig. 3). Expression of CAT, ADH and PDC was induced by both treatments. However, changes occurred earlier in the HS-treated buds, whereas they were more intense in the HC-treated ones. A detailed analysis of expression levels at 6 h to 96 h post-treatment presented solid evidence that the changes in transcript levels following both treatments were temporary, supporting a previous assumption made by Or et al. (2000), based on expression levels recorded at only two time points. The rapid and short-lived changes in gene expression following HS posi-

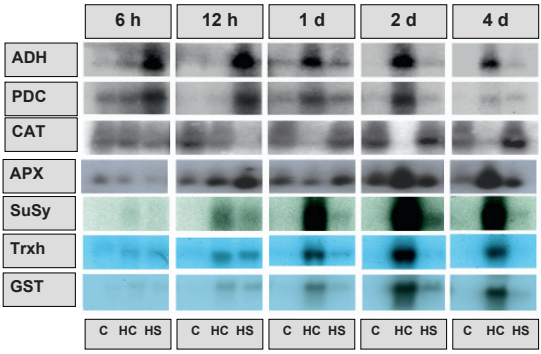

Fig. 3. Effect of controlled bud dormancy release by HC and HS on transcript level of selected genes**.** Equal amounts of total RNA (15 µg) originated from HC-treated (HC), HS-treated (HS) and control (C) buds were sampled at several time points after treatment. Northern blots were probed with radiolabeled PCR products amplified from clones representing *ADH* (AF195866), *PDC* (AF195868), *CAT* (AF236127), *APX* (TC45171) *SuSy* (TC31786), *GST* (TC25208) and *Trxh* (TC35597). Reconstructed from Halaly et al. (2008).

tively correlated with the faster and higher bud break that this treatment exerts. This correlation between molecular and horticultural findings may support the hypothesis that dormancy release is governed, at least in part, by the products of the affected genes, in a process that is controlled at the gene-expression level. Increased expression of *APX*, *GR* and *GST* was also found in response to both treatments, with transient expression characteristics that resembled the above-described profiles. The data indicate that the ascorbate-glutathione cycle is probably induced in response to oxidative stress triggered by both stimuli, and support preliminary indications for activation of the antioxidant machinery during natural dormancy release (Pacey-Miller et al. 2003).

Based on their findings, Halaly and co-workers (2008) proposed that similar cellular processes might be triggered by different stimuli that lead to dormancy release. They also proposed that the evidence for temporary induction of oxidative and respiratory stress in response to both stimuli may indicate that stress-related signals are mechanistically connected to dormancy release.

In accordance with the results reported for grape buds at the transcriptional level (Pacey-Miller et al. 2003, Halaly et al 2008), a temporary enhancement of the activities of APX, ascorbate free-radical reductase (AFR), and DHAR, as well as a steady increase in GR activity, were detected in apple buds in response to application of thidiazuron, which induces apple bud break (Wang et al.1991a). However, these changes were not interpreted as a response to a temporary oxidative burst that may stimulate bud dormancy release. On the contrary, the interpretation was that a decrease in free radical level is required for

dormancy release, based on the preliminary finding that thidiazuron diminished free radical formation several days after its application (Wang et al. 1988). A fast and temporary induction of H_2O_2 in response to thidiazuron might have been missed since the earliest time point analyzed was 2 days. Accordingly, the lower H_2O_2 level at this time point in treated buds may support the high induction of the antioxidant machinery in response to such an early oxidative burst, leading to increased H_2O_2 degradation compared to controls. A similar situation was recently described in sodium azide-treated grape buds measured 2 days after treatment (Perez et al. 2008). Unfortunately, no earlier measurement was carried out in this study either. However, the clear increase of H_2O_2 level in HC-treated buds at 6 h and its leveling off at 48 h may support the suggested scenario (Perez et al. 2008).

Temporary induction of the pentose phosphate pathway in response to oxidative stress was shown in model organisms, and was interpreted as a way of recharging the system with NADPH, required by the induced ascorbate-glutathione cycle (Baxter et al. 2007). In line with this, a temporary increase in glucose-6-phosphate dehydrogenase (*G6PDH*) transcript level was evident in grape buds following application of HC (Or et al. unpublished, Fig. 4) and sodium azide (Perez et al. unpublished).

SuSy and *Trxh* genes, whose induction following HC was previously presented by Keilin and co-workers (2007), appear to be induced by HS as well (Halaly et al. 2008). Support for the temporary induction of glycolysis in response to bud break stimuli was supplied by Wang et al. (1991b), who documented a temporary increase in the activities of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase following application of thidiazuron to apple buds.

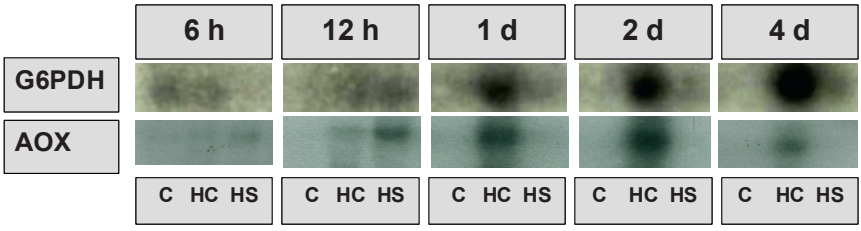

Fig. 4. Effect of controlled bud dormancy release by HC and HS on transcript level of *G6PDH* and *AOX*. Probes for *G6PDH* and *AOX* were amplified from clones representing TC46491 and TC51638, respectively. For experimental details see legend to Fig. 3 (E. Or, unpublished).

5.3.2. A large-scale analysis of changes in the bud trancriptome following HS and HC application

To enable a large-scale comparative analysis of changes in the bud tran-

scriptome following HS and HC application, a grape-bud custom microarray was recently generated (Or et al. unpublished). The array includes 7920 cDNA inserts representing the entire EST collection produced by Keilin et al. (2007) and 1141 *V. riparia* cDNA inserts originated from two libraries produced from paradormant and endodormant buds (Mathiason et al. 2008). RNA samples originating from HC, HS and control buds, collected at 3, 6 12 24, 48 and 96 h after treatment, were used to generate probes for hybridizations. Differentially expressed genes were selected and only clones presenting a significant change following both HS and HC treatments, compared to controls, were selected for further analysis (Or et al. unpublished). The overall transcriptome profile further confirmed the earlier response and milder change in expression following HS, as compared to HC. It also clearly demonstrated the transient nature of the phenomenon, which lasts between 24 and 96 h depending on the inducer. The expression profiles reported by Keilin et al. (2007) and Halaly et al. (2008) were all in full agreement with the profile of the same ESTs on the chip, reconfirming the results and serving as validation for the quality of the data on the chip. Apart from the effects on the respiratory machinery, which are discussed further on, the data supported activation of the cell cycle, protein ubiquitination and small GTP-binding protein-mediated vesicle trafficking, confirming the assumption made previously by Keilin and co-workers (2007). It also indicated that cell-wall loosening may be induced by the treatments while cell-wall synthesis is inhibited (Or et al. unpublished).

Analysis of the array data that has been carried out to date provides clear indications for the induction of both glycolysis and anaerobic pathways on the one hand, and partial inactivation of mitochondrial oxidative phosphorylation on the other (Or et al. unpublished). Transcription of clones representing all the glycolytic enzymes, apart from hexokinase, was generally upregulated by both HC and HS. These results are in agreement with the response of *Arabidopsis* cells to oxidative stress (Baxter et al. 2007). In addition to the induction of functions related to alcohol fermentation, there was also upregulation of lactate dehydrogenase (LDH), involved in lactic acid fermentation, and alanine aminotransferase, which converts pyruvate to alanine *via* fermentation (Ricoult et al. 2006). Combined with the induction of hemoglobin, naphthoate synthase, hypoxia-responsive and submergence-induced genes, the data clearly support that the treatments mimic hypoxic conditions (Sharma et al. 1992, Nie and Hill 1997). A mixed trend was detected for genes encoding TCA-cycle enzymes. Pyruvate dehydrogenase and citrate synthase, which are responsible for entry of pyruvate into the TCA cycle, were induced by both treatments. However, several other enzymes that are involved in subsequent steps, such as aconitase, NADP-dependent isocitrate dehydrogenase and malate dehydrogenase were reduced by both treatments. Interestingly, ATP citrate lyase, which is localized to

the cytosol and catalyzes the cleavage of citrate to acetyl-CoA and oxaloacetate (Hatzivassiliou et al. 2005), was intensively induced by both treatments.

A reduction in transcript level was evident for genes encoding subunits of several complexes in the oxidative phosphorylation pathway, namely Rieske iron-sulfur protein (complex III), the β subunit of the F_1 complex (complex V) and the smaller but vital cytochrome-c oxidase chain 5c. On the other hand, succinate dehydrogenase, which functions in both the TCA cycle and complex II (Buchanann et al. 2000), was induced. It is therefore hypothesized that complex II was induced, but complex III, IV and V were inhibited. Based on this assumption, it was speculated that the alternative electron-transfer pathway might have been activated, leading to increased respiration that is not accompanied by ATP production Two *AOX* genes were cloned from grape buds (TC51638, TC46683), and the expression profile of TC51638, which showed similarity to *AOX1a* that responds to various stresses in other plant species (Clifton et al. 2006), was analyzed. Induction of that gene was clearly evident following both HS and HC application, presenting the same expression pattern described above in terms of timing and intensity (Or et al. unpublished and Fig. 4).

5.3.3. Comparative analyses of the components involved in dormancy release reveal the mitochondrion as a potential sensing center

The coordinated induction of *SuSy*, *PDC* and *ADH* following HC or HS application implied that these stimuli mimic anoxic conditions. Consequently, it was proposed that the mitochondrion may serve as a potential sensing center for the dormancy-release stimuli (Keilin et al 2007, Halaly et al 2008, Perez et al. 2007 2008). Further support for respiratory stress and its direct effect on mitochondrial activity can now be drawn from the parallel and significant changes in expression of many genes that are involved in glycolysis, the TCA cycle, the electron transport chain and the response to anoxia. Additional support can be drawn from biochemical studies that have been recently carried out by Perez and co-workers (2007, 2008 and unpublished), described further on.

The mitochondrion is a central site of metabolism that integrates many aspects of cell function, and its function may be part of a vital mechanism that the cell uses to maintain homeostasis (Molen et al. 2006). In this regard, the production of ROS (Moller 2001) and the induction of AOX activity (Siedow and Umbach 2000, Juszczuk and Rychter 2003) in the mitochondria under stress conditions are of functional importance.

The level of H_2O_2 in the mitochondria is expected to increase if the rate of electrons exiting the mitochondrial electron-transport chain through the terminal oxidases slows down and/or if the rate of electron input increases above and beyond the capacity of the respiratory pathway to process the electrons. Such conditions lead to an over-reduced ubiquinone pool and subsequently, to

increased ROS production (Moller 2001). Excessive mitochondrial ROS generation has downstream consequences for the cell, including altered gene expression and even programmed cell death (Foyer and Noctor 2005). Increasing evidence indicates that AOX induction can prevent the production of excessive ROS by stabilizing the redox state of the mitochondrial ubiquinone pool. However, the induction of such an alternative respiration route is not accompanied by the production of ATP. Based on studies carried out to date on the level of gene expression, it appears that the above scenario may occur in grape buds during dormancy release.

Physiological and biochemical support for this hypothesis has been recently reported. In one study (Perez et al. 2007), buds were sampled from a vineyard located in a temperate zone in Chile from fall (April) to spring (September) at 1-month intervals. ED was maximal from May to June and ED release started between June and July, gradually moving to ECD. The level of H_2O_2, mitochondrial respiratory capacity and cytochrome oxidase (COX) activity were maximal in May, in parallel with maximal ED. While COX activity decreased sharply from May to June, the levels of H_2O_2 and mitochondrial respiratory capacity remained high, significantly decreasing only in the following month (July). The correlation between changes in ED status and the level of the analyzed parameters supported their involvement in dormancy release. The faster decrease of COX activity may be related to chilling-induced inhibition of the normal route of electron transfer in the mitochondria and the expression of an *AOX* gene from May to July may reflect this as well. Although limited, due to the wide gaps of several weeks between sampling dates, these data may agree with those obtained using the artificial stimuli HC and HS, and suggest that chilling may act by a similar mechanism.

In a second study, Perez and co-workers (2008) used the artificial induction of single buds to study the effect of HC application on H_2O_2 level, CAT activity, CO_2 evolution and rates of oxygen uptake by isolated mitochondria within 48 h of treatment. Compared with control buds, the H_2O_2 levels in HC-treated buds were 2-fold higher after 6 h, 50% higher after 24 h and 10% lower after 48 h. In contrast, CAT activity was only 10% lower at 6 h and about 2-fold lower at 24 h and 48 h (Fig. 5).

Application of HC and aminotriazole (AMT), a non-reversible inhibitor of CAT activity, resulted in a significant increase in H_2O_2 levels at 6 h after application and a considerable decrease in CAT activity 24 to 48 h after application. These findings led the authors to suggest that the increase in H_2O_2 level in HC-treated grape buds is due to inhibition of CAT activity. However, the higher H_2O_2 level in HC-treated buds at 6 h and its lower level at 24 and 48 h, compared with the AMT-treated buds, the irreversible nature of the CAT inhibitor and its failure to advance bud break (Or et al. unpublished, Perez et al. unpub-

lished) are issues which must be resolved before direct inhibition of CAT can be postulated as the cause of HC-induced dormancy release. Nevertheless, it is obvious from these studies (Perez et al. 2005, 2007, 2008) that H_2O_2 is involved in dormancy release, although the cascades by which the natural or artificial stimuli are transduced have yet to be determined.

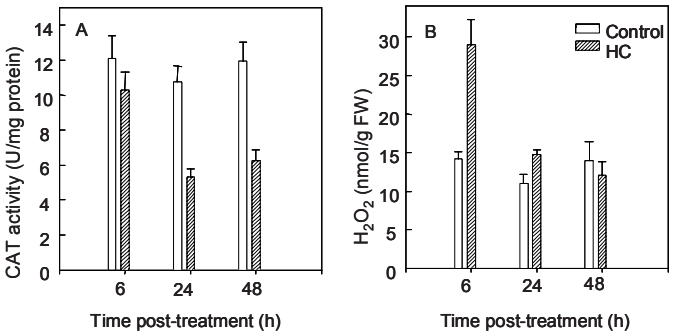

Fig. 5. Changes in CAT activity (A) and in H_2O_2 levels (B) in ED grape buds after HC application. Grape buds after chemical treatment were kept in the greenhouse under forcing conditions and the activity of CAT and levels of H_2O_2 were assessed simultaneously 6, 24 and 48 h post-treatment. Values represent the average of three replicates and bars correspond to standard deviations. Reconstructed from Perez et al. (2008).

CO_2 evolution has been shown to be similar for HC-treated and control buds at 6 h and 24 h, but 2-fold higher in HC-treated buds at 48 h (Perez et al. 2008). A possible shift to a non-phosphorylating electron-transport pathway may lead to a decrease in ATP production, which is in line with HC induction of the glycolysis pathway as a compensating mechanism (Baxter et al. 2007, Halaly et al. 2008, Or et al. unpublished). The influence of the artificial stimuli on the TCA cycle is still questionable but there are preliminary indications that its function might be perturbed as well, which is in line with the induction of anaerobic respiration, induced by the accumulation of pyruvate. Under such conditions, the alternative pathway may be induced, explaining the increased respiration (Buchanann et al. 2000). This may well coincide with the induction of *AOX* transcript from 24 to 48 h (Or et al. unpulished, Fig. 4).

Application of sodium azide, a mitochondrial electron-transport chain inhibitor, leads to enhanced ED release that is comparable to that of HC (Lavee and May 1997, Perez et al. unpublished), supporting the hypothesis that interference in phosphorylative oxidation leads to dormancy release. However, mitochondria isolated from HC-treated buds that were sampled at the time of ED release were fully functional when NADH was supplied as a substrate (Perez et al. 2008). Based on these data, the authors concluded that the stimulatory effect

of HC on bud respiration is related to metabolic changes that lead to increased NADH concentration, rather than to changes in mitochondrion functionality. However, since *AOX* transcript level is induced and the normal electron-transport pathway is inhibited (Or et al. unpublished), an effect on mitochondrial activity is expected. Indeed, very recent data indicate that application of both HC and sodium azide to isolated mitochondria leads to a remarkable decrease in mitochondrial activity (Perez et al. unpublished). The sensitivity of isolated mitochondria to HC, as opposed to the normal behavior of mitochondria isolated from HC-treated buds, indicates the transient and reversible nature of the interference, which might be eliminated during isolation.

Additionally, it was shown that 48h after treatment, sodium azide treated buds contained 40% less H_2O_2 than controls. Although this result may lead to the conclusion that sodium azide does not induce oxidative stress (Perez et al. 2008), the transient induction of H_2O_2 in response to sodium azide might have been missed in the analysis, which was carried at 2, 8 and 14 days after treatment. This is supported by recent indications of increased H_2O_2 level 12 h after application of sodium azide (Perez et al. unpublished). Accordingly, the lower H_2O_2 levels at 48 h may reflect early and intensive induction of the antioxidative machinery, which increased the rate of H_2O_2 degradation in treated buds relative to the controls, as was the case for HS (Halaly et al. 2008, Or et al, unpublished). As already mentioned, a similar situation appeared in apple buds 2 days after thidiazuron application (Wang et al 1991a).

In view of the early and transient nature of the oxidative stress, the respiratory interference and the response of the antioxidant machinery (Keilin et al. 2007, Pang et al. 2007, Halaly et al. 2008, Or et al. unpublished), analysis of the degree of similarity between the different stimuli should probably be carried out at multiple time points within 48 h of their application. Indeed, a recent analysis of the induction of several genes 48 h after application of both HC and sodium azide (Perez et al. unpublished), support this approach.

5.4. Artificially induced dormancy release; current working hypothesis

Intracellular accumulation of H_2O_2 is associated with conditions that increase its production or interfere with its degradation. The question at this point is whether the natural and artificial stimuli of grape-bud dormancy release initially lead to interference in the degradation of normally produced H_2O_2, due to inactivation of CAT, or to an increase in its production *via* perturbation of normal mitochondrial activity. While CAT expression and activity are clearly inhibited by the natural and artificial stimuli, accumulated data question the hypothesis that it is the key and primary cause for the oxidative burst.

In buds exposed to natural chilling, maximal H_2O_2 during ED release coincides with maximal CAT activity (Perez et al. 2007). Additionally, following HC application a significant increase in H_2O_2 levels is evident long before significant inhibition of CAT is recorded (Perez et al. 2008, Fig. 5). These and other data may indicate that chilling, HC, HS, and presumably sodium azide, affect a different primary target that leads to an early increase in H_2O_2 level.

Based on the molecular findings available at the time, Halaly and co-workers (2008) suggested that the phosphorylating electron-transport chain may be the primary target. The new information obtained by Perez and co-workers and Or and co-workers, described above, provides various indications and data supporting this suggestion.

A working hypothesis can currently be suggested (Fig. 6), in which temporary inhibition of oxidative phosphorylation may lead to respiratory and oxidative stress, expressed as decreased production of ATP and a temporary increase in H_2O_2 level. In response, the AOX pathway, glycolysis and anaerobic respiration are induced, in an as yet undefined order, to face the energy crisis, and respiration is enhanced, as reflected by CO_2 evolution. Members of the antioxidant machinery and related pathways (glutathione-ascorbate cycle, GST, thioredoxin, pentose phosphate pathway) are induced in parallel to cope with the oxidative burst. Primary oxidative stress may then be amplified by inactivation of CAT, extending the temporary stress situation. Inhibition of CAT activity in plant tissues in response to oxidative stress has been recorded and related

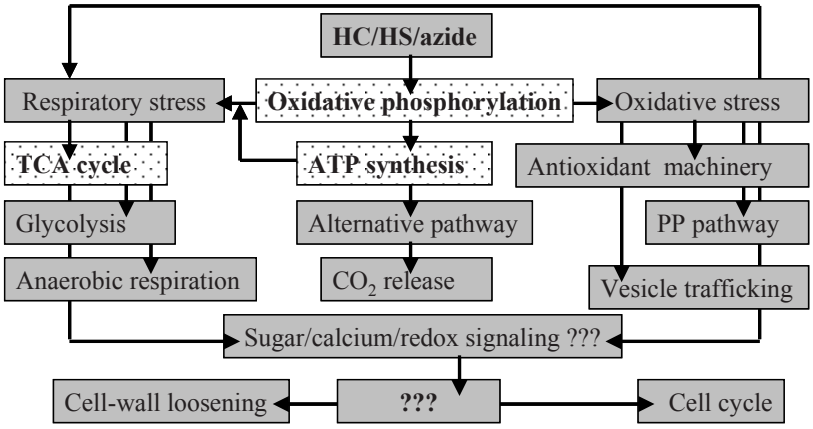

Fig. 6. Current working model for steps during artificially induced dormancy release. Solid gray background represents upregulation and dotted background represents down-regulation. PP: pentose phosphate.

to prior accumulation of salicylic acid (Shim et al. 2003). The increased level of ROS, the change in AMP:ATP ratio, potential sugar sensing, redox signalling

and calcium signalling are all potential candidates that may stimulate the subsequent events in the pathway which appear to involve cell-wall loosening, induction of the cell cycle and induction of ubiquitination-dependent hormonal signalling. Interestingly, redox signalling was recently related to the hormonal regulation of meristem development (Beveridge et al. 2007). These are all challenges that need to be faced in the future study of dormancy release of grape buds.

5.5. Potential similarity in the response to natural and artificial stimuli: lessons from transcript profiling during chilling-induced dormancy release

The aforedescribed cDNA microarrays (Or et al. unpublished) were used to examine changes in the trancriptome of *V. riparia* buds during 2000 h of chilling at 4°C (Mathiason et al. 2008). Cans were sampled at 3-week intervals (500 1000 1500 and 2000 h). Percentage of bud break was 15 at 0 and 500 h, 60 at 1000 h and 100 at 1500 h, suggesting that dormancy release first occurred between 500 and 1000 h. Exposure to 2000 h increased the rate of bud break, reaching 100% a week earlier than the 1500 h time point, suggesting that exposure to chilling after ED release may improve the rate of meristem development and hence the timing of bud break. However, when discussing dormancy release, changes occurring up to 1500 h are informative.

Mathiason and co-workers (2008) compared expression profiles after 1000, 1500, and 2000 h of chilling to that after 500 h of chilling, revealing 1469 ESTs that displayed significant differential expression. The number of changes increased with time of chilling exposure, and there was no way to separate between chilling-related and time-related changes. Reduction of gene expression was first evident at 1000 h *vs*. 500 h of chilling (150 ESTs). At 1500 h, 601 ESTs exhibited a reduction, including almost all of the ESTs that were down-regulated at 1000 h. Only 27 ESTs were induced during dormancy release. On the other hand, nearly 400 additional ESTs were reduced and 300 were induced from 1500 to 2000 h, suggesting that more than half of the differential ESTs are not related to the event of dormancy release.

The small number of genes induced during dormancy release is surprising, in light of the data recorded following the application of artificial and natural stimuli in both grape and raspberry buds (described earlier). Interestingly, several genes that are significantly induced in response to natural and artificial stimuli showed a significant change in the opposite direction in this study. It is therefore speculated here that this is not accidental; rather, it may indicate that a transient and significant change that occurred between 500 and 1000 h was

missed, and that the opposite change represents the termination stage of the transient change and beyond. This is true for GST, ABC-transporter, citrate synthase, SuSy, ATP citrate lyase and GAPDH. This disagreement might be settled by a very detailed analysis of the expression of candidate genes that have now been identified at very short sampling intervals between 500 and 1000 h. A preliminary study that used the same platform with chilled *Vinifera* buds, which require less chilling than *V. riparia*, revealed clear transient induction of SuSy between 200 h and 600 h, representing dormant and partial release status, respectively. This result may support the above speculation (Or et al. unpublished).

Apart from the contrasting findings, there are many similarities in the responses to HC, HS and controlled chilling stimuli, such as reduction in dehydrin, pathogenesis related proteins, xyloglucan endotransglycosylase, ribulose bisphosphate carboxylase, glucosyl transferase and auxin/IAA, and induction of peroxidase, metalothionin, expansin, PIP2 aquaporins and actin. Similarity can also be inferred from indications of transient oxidative stress during natural dormancy release (Pacey-Miller et al. 2003, Perez et al. 2007). Another finding, shown by Mathiason and co-workers and shared by all grape systems studied to date, as well as by studies carried out with raspberry buds and leafy spurge, is the large proportion of cell defense/stress response genes among those that are significantly induced. This may indicate that artificial stimuli and chilling induce a similar mechanism, and that a temporary disturbance in respiration and oxidative stress may be part of this mechanism. Interestingly, the finding that most of the ESTs that are significantly affected by chilling belong to carbohydrate, energy and amino acid categories (Mathiason et al. 2008) is in line with the scenario described for *Arabidopsis* cells exposed to oxidative stress (Baxter et al 2007).

While Mathiason and co-workers (2008) concluded, based on their data, that carbohydrate metabolism is inhibited and buds are less metabolically active during dormancy release, Mazitelly et al. (2007) brought data indicating the high import of sugars during dormancy release, which was supported by massive induction of sucrose degradation and glycolysis in response to HC and HS (Halali et al. 2008, Or et al. unpublished). This discrepancy may be explained by both the working hypothesis presented above and the response of *Arabidopsis* cells to oxidative stress, where both upregulation of glycolysis and downregulation of the TCA cycle are expected and in fact, have been shown (Baxter et al. 2007).

The behavior of the buds between 1500 and 2000 h, representing the ECD stage, is of special interest. The inductive effect of chilling on the rate of meristem development once transferred to growth-permissive conditions is in line with the phenomenon of leaf rosette formation after dormancy release of

Rosacea buds in response to sub-optimal chilling period. It may testify to the advantage of a preparatory stage of ECD under chilling, which might allow optimal induction of growth-regulating mechanisms. Among the ESTs upregulated at this stage were aquaporins, which are involved in transport, expansin, involved in cell-wall loosening, histone, involved in the cell cycle and rhodopsin receptor, involved in light perception; all support a growth-related preparatory scenario (Mathiason et al. 2008) and emphasize the importance of the genes that are first regulated between 1500 and 2000 h, which deserve special attention in future analyses of such preparatory events.

6. CONCLUDING REMARKS

Studies performed to date analyzed mainly changes in gene expression. While this analysis is insufficient to completely understand dormancy release, it serves as a powerful tool to reveal candidate pathways and events that might be mechanistically connected to this complex process. At this stage, a detailed study of the selected pathways/candidate factors that have been revealed may further clarify their contribution in the overall mechanism. Further understanding may benefit from functional analyses of key genes, which remain an experimental challenge in grape due to the difficulty in producing transgenic grapevines. Mass analysis of metabolic changes, which is now technically feasible, may also be a valuable step. The events in the cascade that mediate between the initial shock and the induction of cell-cycle and cell-wall metabolism, which may be regulated by redox and/or sugar signalling, probably deserve our attention in the future.

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2

GRAPEVINE & SULFUR: OLD PARTNERS, NEW ACHIEVEMENTS

S. Amâncio, S. Tavares, J.C. Fernandes & C. Sousa

DBEB/CBAA, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Lisboa 1349-017, PORTUGAL

1. INTRODUCTION

1.1. The central role of sulfur in biological functions

Sulfur (S) is the 14th more abundant element on earth crust (Charlson et al. 1992), the 9th and least abundant essential macronutrient in plants (Saito 2004) and the 6th element in the cytoplasm (Xavier and LeGall 2007). The interconversion of oxidized and reduced sulfur states, the biogeochemical sulfur cycle, depends mainly on microorganisms (Falkowski et al. 2008) and plants. The inorganic forms of S in soil consist mainly of sulfates (SO_4^{2-}) (Mengel and Kirkby 1982). Assimilatory reduction of sulfate ion integrates, together with O_2 bioproduction, CO_2 fixation, nitrate ion reduction and N_2 fixation, the biological processes essential to aerobic life. In the reduced state, S is the key element of the amino acids cysteine and methionine. The stability of the C-H and N-H covalent bonds strongly limits the carbon and nitrogen reactivity; this is not the case for S-H since the affinity of sulfur for hydrogen is low, although it is higher than for carbon (Xavier and LeGall 2007). It is accepted as a biological principle that the key function of sulfur is to provide disulfide bonds between amino acids within proteins. The thiol group of cysteine radical is fundamental for protein structure and function. Other thiol compounds more stable than cysteine, e.g. the tripeptide glutathione (GSH), concur to the cell redox regulation (Rouhier et al. 2008). Plants, yeasts and some bacteria can reduce sulfur from the oxidation/reduction state of SO_4^{2-} (+6) to the sulfide (S^{2-}) state (-2).

Sulfur plays a major role in plant growth due to its involvement in essential metabolic pathways. Plants are able to reduce SO_4^{2-} to S^{2-} and to incorporate it into cysteine; the greater part of sulfate taken up by plants is used for protein synthesis. Therefore the efficient use of carbon and nitrogen in plant growth depends on the absorption and assimilation of appropriate amounts of sulfate

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(Brunold 1976). Conversely, animals are unable to reduce sulfate depending mainly on plants and on specific microorganisms to obtain S-amino acids. Man and other mammals depend on methionine supplied in their diet, which is synthesized by plants from cysteine through a trans-sulforylation pathway (Ravanel et al. 1998, Hesse and Höefgen 2003). Organic sulfur is also found in the form of GSH, the thiol tripeptide formed by cysteine, glutamate and glycine that mediates redox reactions by the interchange of dithiol-disulfide. The proteins thioredoxin and glutaredoxin together with GSH and ascorbic acid are the main regulators of cell redox system (Rouhier et al. 2008).

1.2. Sulfur nutrition and plant defense against pathogens

Whole plants do not accumulate nor remobilize S-reserves (Mengel and Kirkby 1982). In the past, the sulfur used by crop plants resulted from sulfur-containing fertilizers and/or sulfur in rainfall (Jolivet 1993). Due to environmental policies both these sources were significantly reduced in the last 25 years: atmospheric sulfur deposition significantly decreased and many of the currently used mineral fertilizers lack sulfur (Blake-Kalff et al. 2000). Recent studies indicate that sulfur deficiency can be a limiting factor to crop yield and quality (Saito 2004, Hawkesford 2005). Therefore, former research on plant adaptation to excessive inputs of sulfur due to aerial pollution moved now into the effects of S-deficiencies.

A positive influence of sulfur nutrition on plant health was demonstrated in western Europe in the fourth quarter of last century, when the decrease in atmospheric sulfur emissions was accompanied by an increase in the incidence of fungal diseases of annual crops including oilseed rape, a high S-demanding specie (Bloem et al. 2007). Elemental sulfur (S^0) is probably the oldest pesticide, with references as old as 1000 BC (Williams and Cooper 2004). Unexpectedly for eukaryotes, it was unravelled that some plant species produce S^0 as a component of the defense system against vascular pathogens (Williams et al. 2002). S^0 was then identified as the only inorganic phytoalexin recorded to date (Williams and Cooper 2004).

In addition, several S-secondary plant metabolites play key roles in defense against pathogens (Hell and Kruse 2007), namely glucosinolates and allins (Schnug 1997). Glucosinolates are S-containing glucosides produced mostly by members of the *Brassicaceae*. In response to plant tissue damage, glucosinolates are hydrolyzed by myrosinases releasing volatile isothiocyanates which have a wide range of biological effects; they are inhibitors of microbial growth, attractants for specialist insects, herbivore toxins and feeding repellents (Mikkelsen et al. 2003). Plant defensins are small (45 to 54 aminoacids), basic peptides that have a characteristic three-dimensional folding pattern stabilized by

eight disulfide-linked cysteines (Thomma et al. 2002). Several defensin gene sequences from different plant species are now available; *y*-thionin represents an example of a plant defensin structurally related to defensins in general, including human defensins. Most plant defensins exhibit activity against a broad range of fungi, including various plant pathogens, and also against insect pests. Although defensins accumulate in response to pathogens and their elicitors (Thomma et al. 2002), they can also be developmentally regulated. For the first time in grapes, genes encoding defensins were differentially expressed among cultivars, when using ESTs derived from flower-berry libraries: one sequence which encodes a putative *γ*-thionin is expressed exclusively in Cabernet Sauvignon ripening berries, while it is expressed at pre-véraison, véraison, and ripening berries of Chardonnay, suggesting distinct patterns of gene expression between genotypes (Goes da Silva et al. 2005).

1.2.1. Sulfur in grapevine nutrition and health

References on sulfur use are found in the Bible and in Greek and Roman literature. As early as more than 2000 years ago, Romans discovered the beneficial effects of sulfur as a potent antidote against plant pathogens and refer the application of elemental sulfur in the vineyards (Rausch 2007). To date, the reports on SO_4^{2-} nutrition or supply or symptoms of sulfur deficiency in grapevine are scarce.

The effect of sulfur on plant growth, productivity and product quality mostly relates sulfur nutrition in interaction with nitrogen (Brunold 1976, Byers et al. 1987, Schnug 1997). In grapevine xylem sap, sulfate, chloride and phosphate increased by N fertilizer treatment, while nitrate was the major anion followed by malate (Peuke 2000). The protective effect of elemental S against pests and diseases has been mostly reported after foliar application (Bloem et al. 2007). In the decade of 90's field trials showed that S fertilization could substitute for fungicide application in crop yield protection from pests attack. Then the concept of Sulfur Induced Resistance (SIR) was introduced to describe the natural resistance of plants against fungal pathogens through the stimulation of sulfur metabolic processes by fertilizer application strategies (Haneklaus et al. 2007), although S-containing metabolites responsible for SIR have not been fully elucidated (Bloem et al. 2007).

In grapevine organic farming, incorporation into the soil of fine-granule sulfur and *Thiobacillus* microorganisms, restricted powdery mildew infected berries and leaves by more than 80%, in comparison with 90% after treatment with a conventional fungicide used as control (Bourbos et al. 2000). In fact, after the introduction of powdery mildew (*Erysiphe necator*, previously *Uncinula necator*) in Europe, in the XIX century, it was noticed that sulfur applied to vine leaves and berries significantly protected from powdery mildew infection. Tra-

ditionally, treatments against this disease used copper sulfate as Bordeaux mixture, a high S content fungicide, or S^0 applied as a dust or a wetable powder (Williams and Cooper 2004). Aqueous solution of lime sulfur was also used as a spray and when applied to dormant grapevines it delayed the epidemic development of powdery mildew (Gadoury et al. 1994). $CuSO_4$ alone, especially at low concentrations, elicited a substantial production of phytoalexins in grapevine leaves. Evidence is also provided that chitosan (CHN), a deacetylated derivative of chitin, which significantly reduces the infection of grapevine leaves by *Botrytis cinerea* and *Plasmopara viticola*, in combination with $CuSO_4$ confers protection against both (Aziz et al. 2006). S^0 has been used with caution due to eventual inhibition of the microbial activity during the grape must fermentation and residues remaining in the wine (Schutz and Kunkee 1977).

However, residual sulfur levels decrease rapidly following the last dusting (Thomas et al. 1993) and late S^0 application does not interfere with alcoholic fermentation neither affects wine analytical parameters (Jolivet 1993). Despite the advantages of elemental sulfur due to its target activity against powdery mildew, low cost and organic production, vineyard fungicides are increasingly substituted for chemicals without any sulfur; besides, last generation fertilizers contain low S contamination. Nevertheless, many compounds responsible for typical aromas of *Vitis vinifera* wines are sulfur containing substances (dePinho et al. 1997).

The availability of sulfur in the vineyards, grapevine sulfate nutrition and sulfur as biotic stress antagonist are important topics for separate review articles. In the present review we report advances in the last few years on the expression and regulation of *Vitis* genes encoding for sulfur transporters and sulphur assimilating enzymes.

2. SULFATE AS THE MAIN SULFUR SOURCE

Sulfate is acquired by plant roots from the soil by a multiphase rate mechanism (Clarkson et al. 1993). The soil solution is usually poor in sulfate (micromolar range), so the acquisition of this anion is strongly dependent on a low K_m uptake system, an active transport that requires energy due toe high electrochemical gradient trough the plasma membrane. Permeases mediate the transfer of sulfate from the soil solution into the cytosol of root cells (Yoshimoto et al. 2002).

2.1. Grapevine sulfate uptake and sulfate transporters

Two kinds of transporters mediate the initial uptake and the distribution

of sulfate throughout the plant: one with low K_m (10 μM) assuring a high-affinity sulfate transport (HAST), and another with much higher K_m responsible for the low-affinity sulfate transport (LAST).

Plants have evolved mechanisms to regulate sulfate assimilation in response to sulfur availability and for coordination of sulfate assimilation with growth and nitrogen assimilation (Stulen and De Kok 1993). The primary response of numerous plant systems under sulfur depletion is a clear upregulation (or derepression) of HAST, at the transcription level (Smith et al. 1995 1997, Leustek et al. 2000, Takahashi et al. 1997 2000, Shibagaki et al. 2002, Yoshimoto et al. 2002). The increase in uptake capacity is also ascribed to a raise in the expression of sulfate transporter protein (Hawkesford 2000). Conversely, sulfate repletion leads to the down-regulation (or repression) of the transporters transcription (Maruyama-Nakashita et al. 2004). Apparently, the regulation imposed by sulfur-status at the molecular level is highly coordinated with the physiological responses, either in cells (Hatzfeld et al. 1998, Clarkson et al. 1999) or at whole plant level (Clarkson et al. 1993, Smith et al. 1997).

The first plant gene encoding for sulfate transporter was isolated from the tropical forage legume *Stylosanthes hamata* and confirmed by functional complementation in a mutant yeast *Saccharomyces cerevisae* (Smith et al. 1995). Since then many sulfate transporter sequences from different plant species are available in public databases, allowing the alignment of sulfate transporters into 5 groups based on the predicted protein sequences (Hawkesford 2003). The grapevine genome release (Jaillon et al. 2007, Velasco et al. 2007) made it possible to identify nine protein sequences related to the sulfate transporter family. Phylogenetic analysis showed that these sequences can be assorted to three of the five sulfate transporter family groups (Fig. 1).

Group 1 comprises the genes for high-affinity sulfate transport regulated by S external conditions. In *Arabidopsis thaliana* genome, three different sulfate transporters were identified, *AtSultr1;1* and *AtSultr1;2* mainly localized to root cells and *AtSultr1;3* to the phloem (Yoshimoto et al. 2003). In *Vitis vinifera* cv Pinot noir genome only one sequence is assigned to Group 1 (Fig. 1). Previously to *Vitis* genome release, an homologous sequence from *V. vinifera* cv Touriga Nacional *VvST* (EF155630) was obtained by RT-PCR using degenerated primers designed after the alignment of sulfate transporter sequences from public databases (Tavares et al. 2008). Cell suspensions and roots of *in vitro Vitis vinifera* were selected to study the role of *VvST*, after sulfate removal (-S). Sulfate uptake by *V. vinifera* cells was significantly affected (Fig. 2A) under a time scale similar to that described previously for maize cells (Clarkson et al. 1999). In the more complex root system, after 15 days without S, the influx was twice the value of +S plants (Fig. 2D). The abundance of *VvST* mRNA matched the derepression of sulfate uptake capacity (Fig. 2C), suggesting a transcrip-

tional regulation of the sulfate transport in response to S availability (Maruyama-Nakashita et al. 2004). Recent reports demonstrated that sulfate transporters are transcriptionally regulated at the promoter level at the SURE *cis*-factor (Maruyama-Nakashita et al. 2005) or by the SLIM *trans*-factor (Maruyama-Nakashita et al. 2006).

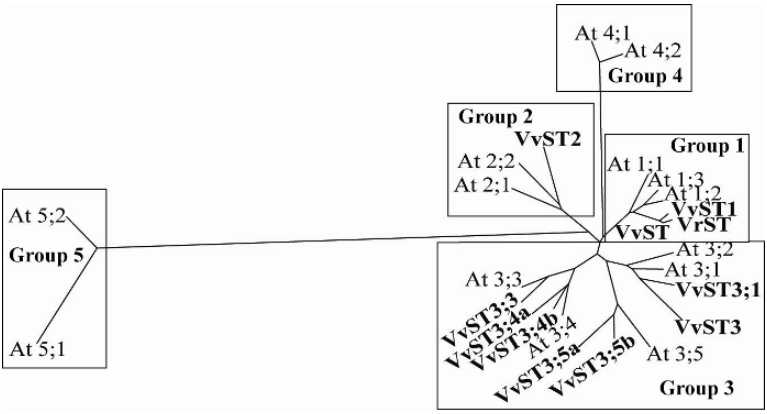

Fig. 1. Phylogenetic analysis of selected plant sulfate transporter amino acid sequences. Accession numbers from NCBI: *Arabidopsis*: AtSultr1;1, BAA33932; AtSultr1;2, BAA95484; AtSultr1;3, BAB16410; AtSultr2;1, BAA20085; AtSultr2;2, BAA12811; AtSultr3;1, BAA21657; AtSultr3;2, BAA20282; AtSultr3;3, NP173722, AtSultr3;4, BAB21264; AtSultr3;5, BAB55634; AtSultr4;1, BAA23424; AtSultr4;2, BAB19761; AtSultr5;1, NP_178147; AtSultr5;2, NP_180139; *Vitis vinifera*: VvST, EF155630; hypothetical proteins of *Vitis vinifera* with homology to sulfate transporters, VvST1, CAN75170; VvST3;1, CAN61400; VvST3,4a, CAO45933; VvST3;4b, CAN83977; VvST3;3, CAN77009; VvST2;1, CAN66052; VvST3;5a**,** CAN84174; VvST3,5b, CAN74632; VvST3, CAN70402; *Vitis rupestris*: VrST, EF155629. Alignments performed using the T-COFFEE web service (Notredame et al. 2000) and the resulting un-rooted tree drawn using TreeView version 1.6.6 (Page 1996).

A strong repression of sulfate influx was induced by sulfate repletion (+S), now at hour time scale (Fig. 2B). The abundance of *VvST* reflected a marked decrease (Fig. 2C), although less pronounced than the response at the protein level responsible for the repression of sulfate influx. Thus, the regulation of *V. vinifera* high affinity sulfate transporter by S starvation and S resupply occurs at the mRNA expression level and also at *de novo* protein synthesis, as reported for *Arabidopsis* (Maruyama-Nakashita et al. 2004). *VvST* must also be the main responsible for the upregulation of sulfate influx in -S roots, considering that its expression increased significantly. In conclusion, *VvST* was confirmed as a sulfate transporter of group 1, considering the protein (and nu-

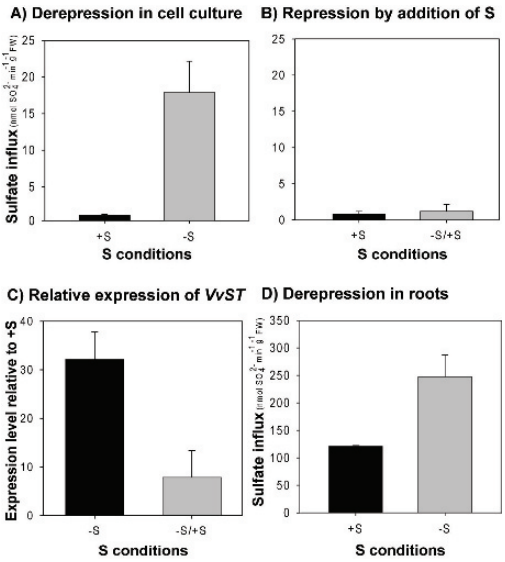

Fig. 2. Sulfate influx and *VvST* expression in *Vitis vinifera* cv Touriga Nacional cells and roots under full sulfate (+S) and sulfate deprivation (-S) conditions A) Sulfate influx in cell suspensions 5 days in +S and –S measured as $^{35}SO_4^{2-}$ uptake. B) Sulfate repression of sulfate influx in cells de-repressed by sulfate deprivation (-S). C) Relative expression of *VvST* in -S cells and repression of *VvST* by sulfate. mRNA of cell suspensions in –S or +S for 5 days analyzed by SYBR-green RT-PCR (Bovy et al. 2002). Each point is the mean of three replicates from independent cultures ± SD. RNA levels for *VvST* were normalized against the expression of *Act2* RNA. D) Effect of sulfate conditions on root-uptake.

cleotide) sequence but also the molecular and physiological data.

The common characteristic among group 2 sulfate transporters is its low affinity for sulfate, expressed mainly in vascular tissue of both root and shoots tissues. From the two isoforms of *Arabidopsis thaliana*, AtSultr2;1 and AtSultr2;2, the first isoform is strongly up regulated by low sulfur availability in roots (Takahashi et al. 2000). *V. vinifera* possess only one isoform (VvST2, Fig. 1) that fits into this group. *VvST2* is expressed in roots and cells, with an upregulation only visible after 7 days in –S conditions (Fig. 3).

A large and diverse number of sulfate transporters isoforms has been assigned to Group 3 (Hawkesford and De Kok 2006). *AtSultr3;5*, the only isoform characterized to date, is unable to complement the yeast mutant for sulfate transporter and does not respond to S conditions. Apparently, it is not a functional transporter but can contribute to sulfate uptake when co-expressed with AtSultr2;1 (Kataoka et al. 2004a). In *V. vinifera* genome six sequences were assigned to this group (Fig. 1), confirming an apparent redundancy. Excluding VvST3;5a,b, all *V. vinifera* sulfate transporters were expressed in roots and culture cells. An homolog to *VvST3;5a,b* was preferentially expressed in the seed

of Cabernet Sauvignon berry indicating a probable specific localization and role in the development of the seed (Grimplet et al. 2007). After 4 days in –S conditions, *VvST3* is the only transcript showing a mild upregulation, although at day 7 *VvST3;3* and *VvST3* responded to the mineral stress (Fig. 3). *VvST3;4a,b* was not affected by –S conditions, behaving like other group 3 sulfate transporters identified to date in other species. The last isoform was also expressed in the pulp of Cabernet Sauvignon grape berry, probably contributing to the overall supply of S under normal conditions (Grimplet et al. 2007).

Sulfate transporters belonging to group 4 were localized to the tonoplast and may play a role in mobilizing SO_4^{2-} from the vacuole (Kataoka et al. 2004b). The functions and localization of group 5 transporters are still unknown (Hawkesford and De Kok 2006). Apparently group 4 and 5 are not represented in *V. vinifera* genome (Fig. 1), probably substituted for sulfate transporters from group 3.

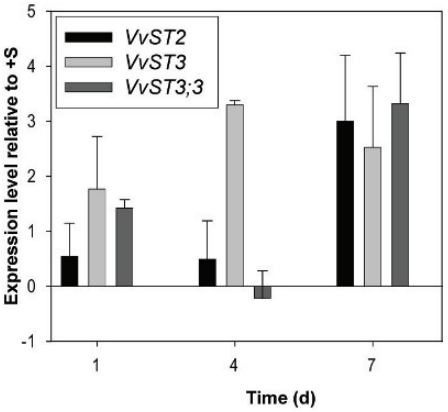

Fig. 3. Relative expression of sulfate transporters *VvST2*, *VvST3 and VvST3;3*, in *Vitis vinifera* cells in full sulfate (+S) and sulfate deprivation (-S) conditions. Analysis of mRNA was analyzed as in Fig. 2 (Tavares et al. unpublished results).

3. SULFATE ASSIMILATION

Sulfur assimilation in plants plays a key role in the biological sulfur cycle (Saito 2004). SO_4^{2-} in the soil and SO_2 in the air are assimilated into cysteine, which can be converted to methionine. S in cysteine and methionine are mainly incorporated into proteins and represent up to 70% of the total sulfur content of plants (Hawkesford and De Kok 2006). The free thiol pool comprises cysteine and GSH, where GSH is a less reactive storage and transport form of thiol compounds (Höefgen and Hesse 2007). It was demonstrated that root cells reduce and assimilate sulfate at a rate that varies between species, as it occurs with ni-

trate (Pate 1980), if root cells were unable to reduce the sulfate ion, only oxidized sulfur would circulate in xylem vessels.

3.1. Enzymatic milestones

The assimilation of sulfate is, with carbon fixation and nitrogen assimilation, one of the basic pathways used for the incorporation of inorganic elements into the organic molecules that drive cell metabolism. SO_4^{2-} assimilation in plants involves three main steps which are shown in Fig. 4.

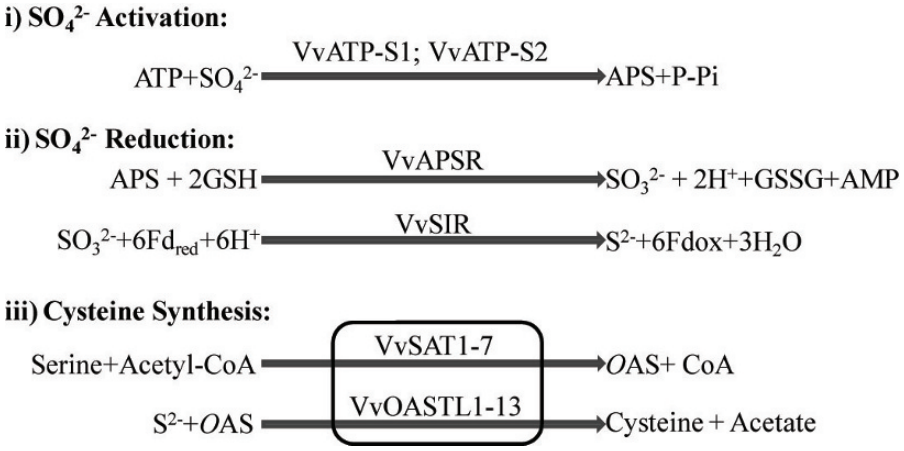

Fig 4. Flow chart of reactions of sulfate assimilation in *Vitis vinifera*: VvATP-S1,2 - sulfate-ATP: adenylyl transferase (E.C. 2.7.7.4, APS sulfotransferase); APS - adenosine 5′-phosphosulfate; VvAPSR - GSH:APS sulforeductase (E.C. 2.8.2n, GSH-APS reductase); VvSIR - S^{2-}:ferredoxin oxidoreductase (E.C. 1.8.7.1, sulfite reductase, SIR); VvSAT - serine acetyl transferase (E.C. 2.3.1.30, SAT); *O*AS – *O*-acetyl-serine; Vv*O*ASTL1-13 - *O*-acetyl-serine sulfydrilase (E.C. 4. 2.99.8 *O*-acetyl–L-serine(thiol)-lyase, *O*ASTL).

3.1.1. Sulfate activation

Sulfate is activated to adenosine phosphosulfate (APS) by a phosphate bound with consumption of ATP and release of pyrophosphate by ATP sulfurylase (ATP: sulfate adenylyl transferase, E.C. 2.7.7.4, ATP-S; Fig. 4,i). Since the reaction equilibrium of ATP-S favors the reverse reaction (ATP and SO_4^{2-} formation) (Saito 2004) and in plants pyrophosphate concentration is around 0.3 mM, *in vivo* activity of ATP-S depends on the consumption of APS in further reduction or phosphorylation reactions. *Arabidopsis ATP-S* genes are chloroplast targeted therefore the metabolic steps that reduce sulfate to sulfide are exclusively chloroplast localized, although ATP-S activity can also be measured in the cytoplasm. One hypothesis is that APS generated in the cytoplasm is used

in sulfation reactions (Rotte and Leustek 2000) which occur, for instance, in glucosinolate biosynthesis.

3.1.2. Sulfate reduction

In plants, recent lines of evidence demonstrate that the two step-reduction of SO_4^{2-} (S:+6) to S^{2-} (S:-2) is carried out by GSH-APS reductase (two electrons) (GSH:APS sulforeductase, E.C.2.8.2.n, APSR; Fig. 4ii) and the plastid enzyme sulfite reductase (S2-: ferredoxin oxidoreductase, E.C. 1.8.7.1., sulfite reductase, SIR) (Fig. 4,ii). The first reduction step is equivalent to the bacterial formation of free sulfite (SO_3^{2-}) as a free intermediate, through PAPS reductase activity. It was demonstrated that *Arabidopsis thaliana* cDNA is able to complement functionally an *E. coli* mutant defective in PAPS reductase activity, and encode proteins with a thioredoxin/glutaredoxin-like domain (Gutierrez-Marcos et al. 1996, 1997). In fact, the chloroplast mature enzyme contains an N-terminal reductase domain and a C-terminal GSH dependent glutaredoxin domain. In brief, in plants APSR reacts with APS by the thiol group of one cysteine to form an intermediate enzyme–Cys-SSO_3^{2-} with AMP release by hydrolysis; this intermediate is reduced with GSH and sulfite and the complex enzyme-Cys-S-SG are liberated. The enzyme is regenerated by reduction with a second GSH (Bick and Leustek 1998). Reduction is completed by the production of sulfide S^{2-} by the transfer of 6 electrons from reduced ferredoxin to sulfite to form sulfide, in a reaction catalyzed by sulfite reductase. In plants this step is apparently not targeted for regulation of sulfate assimilation and, unlike bacteria, the plant enzyme has not been the object of much research.

3.1.3. Sulfide assimilation and cysteine synthesis

S^{2-} is incorporated into cysteine by the activity of the complex serine acetyl transferase (E.C. 2.3.1.30, SAT) and *O*-acetyl-serine sulfydrilase (*O*-acetyl–L-serine(thiol)-lyase, E.C.4.2.99.8, *O*ASTL) (Fig. 4iii). The level of free cysteine in plants is very low but the flux can be quite high (Höfgen and Hess 2007). The only direct carbon/nitrogen precursor for cysteine is *O*-acetyl serine (*O*AS), which results from the acetylation of serine by SAT. This substrate incorporates S_2^- into the β-position of cysteine in a reaction catalysed by *O*ASTL. The free recombinant SAT forms instable multimeric aggregates which are prevented in the presence of *O*-acetyl–L-serine(thiol)-lyase. In the presence of *O*ASTL the protein-protein interaction between the two enzymes forms the pyridoxal phosphate containing cysteine synthase complex. *O*AS triggers the dissociation of the complex while sulfide counteracts this effect (Droux et al. 1998). SAT and *O*ASTL are present in three cell compartments, plastids, mitochondrion and cytoplasm, eventually to adjust the cysteine pool to protein syn-

thesis (Saito et al. 1997).

3.1.4. Regulation by sulfur availability

Sulfate acquired from the soil solution by plant roots can be stored in vacuoles or enter the assimilatory pathway. Removal of S supply cause an increase in the protein activity or mRNA pools of some enzymes responsible for the uptake and the assimilatory pathway, such increase is observed after several days in whole plants (Buchner et al. 2004, Hell et al. 1997) or several hours in cell suspensions (Hatzfeld et al. 1998). Following resupply of SO_4^{2-} all fall in parallel with cysteine and GSH increase. In most plant systems analyzed so far, sulfate, cysteine, and GSH are described as negative regulators and *O*AS as positive regulator of sulfur genome (Droux 2004). GSH-APS reductase is thought to be a prime regulation point of the pathway (Vauclare et al. 2002), since its activity and RNA increase concomitantly with S starvation and with stresses that increase the demand for GSH and then for cysteine. The control of gene expression limits excess uptake and assimilation with negative regulation by downstream S-compounds and a positive regulation by the serine derivative *O*AS. Therefore the efficient use of N in plant growth depends on the absorption of appropriate amounts of S.

3.2. Grapevine genes for sulfur assimilation enzymes

3.2.1. Identification of sulfur assimilation genes

Partial sequences of *V. vinifera* genes encoding for APR (EU275236), SAT (EU275238) and *O*ASTL (EU275237) were cloned and deposited at Gene Bank. Subsequently to grapevine genome sequencing (Jaillon et al. 2007, Velasco et al. 2007), all genes for putative isoforms of sulfate assimilation enzymes were identified in databases.

The first enzyme of the sulfur assimilation pathway responsible for sulfate activation in *V. vinifera* has two isoforms, VvATP-S1 and VvATP-S2 (Fig. 5), while the enzymes responsible for the reduction of sulfate show one isoform each, VvAPSR and VvSIR, respectively unlike *A. thaliana*, which has four different APSR isoforms (Fig. 6).

The position of VvATP-S1 in the phylogenetic tree ascribes a high homology to AtATP-S4, AtATP-S3 and AtATP-S1 and to isoforms from *Brassica spp*, *Camelia sinensis* and *Solanum tuberosum*; VvATP-S2 isoform is close to *Populus* ATP-S and AtATP-S2. The nucleotide sequences from *Vitis vinifera* genome, *VvAPT-S1* and *VvAPT-S2*, reproduce the homology depicted at amino acid level. A sole VvAPSR isoform clustered close to AtAPSR1, and the amino acid sequence confirms the protein structure (Bick and Leustek 1998): a domain of the adenine nucleotide alpha hydrolases (AANH) superfamily, which binds

to adenosine nucleotide, a plant-type APS reductase containing a C-terminal redox active TRX domain, and an N-terminal reductase domain. Since plant-type APS reductase uses GSH, as its electron donor, the C-terminal domain functions like glutaredoxin, a GSH-dependent member of the TRX superfamily.

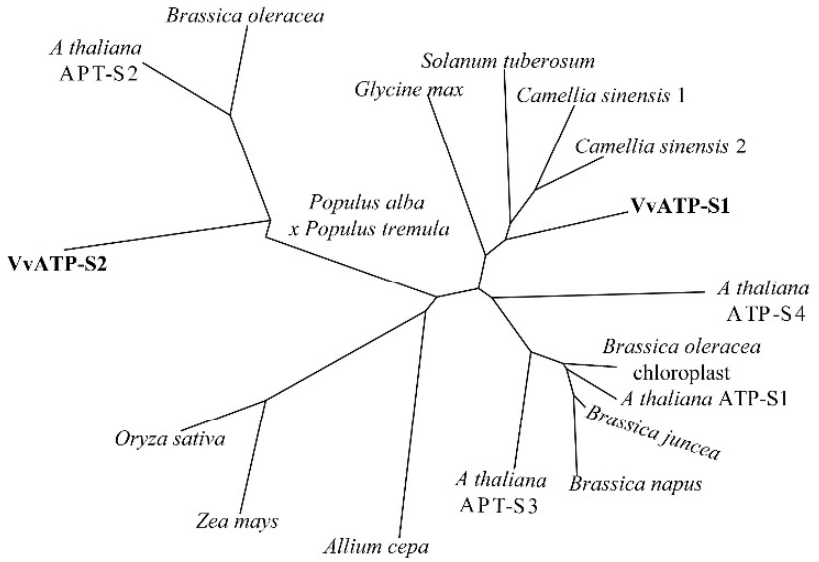

Fig. 5. Phylogenetic analysis of selected ATP sulfurylase amino acid sequences. Accession numbers from NCBI: *Allium cepa*, AAL61615.1; *Arabidopsis thaliana*: AtATP-S1, NP_188929.1; AtATP-S2, NP_564099.1; AtATP-S3, NP_193204.1; AtATP-S4, NP_199191.1; *Brassica juncea*, CAA11417.1; *Brassica oleracea* chloroplast, ABY59052.1; *Brassica oleracea*, AAB67995.1; *Brassica napus*, AAB53100.1; *Camellia sinensis*: CsATP-S1, ABF47291.2; CsATP-S2, ABE01402.1; *Glycine max*, AAL74418.2; *Oryza sativa*, BAA36274.1; *Populus albaxPopulus tremula*, AAQ57203.1; *Solanum tuberosum*, CAA55655.1; hypothetical proteins of *Vitis vinifera* with homology to ATP sulfurylase, VvATP-S1, CAN67181.1, VvATP-S2, CAN84072.1; *Zea mays*, NP_001104877.1. Alignments performed using the T-COFFEE web service (Notredame et al. 2000) and the resulting unrooted tree drawn using TreeView version 1.6.6 (Page 1996).

The sole isoform of sulfite reductase present in *Vitis vinifera*, such as in *Arabidopsis*, contains two main domains: an iron-sulfur (4Fe-4S) cluster and siroheme domain and the ferredoxin–binding domain. Two copies of these sequences are found in sulfite reductase and form a single structural domain (Nakayama et al. 2000).

Serine acetyltransferases are proteins containing hexapeptide repeats characteristic of transferase enzymes. It has been proposed that secondary structures formed by these repeats are involved in the interaction of SAT with *O*-acetylserine-(thiol)-lyase (Howarth et al. 1997). The *Vitis vinifera* genome database contain seven sequences although after homology analysis only four can be

considered different, a number close to the five genes found in *Arabidopsis* genome (Hell et al. 2002).

Eight isoforms of *O*-acetylserine-(thiol)-lyase nucleotide sequences resulted from the homology analysis out of the 13 sequences identified (Fig. 7), which are translated into proteins with the highly conserved pyridoxal-phosphate cofactor domain. A close number (nine) of *O*-acetylserine-(thiol)-lyase is also found in *Arabidopsis* genome (Saito 2004).

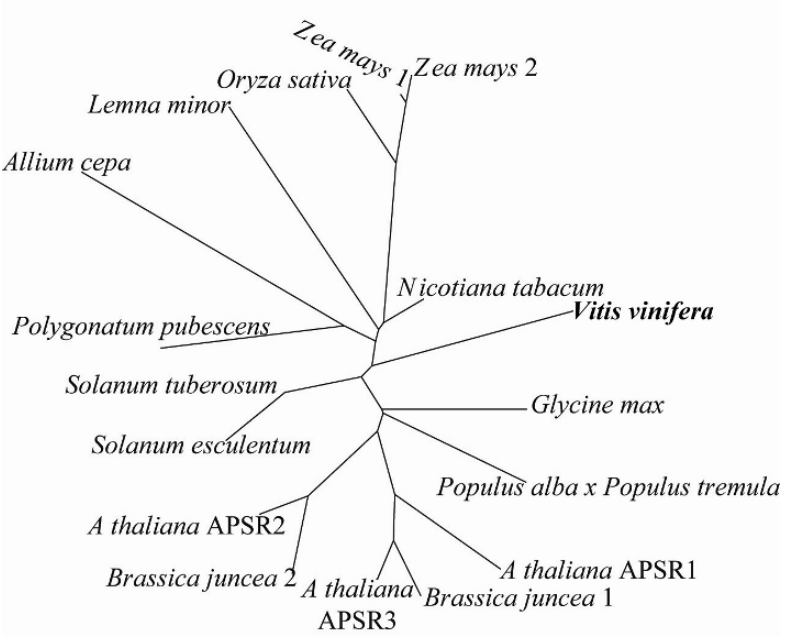

Fig. 6. Phylogenetic analysis of selected APS reductase amino acid sequences. Accession numbers from NCBI: *Allium cepa*, AAF18999.1; *Arabidopsis thaliana*: AtAPSR1, NP_192370.1; AtAPSRS2, NP_176409.1; AtAPSRS3, NP_193930.1; *Brassica juncea*: BjAPSR1, CAA04611.1; BjAPSR2, CAA04610.1; *Glycine max*, AAL66290.1; *Lemna minor*, CAB65911.1; *Nicotiana tabacum*, AAT67174.1; *Oryza sativa*, NP_001059750.1; *Polygonatum pubescens*, AAZ75685.1; *Populus albaxPopulus tremula*, AAQ57202.1; *Solanum esculetum*, AAU03359.1; *Solanum tuberosum*, CAA55655.1; hypothetical protein of *Vitis vinifera* with homology to APS redutase, VvAPSR, CAO24008.1, EU275236; *Zea mays*, ZmAPSR1, NP_001105764.1; ZmAPSR2, NP_001105805.1. Alignments performed using the T-COFFEE web service (Notredame et al. 2000) and the resulting unrooted tree drawn using TreeView version 1.6.6 (Page 1996).

3.2.2. Expression of *V. vinifera* sulfur assimilation genes

A reverse correlation between sulfate availability and sulfate assimilation gene expression is well documented for model plants and some other species; grapevine data proceed from ongoing research (Tavares et al. unpublished results). The analysis of the expression of genes for sulfate metabolism enzymes

in response to sulfate depletion in grapevine cells (Fig. 8), but also in roots and leaves, showed that the relative abundance of *VvATP-S1, VvSr* and particularly *VvAPR,* is up-regulated in the three systems, confirming the crucial role of APR in sulfur metabolism pathway (Vauclare et al. 2002). As in other species, the up-regulation of *VvATP-S* and *VvAPR* is significantly amplified in the cell system when compared with the whole plant analysis. The expression of *VvSr* is equivalent in cells and root. *SAT* transcripts namely *SAT6,* is significantly derepressed in cells at day 7 in roots and leaves (Fig. 8). This late up-regulation seems more related to long-term S-deficiency response (Hirai et al. 2003).

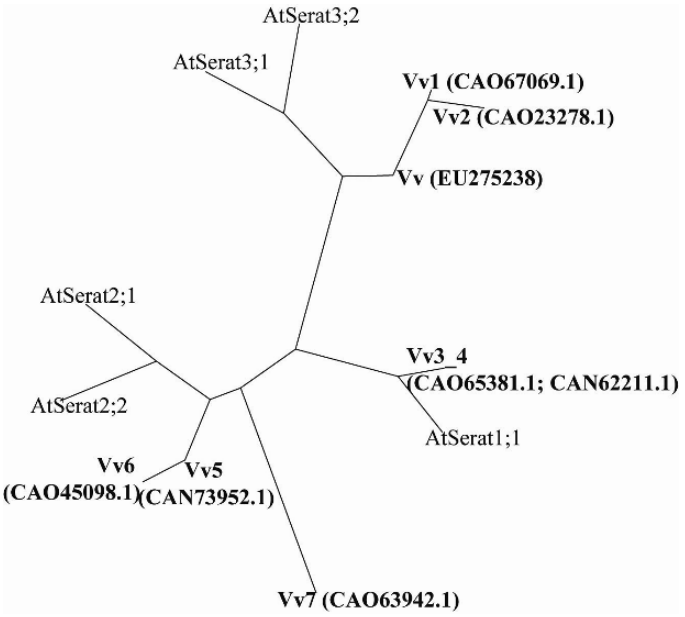

Fig. 7. Phylogenetic analysis of serine acetyl transferase amino acid sequences from *Arabidopsis thaliana* and hypothetical protein sequences of *Vitis vinifera* with homology to *A. thaliana*. Accession numbers from NCBI: *Arabidopsis thaliana*: AtSerat1;1, NP_200487.1; AtSerat2;1, NP_175988; AtSerat2;2, NP_187918; AtSerat3;1, NP_565421.1; AtSerat3;2, NP_195289.3; *Vitis vinifera*: Vv1-7, each sequence is indicated in the tree by the correspondent NCBI accession number. Alignments performed using the T-COFFEE web service (Notredame et al. 2000) and the resulting unrooted tree drawn using TreeView version 1.6.6 (Page 1996).

3.2.3. Negative and positive regulators

In most plant systems analyzed so far sulfate, cysteine and GSH are described as negative regulators and *O*AS as positive regulator of sulfur genome (Hell 1997, Droux 2004). Analysis of the abundance of *ATP-S1* and *APS* reductase mRNAs in *Vitis vinifera* cells as a response to sulfate deficiency, sulfate

re-supply, GSH, cysteine or OAS addition (Fig. 9) confirmed that SO_4^{2-} and the thiol metabolites cysteine and GSH are strong negative regulators of *APR* expression (Vauclare et al. 2002). *O*AS has been considered as a general regulator of gene expression in response to sulfur nutrition (Hirai et al. 2003). Supply of exogenous *O*AS to various higher plant systems showed that it can have a limiting effect on cysteine synthesis (Neuenschwander et al. 1991) and it can also cause de-repression of sulfate reduction pathway (Hatzfeld et al. 1998). In *Vitis* cells growing in sulfur medium the effect of *O*AS was responsible for a 4-fold de-repressing of *APSR* expression and a 3-fold in *ATP-S*. The up-regulation of grapevine *ATP-S* is of the same order of magnitude as ATP-S activity in maize cells treated with *O*AS (Clarkson et al. 1999).

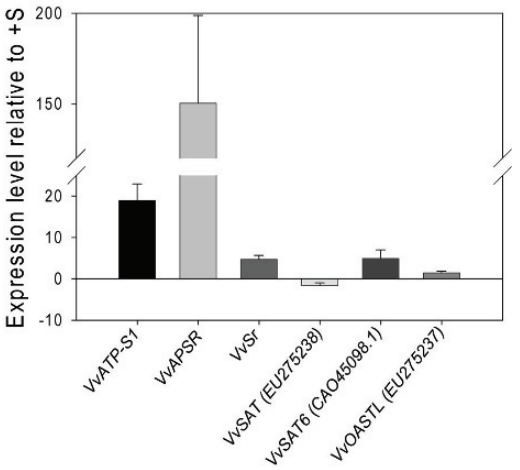

Fig. 8. Relative expression of the genes for of the sulfate assimilation enzymes in *Vitis vinifera* cv Touriga Nacional cells after 7 days under sulfate deprivation (-S) conditions. mRNA was analyzed as in Fig. 2 (Tavares et al. unpublished).

4. CONCLUDING REMARKS

Ongoing research is attempting to unravel the *Vitis* primary sulfur pathway, from root sulfate uptake to cysteine synthesis. The expression of genes of some sulfate transporters has been analyzed for the first time (Tavares et al 2008) and genomics of the assimilatory enzymes is in progress.

Recently, the release of grapevine genome (Jaillon et al. 2007, Velasco et al. 2007) disclosed large families of genes associated with primary sulfur metabolism, sulfate uptake and assimilation, methionine and GSH metabolism, as well as with secondary metabolism pathways directly connected with sulfur

availability. The role of GSH in photosynthetic organisms and the emerging functions for glutaredoxins was reviewed recently (Rouhier et al. 2008). GSH is the most abundant low-molecular-weight thiol in cells and it constitutes a redox buffer that keeps the intracellular environment reduced. It can be oxidized to GSSG by some reactive oxygen species (ROS), such as H_2O_2; thus playing a major role in the detoxification of ROS. GSH also participates in the glutathione/ascorbate cycle, in which GSH allows regeneration of reduced ascorbate, the other major antioxidant in plant cells. GSH also provides electrons to peroxidases of the peroxiredoxin family, which use GSH alone or with glutaredoxin. Other GSH-dependent enzymes, such as glutathione S transferases (GST) form GSH conjugates. In grapevine, stress conditions which up-regulate anthocyanin biosynthesis can lead to anthocyanin levels toxic to the plant cells. This toxic effect can be avoided by the transport of anthocyanins into the vacuole after conjugation with GSH by GSTs (Boss and Davies 2009, Xiang et al. 2001).

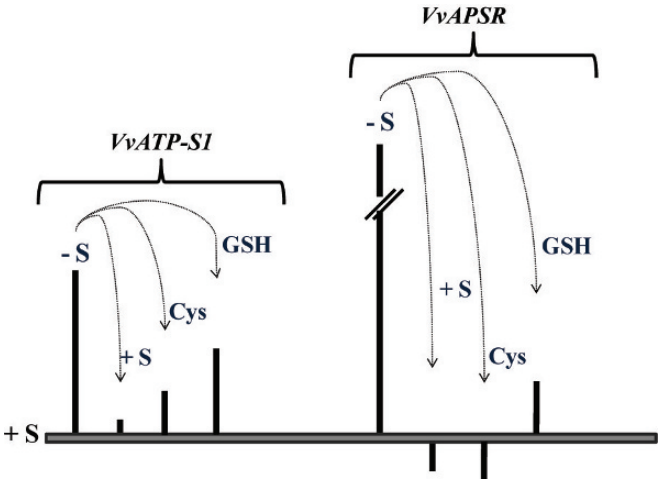

Fig. 9. Relative expression of *VvATP-S1* and *VvAPSR* in S-depleted cells transferred to + S (1.5 mM) or receiving 1 mM GSH or 1 mM cysteine for 24 h, analyzed by real-time PCR as in Fig. 2 (Tavares et al. unpublished).

Phenylpropanoid compounds are induced in plants by biotic and abiotic stresses. Low nitrogen is related with the accumulation of flavonoids, low phosphorous with anthocyanins and low iron with phenolic acids (Dixon and Paiva 1995). Anthocyanins are secondary metabolites present in almost all higher plants, namely in the skin of red grapes. It is reported that nitrogen supply affects the metabolic pathway of anthocyanins: berries from high N supplied vines had a lower anthocyanin content which decreased significantly during

maturation (Hilbert et al. 2003). Stilbenes are stress-induced phenylpropanoids which result from the condensation of *p*-coumaroyl-CoA or cinnamoyl-CoA with malonyl-CoA by the action of stilbene synthase. In grapevine, resveratrol is a low molecular weight stilbene acting as a phytoalexin (Bavaresco et al. 2009). Its synthesis is elicited in leaves, berries and cell suspensions in response to biotic, abiotic and nutritional stress. N supply decreased resveratrol in leaves and berries (Bavaresco et al. 2001). However, the effects of sulfur availability on phenylpropanoid compounds, including anthocyanins and resveratrol, have not been elucidated. These compounds have important roles in the resistance of grapevine to biotic and abiotic stresses and can confer antioxidant properties to red wine; therefore the cross talk between sulfur and phenylpropanoid metabolism is an important research avenue to investigate.

A point deserving future attention is the hormonal control of sulfur assimilation and the effect of sulfur status mediated by hormonal signalling. *Arabidopsis* transcriptome analysis of sulfur-deficiency stress suggests that auxin is involved in the activation of nitrilase at expenses of glucosinolate biosynthesis leading to changes in root morphology (Kutz et al. 2002, Hirai and Saito 2004). Cytokinin is reported as a negative regulator of sulfur acquisition (Maruyama-Nakashita et al. 2004). Based on the hypothesis of evolutionary conservation of cytokinin signalling pathway (Müller and Sheen 2007) the effect of S-depletion on the genomics of cytokinin transduction signalling was tested in grapevine cells. The expression of *VvCyt*, the grapevine ortholog to the histidine kinase cytokinin receptor CRE1/AHK4, is not affected by S-depletion while a tendency for a down-regulation of *VvHP*, a histidine phophotransmitter and *VvRRa*, an isoform of A-type RR transcription factor, was observed after 5-7 days in S-deficiency (Fernandes and Tavares, unpublished results).

The basic knowledge obtained in the last ten years with the model plant *Arabidopsis* is now applied to crop plants (Saito 2004). The grapevine genome sequencing (Jaillon et al. 2007, Velasco et al. 2007) and the high number of *Vitis* ESTs deposited at database (Adam-Blondon et al. 2007) makes it possible to investigate the coordination between sulfur primary metabolism and secondary metabolic pathways and, last but not least, to obtain further information on the fine-tuning of sulfur genome regulation at the level of promoter *cis* elements, transcription factors and small RNAs.

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3

GRAPE BERRY MINERAL COMPOSITION IN RELATION TO VINE WATER STATUS & LEAF AREA/FRUIT RATIO

F. Etchebarne[1,2], H. Ojeda[2] & A. Deloire[1,3]

[1]INRA, UMR 1083 Sciences for the Enology, 2 Place P. Viala, F-34060 Montpellier, FRANCE

[2]INRA, UE 0999 Pech Rouge, F-11430 Gruissan, FRANCE

[3]SupAgro, Plant Sciences Department, 2 Place P. Viala, F-34060, Montpellier, FRANCE

1. INTRODUCTION

The use of irrigation in Viticulture is considered as a standard practice, and it is performed mainly during drought periods as an effective means for regulating water availability to grapevines. Grape development and ripening have been extensively studied, both in terms on the factors involved in fruit growth and the evolution of primary and secondary metabolites (Ribéreau-Gayon 1975, Possner and Kliewer 1985, Coombe 1987, Iland and Coombe 1988, Gutiérrez-Granda and Morrison 1992, Ollat et al. 2002). The influence of water supply on plant development and physiology has been widely described in the literature (Smart et al. 1974, Hardie and Considine 1976, Becker and Zimmermann 1984, Van Zyl 1984, Matthews et al. 1987, McCarthy 1997, Ojeda et al. 2001, 2002).

The supply of mineral elements affects the plant's development and physiology, with a balanced mineral supply being paramount in the vineyard to avoid excessive vigour or mineral deficiency, both needed to sustain the plant's equilibrium. An understanding of the mineral nutrition of vines depends on information from numerous scientific disciplines. Physiologically, the definition must be broadened to include the selective *acquisition* of these materials from the environment and its internal *distribution* to places where it is needed (Clarkson and Hanson 1980). Grape berry growth is supported by imports of carbohydrates, water and mineral nutrients. The description of these imports provides some information about the compounds required to support berry development.

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It also increases understanding of the physiological mechanisms underlying these imports (Ollat et al. 2002).

Grape berry mineral composition is also important in that it is involved in wine chemical composition. From a technological point of view, potassium influences the pH of musts and wines and thereby their chemical and microbiological stability, in addition to the perception of wine flavour (Hale 1977, Morris et al. 1983, Donèche and Chardonnet 1992, Gutiérrez-Granda and Morrison 1992, Mpelasoka et al. 2003). Grape berry mineral composition also plays a role in fruit development. Calcium deficiency in fleshy fruit can cause physiological disorders such as bitter-pit in apples, blossom-end rot in tomatoes, tip-burn in strawberries, or watercore in melons (Shear 1975, Odet and Dumoulin 1993, Wills et al. 1998).

The calcium content of grape-berry skins is also linked to the fruit's resistance to pathogenic bio-aggressors (Chardonnet and Donèche 1995). Furthermore, deficiencies in phosphorus, zinc, manganese and molybdenum result in a reduction of fruit set and a deficiency in potassium results in unevenly ripened berries (Mullins et al. 1996). Potassium may be involved in the translocation of solutes into the berry through its roles in phloem loading and unloading (Lang 1983). According to Mullins et al. (1996), potassium is associated with apoplastic sugar transport. Sugars are the principal solutes accumulated in grapes during the second period of berry growth, and as compounds that are as mobile as they are abundant, they may be regarded as important constituents in osmotic control, notably in situations of low soluble sugar accumulation (Mpelasoka et al. 2003). Other mineral elements such as sodium, calcium, magnesium, copper, manganese and phosphate also play a role in osmotic balance. The latter, however, are minor contributors given their low concentrations in the grape, in addition to their low mobility (Mpelasoka et al. 2003).

Even though the scope of this review is somewhat more restricted than its title suggests, original results are presented with regards to the current knowledge about the flux of mineral nutrients and the modifications source-sink relationships in which they are acquired and accumulated by berry growth and ripening.

Broadly, the approach which we have adopted in the present review has been to ask the following questions about berry mineral nutrient accumulation: How are nutrients transported by the sieves within the vascular system of the plant? How effectively are nutrients accumulated on a 'whole berry' level? How are nutrients partitioned inside the berry? We shall be considering two important, but not frequently reviewed aspects of the subject: the influence of vine water status and ratio leaf area/fruit.

The results presented in this review come from an original study on the accumulation of potassium, calcium, magnesium and sodium in berries on vines

submitted to different water status treatments. This study was carried out during the 2006 season on young (4 year old) vines of Grenache Noir (clone 134) grafted onto Richter 110 grown on clay limestone soil (dry Mediterranean limestone marl) at the Pech Rouge experimental vineyard at INRA-Gruissan, France. The vineyard was planted with a north-south row orientation, 1 m (vine spacing) x 3m (row spacing), trained according to the lyre trellis system, pruned in single cordon of Royat and watered by a drip irrigation system. Two levels of water status, irrigated (I) and non-irrigated (NI) were investigated. For each level of water status, two levels of total leaf area were compared: uniform growth of primary shoots was obtained by topping at 10 primary leaves or 18 primary leaves and by removal of secondary shoots (side shoots) and tendrils from berry-set onwards. Second level bunches were removed to leave only one bunch per shoot. To implement the two water level treatments, irrigation commenced from bunch closure onwards (stage 32, Coombe 1995).

2. VINE WATER STATUS

Water availability to vines is an important factor in the terroirs of grape-growing regions (Seguin 1975, Van Leeuwen et al. 2004, Deloire et al. 2005). The vine obtains water from rainfall and the water table, therefore when it is in short supply, it is necessary either to irrigate or to accept the effects of water deficit. Depending on the intensity of the water deficit and the period during which it occurs, it may or may not be favourable for the harvest and the wine produced. The objective of this section is to provide some information on the difference that exists on berry-level with regard to the relationship between vine water status and leaf area. The plant's water status was measured with the pre-dawn leaf water potential (Ψ_{pd}) technique (Scholander et al. 1965), monitoring its evolution during the vegetative cycle for the two treatments, e.g. plants irrigated and non-irrigated, as a function of water reserves in the soil readily available to the roots. Figure 1 shows that eight days after anthesis (8 daa) the values of Ψ_{pd} were -0.1 to -0.2 MPa showing that water deficit was low to absent for the four treatments according to the threshold values established by Carbonneau (1998).

For the I treatment, irrigation commenced at bunch closure (32 daa) and a few days before véraison (60 daa) a progressive difference between I (irrigated plants) and NI (non-irrigated plants) could be observed. The I treatments maintained a water status close to -0.2 MPa until 60 daa, then dropping to -0.3 to -0.5 MPa as a result of a period of severe drought during August (corresponding to the stages of véraison-ripening). In parallel, the Ψ_{pd} values for the NI treatments dropped to approximately -0.9 MPa some days after véraison. At 70 daa

the parcel was supplied with 8 mm of water to improve the general water status of the vines. This watering in addition to the 43 mm of rain that fell on 78 daa re-hydrated the plants, particularly the NI treatments. Consequently the Ψ_{pd} values for I were close to -0.2 MPa and for NI to -0.3 MPa at the end of ripening (100 daa). Nonetheless there was no difference between the treatments with differing leaf area/fruit ratio during the vegetative cycle.

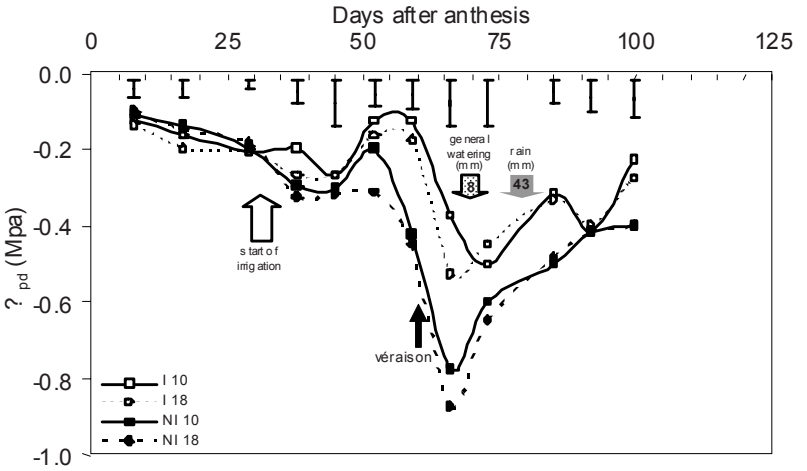

Fig. 1. Evolution of pre-dawn leaf water potential (Ψ_{pd}) of cv Grenache Noir from vines undergoing different irrigation and leaf area treatments during 2006 season. I and NI indicate the irrigated and non-irrigated treatments; 10 and 18 refer to leaf area/fruit ratio. Bars represent ± limits of significance ($\alpha = 0.05$).

3. SOURCE-SINK RELATIONSHIPS

There is strong competition between the different parts of the vine, and the bunches are weak sinks until ripening commences (Hale and Weaver 1962). Post-véraison, carbohydrates are imported into the berry at a higher rate and clusters become very competitive sinks. However, shoot growth during ripening may slow sugar accumulation in the berry. At any stage, berry growth and development are affected by modification of the carbohydrate supply to the berry. These modifications are brought about by adjusting the number of bunches in relation to the leaf area in order to achieve the required grape maturation. With regard to leaf removal, a large decrease in leaf area just after fruit set can induce a reduction in berry growth. In this situation, Kaps and Cahoon (1992) showed that berry weight at maturity is related to the ratio leaf area/fruit weight. Noteworthy in our dataset for fresh weight and berry water content (Fig. 2a,c), val-

ues compared between two levels of water status and two leaf area treatments showed significant differences for vine water status, especially during the latter part of the season, and any difference for the ratio leaf area/fruit. Berry dry weight was less influenced (Fig. 2b).

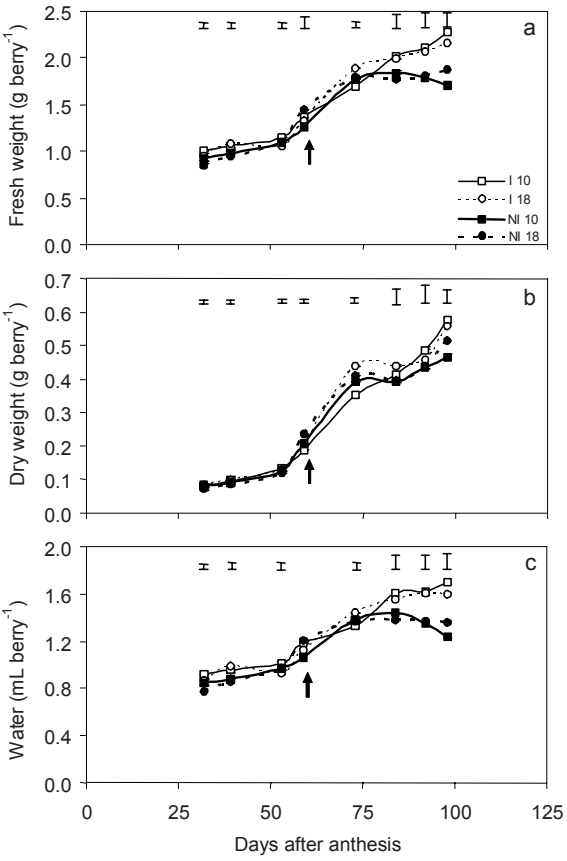

Fig. 2. Evolution of fresh (a) and dry (b) weight in g berry^{-1} and the water content (c) of the berry in mL berry^{-1}, during growth and ripening (cv Grenache noir) from vines undergoing different irrigation treatments and leaf area during 2006 season. Labels are described in Fig. 1. Arrows indicate véraison and bars represent ± limits of significance ($\alpha = 0.05$).

4. BERRY GROWTH AND DEVELOPMENT

Berry growth occurs in two principal phases. A first herbaceous growth phase followed by the ripening phase, the onset of which is called véraison. Be-

tween these two phases there is a relatively short lag phase during which the berry weight and volume remains constant. Véraison is characterised by berry softening and colour changes, and berries undergo major changes in chemical composition, rapid increase sugars accumulation and organic acid depletion (Coombe 1992). The conduction of sap within the fruit is *via* the phloem and xylem networks, in a double vascular system, central and peripheral. The fruit is connected to the bunch by a pedicel.

Water availability to the plant is an important factor in organ enlargement, but it also interacts closely with photosynthesis and berry carbohydrates supply (Ollat et al. 2002). Berry size is affected by water deficit from anthesis to maturity; however, final berry size is more influenced by water deficits between flowering and véraison (Becker and Zimmermann 1984, Poni et al. 1994, Ojeda et al. 2001). Ojeda et al. (2001) observed that water limitation during the first growth phase of the berry (before véraison) does not affect cell division but decreases cell volume. The results show that vine water status is a more important factor than leaf area with regards to determining final berry size (Fig. 2).

5. MINERAL NUTRIENTS

Grape berry development is concomitant with an influx of water, carbon and a number of mineral nutrients. There are 17 nutrients which are considered to be essential for the growth and development of plants, with some having a role in the structure of compounds while others are involved in enzyme activation, or as a charge carrier and osmoregulator (Marschner 1995). In this section we attempt to review the general properties of K^+, Ca^{++}, Mg^{++} and Na^+. These mineral nutrients are essential to plant life amongst other functions in the natural environment. Moreover, we also present original results concerning the pattern of accumulation and partitioning of K^+, Ca^{++}, Mg^{++} and Na^+ into the berry. These cations have osmotic and ion balance roles, as well as specific functions in enzyme conformation and catalysis (Clarkson and Hanson 1980).

The mineral nutrient status of the grape berry is of concern not only to the viticulturist, but also to the oenologist because there is a direct impact of berry nutrition on must and wine composition. Many factors may affect the net accumulation of mineral nutrients in the berry through their effects on root cation uptake, translocation from root to shoot, re-translocation of cations from shoot back to root, the mineral nutrient reserve, and the number of berries and berry growth rates in relation to vine vigour. These factors include the soil, plant functioning, vine microclimate and cultural practices. Inter-relationships amongst the effects of these factors are likely to complicate any simple explanation of the regulation of cation accumulation in grape berries. Our results docu-

mented the influence of two important factors in grape berry quality, e.g. vine water status and the ratio leaf area/fruit on the primary shoots.

5.1.Potassium

5.1.1. Functions of K^+ in plants

K^+ is the most abundant cellular cation. The classical reviews by Evans and Sorger (1966) and Suelter (1974) advanced the idea that high concentrations of K^+ are required for the active conformation of many enzymes participating in intermediary metabolism and biosynthesis. High concentrations are also needed to neutralize the soluble and macromolecular anions of a cytoplasm which has few organic cations. In this role, K^+ contributes much to the osmotic potential. A corollary of these requirements is that K^+ must be readily transported along with acidic metabolites, in order to balance their formation.

Transport appears to lie with a K^+-activated Mg^{++}- requiring ATPase of cell membranes (Hodges 1973, Poole, 1978). Frequently, K^+ appears to be in electrochemical equilibrium indicating passive flux (Higinbotham 1973), but this is not true for low external K^+ concentrations, and models based on a H^+/K^+ exchanging ATPase have been proposed (Hodges 1973, Lin and Hanson 1976, Cheeseman and Hanson 1979). Whatever the mechanism, transport of K^+ appears to be implicated in several physiological functions: phloem transport (Ashley and Goodson 1972, Hartt 1970, Mengel and Viro 1974), guard cell turgor (Raschke 1977), leaf movements (Satter et al. 1974, Schrempf et al. 1976), and cell growth (Cram 1974). Thus the nutritional need for K^+ centres on four physiological-biochemical roles: enzyme activation, membrane transport processes, anion neutralization, and osmotic potential (Clarkson and Hanson 1980).

5.1.2. Pattern of berry K^+ accumulation

Potassium is the principal cation accumulated by the berry during the entire growth period (pre- and post-véraison) (Conradie 1981, Possner and Kliewer 1985, Donèche and Chardonnet 1992, Schaller et al. 1992, Creasy et al. 1993, Boselli et al. 1995) with a sharp increase at the onset of ripening (Ollat and Gaudillère 1996, Rogiers et al. 2001, 2006a,b); also, the accumulation of K^+ in the berry seems to be linked to the accumulation of fruit dry matter and flesh weight in relation to plant water status (Fig. 3a). K^+ is the principal osmotically active cation in the berry's phloem and would appear to contribute to the flow of phloem sap (loading of soluble sugars), thus helping to establish an osmotic gradient between the leaves (source) and the berries (sink) (Véry and Sentenac 2003).

In contrast to these findings, a decrease in the concentration of K^+ per dry matter ($mg.g^{-1}$ DW) was found for both situations of vine water status during

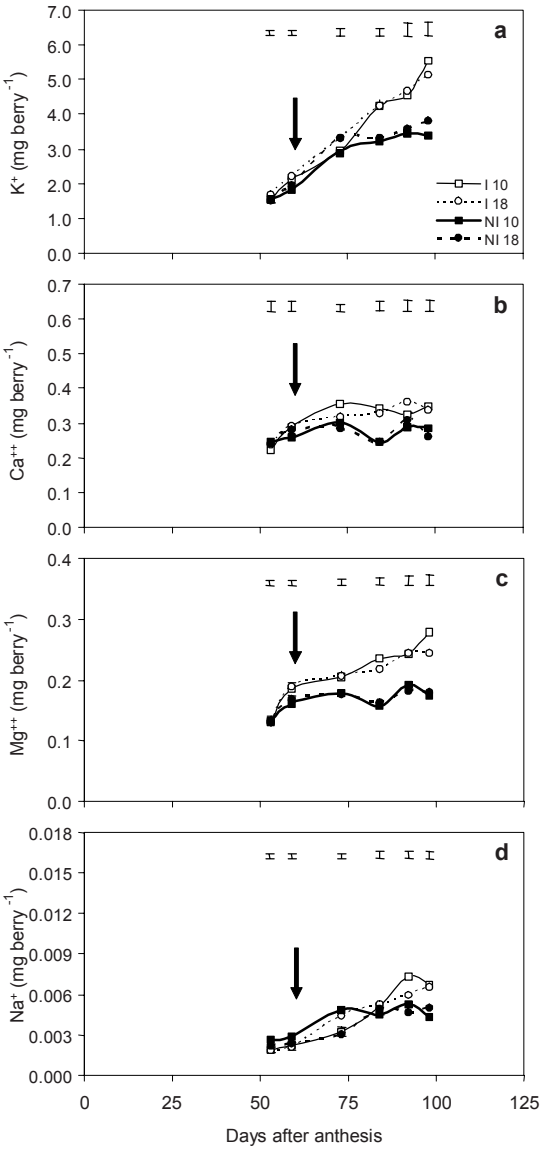

Fig. 3. Accumulation of potassium (a), calcium (b), magnesium (c) and sodium (d) in berry of Grenache noir during ripening. The most abundant cation per berry is K^+. The accumulation of Mg^{++} and Ca^{++} is significant until véraison; water deficit inhibits the accumulation of these cations post-véraison. K^+ and Na^+ continue to accumulate in the fruit after véraison in spite of water stress. Labels are described in Fig. 1.The arrows indicate véraison. Results are expressed in mg $berry^{-1}$. The bars represent ± limits of significance ($\alpha = 0.05$).

ripening (data not shown). Other authors mostly observed an augmentation of this parameter per unit fresh weight (Hale 1977), or found it to remain relatively con- stant (Boselli et al. 1995). The concentration of K^+ in the berry increases if the level of K^+ accumulated is greater than the growth rate of the berry (Mpelasoka et al. 2003).

5.1.3. Pattern of K^+ partitioning into berry

Information on the partitioning of K^+ within a berry is important for winemaking process. Our results suggest that the rate of berry K^+ accumulation during ripening is related to plant water status. This trend was also observed separately in the skin, flesh and seeds (Fig. 4a1, a2, a3). At maturity the skin contains 37% of berry K^+, thus confirming the observations made by Iland and Coombe (1988) for Syrah and by Donèche and Chardonnet (1992) for Cabernet Sauvignon. The flesh contained 60% and the seeds 3%, respectively of berry K^+ at maturity and these percentages are not modified by plant water status, nor by

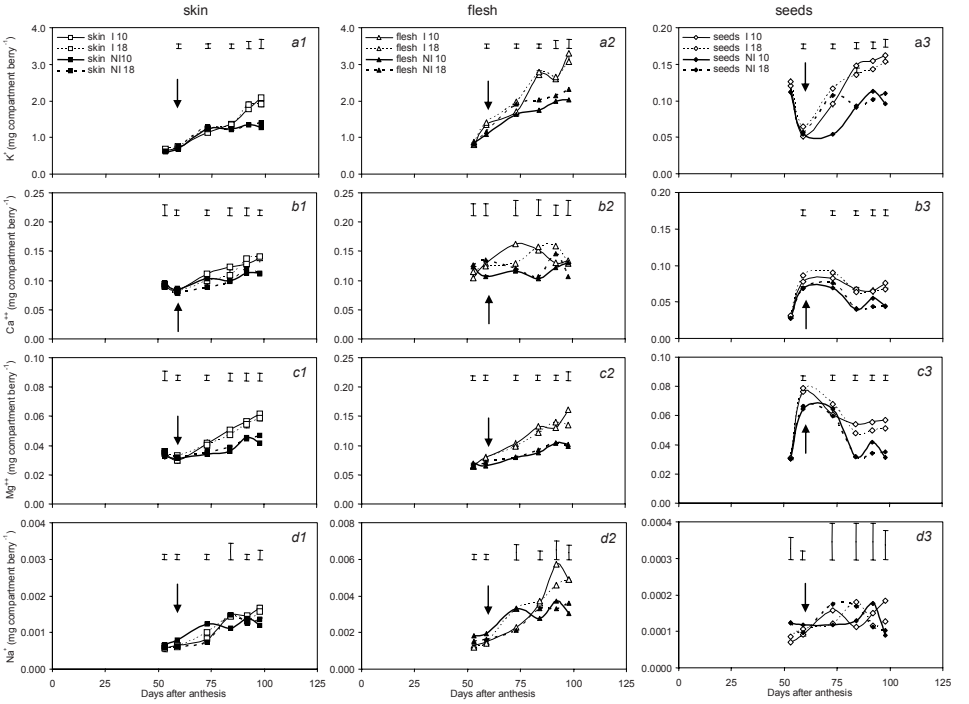

Fig. 4. Accumulation and distribution of (a) potassium, (b) calcium, (c) magnesium and (d) sodium in the different berry compartments, respectively skin, flesh, seeds of Grenache noir during ripening. Water deficit occurring from véraison onwards modifies the accumulation of mineral elements in the different berry compartments. Labels are described in Fig. 1. Arrows indicate véraison. Results are expressed in mg berry compartment^{-1} (skin, flesh and seeds). The bars represent ± limits of significance ($\alpha = 0.05$).

the ratio of leaf area/fruit in the conditions of this study.

According to Rogiers et al. (2006b) the predominant accumulation of K^+ in the flesh and in the skin confirms the role of this cation in cellular expansion. Within berries of cv Grenache noir, the seeds were the weakest sinks for K^+. Moreover, it has been shown that seeds continue to accumulate K^+ during fruit ripening. This result implies that seeds continue to be supplied by the central vascular system of the fruit during ripening. According to Welch (1986) in legumes and grains, most of the nutrients are delivered to the seeds by the phloem, because a discontinuity occurs between xylem vessels and the seeds. Furthermore, it was reported that at véraison the flow in the peripheral vascular system is interrupted, whereas the flow of the axial xylem continues, especially with regards to the seeds (Düring et al. 1987). Factors that affect mineral accumulation and partitioning in the fruit include climatic conditions, available soil K^+ as well as the relationship between root absorption and the distribution of this cation in the plant (sink-source competition).

The differences in K^+ accumulation among different compartments may be attributed to differences in cell structure and/or to the different K^+ roles in the tissues. Compared to flesh cells, skin cells are smaller and have thicker walls and a smaller cytoplasm (Harris et al. 1968, Considine and Knox 1979, Nii and Coombe 1983).

5.2. Calcium

5.2.1. Functions of Ca^{++} in plants

Compared to Mg^{++}, there are four striking attributes of Ca^{++}: (1) the cytoplasmic ionic activity is low in all eukaryotes investigated; (2) Ca^{++} has low physiological mobility, which is reflected in low rates of accumulation, cell-to-cell transport, and phloem transport; (3) there is a critical requirement for Ca^{++} outside the protoplast, on the exterior surface of the plasmalemma and in the cell wall; (4) Ca^{++} appears to have a limited role as an enzymatic cofactor (Clarkson and Hanson 1980). Contrary to this is the observation that Ca^{++} is accumulated by plants, especially in leaves where it can be irreversibly deposited in concentrations rivalling that of K^+. Physiologically, Ca^{++} serves to prevent cell membrane damage and leakiness (Poovaiah and Leopold 1976, Simon 1978), as well as to delay senescence and abscission (Poovaiah and Leopold 1973). It thus has a well-recognized but unidentified structural role in membrane integrity. Ca^{++} also strengthens cell wall structure, a result particularly noticeable in soft fruits (Simon 1978), and is the major cation integral to the protein-pectin “cement” of the middle lamella (Ginzburg 1961, El Hinnaway 1974). In addition, there are finite requirements for intracellular Ca^{++} which are physiologically manifested in cell division and enlargement (Burström 1968).

5.2.2. Pattern of berry Ca^{++} accumulation

Calcium is considered to have low phloem mobility (Welch 1986) and therefore most likely enters the berry through the xylem. The accumulation of Ca^{++} principally takes place during herbaceous growth of the fruit (before véraison). During ripening, the accumulation of this cation is low to absent and depends on plant water status. Several studies have shown that Ca^{++} is accumulated in the berry throughout its development (Schaller et al. 1992, Ollat and Gaudillère 1996, Rogiers et al. 2000, Cabanne and Donèche 2003). However, others indicate that the accumulation of Ca^{++} ceases after véraison (Hrazdina et al. 1984, Possner and Kliewer 1985, Creasy et al. 1993). It appears that in grapes, as in apple fruit (Saure 2005), Ca^{++} accumulation patterns are variable. This may be due to seasonal differences in vapour pressure gradients and thus transpiration (Marschner 1995). Since Ca^{++} is carried along the transpirational stream, any environmental factor which influences the flow rate of this stream would also influence the accumulation of Ca^{++} into the transpiring organ. Our results suggests that the Ca^{++} content per berry may have a steady upward trend during post-véraison enlargement under certain conditions, e.g. non-restrictive water supply to the plant (Fig. 3b). Climatic conditions as well as soil characteristics (including moisture levels and mineral content), may also play significant role in determining nutrient translocation to the berry.

On the other hand, Ca^{++} concentration expressed per unit of dry matter ($mg.g^{-1}$ DW) showed a quite different trend in comparison to plots of the total amounts per berry (data not shown). Between véraison and maturity, the concentration of Ca^{++} decreased significantly (around 35%). This decline occurred due to post-véraison accumulation rates not exceeding pre-véraison rates. In all cases, the differences between the irrigated and non-irrigated vines were not statistically significant (data not shown).

5.2.3. Pattern of Ca^{++} partitioning into berry

The evolution of the Ca^{++} content in the various compartments can vary from year to year or with the grapevine cultivar (Cabanne and Donèche 2001). It appears that the Ca^{++} content of the pericarp increases until véraison, then decreases during ripening (Cabanne and Donèche 2003). At véraison, rupture of the xylem vessels occur in the pericarp and this is probably responsible for the halt of Ca^{++} accumulation in this compartment (Düring et al. 1987). On the other hand, our results show that Ca^{++} accumulation was not observed in the flesh nor in the seeds, contrary to what was observed for the skin during the post-véraison phase, in both water status treatments (Fig. 4b1,b2,b3). According to Donèche and Chardonnet (1992), there is a dramatic reduction in the concentration of Ca^{++} in cells of the flesh, whereas it is abundant in the skin area.

In Grenache noir berries, the seeds were the smallest sink for the Ca^{++}. It was accumulated into the seeds of the berry prior to véraison, not during ripening. Moreover, Cabanne and Donèche (2003) and Rogiers et al. (2006b) reported that the seed's Ca^{++} content increases during ripening in several other grape varieties (Cabernet Sauvignon, Semillon, Merlot, Sauvignon and Syrah). In the pre-véraison phase, xylem tracer studies indicated that xylem flow occurred along not only the bundles supplying the skin and flesh of the berry, but also the central bundles leading to the seeds (Rogiers et al. 2001), again suggesting continuous xylem flow to the seeds. During the post-véraison phase, however, the xylem tracers were limited to the brush region of the central bundles (Düring et al. 1987, Findlay et al. 1987, Creasy et al. 1993, Rogiers et al. 2001).

The metabolism of the grape berry is now known to be a complex process where considerable compartmentalisation occurs between different types of tissues (Famiani et al. 2000). The Ca^{++} content and its evolution must be considered in the same way (Cabanne and Donèche 2003). Indeed, while Ca^{++} accumulation ceases at the onset of ripening in the flesh and seeds, it persists in the skin. At the same time, Ca^{++} is probably translocated to skin cells during ripening by apoplastic and/or symplastic movement or by partial functioning of the xylem after véraison.

5.3. Magnesium

5.3.1. Functions of Mg^{++} in plants

Concentrations of Mg^{++} in plant tissue are variable but always rather high. Since 70% or more is freely diffusible (Mengel and Kirkby 1978), Mg^{++} must have the highest chemical activity of any divalent cation in the cytoplasm. Depending on the relative abundance of K^+, it will also contribute to neutralization of sugar phosphate, sugar nucleotides, and organic and amino acids. The first important property of Mg^{++} is the relatively high solubility of its salts. Mg^{++} has a structural role in chlorophyll, is required for ribosomal integrity, and undoubtedly contributes to the structural stability of nucleic acids and membranes. There is evidence that fluxes of Mg^{++} can serve to regulate enzyme activity, as occurs with the light-activated increase in CO_2 fixation by chloroplasts (Walker 1974). The mobility of Mg^{++} is a distinguishable property (Clarkson and Hanson 1980).

5.3.2. Pattern of berry Mg^{++} accumulation

Mg^{++} is generally considered to be a phloem-mobile element (Welch, 1986). The accumulation of Mg^{++} in the berry occurs in two successive phases, before and after véraison (Donèche and Chardonnet 1992). According to several

authors, the high rate of accumulation of this element post-véraison is thus consistent with flow through the phloem into the grape berry after véraison (Lang and Thorpe 1989, Greenspan et al. 1994). In Grenache noir berries we have observed that Mg^{++} entered in the berry mostly before véraison, and thereafter the accumulation of this cation remained more or less constant after véraison in NI treatments. It however appeared to increase in the I treatments (Fig. 3c).

By contrast, the concentration of Mg^{++} expressed per unit of dry matter (mg g^{-1} DW) declines markedly to below that occurring prior to veraison (data not shown). In all likelihood, the decrease in concentration of this element could be attributed directly to the gain in berry size and solutes accumulation in post-véraison phase.

5.3.3. Pattern of Mg^{++} partitioning into berry

The strongest berry compartment sinks for the phloem-mobile nutrients varied for each element. In Grenache noir berries, Mg^{++} accumulation at véraison was identical in the flesh and the seeds. During ripening, Mg^{++} accumulation in the flesh was much higher than in the skin and seeds (Fig. 4c*1*, c*2*, c*3*). The flesh is always richer in Mg^{++} than the skin, which concurs with the results of Donèche and Chardonnet (1992) and Rogiers et al. (2006b). On the other hand, seeds essentially stopped accumulating Mg^{++} at véraison, leading to drastically reduced levels during ripening. This suggests that, in Grenache noir berries, the seeds became isolated in some way after véraison with regard to Mg^{++} accumulation. This isolation, however, appeared to be limited to the seeds, since the flesh and skin continued to accumulate most of the Mg^{++} after this time. The quantity of this element increased during ripening but at different rates depending on the response of berry to vine water deficit rather than to the ratio of leaf area/fruit.

5.4. Sodium

5.4.1. Functions of Na^+ in plants

In the sense of Nicholas's definition (1961), a case can be made for considering Na^+ a *functional* nutrient element in all terrestrial plants. To some degree Na^+ occurs in all soils and in all plants grown in these soils, and it participates in metabolism not only through mechanisms which actively secrete it from roots (Jeschke 1973, Ratner and Jacoby 1976). Whenever soils are saline and soil water availability is low, a diverse group of plant families have evolved, the halophytic species, which accumulate sufficient Na^+ salts in vacuoles to maintain turgor and growth (Rains 1972, Cram 1976, Jennings 1976, Hellebust 1976, Flowers et al. 1977). However, Na^+ is not an *essential* element except in certain salt-tolerant plants with C_4 metabolism (Brownell and Cross-

land 1972, Shomer-Ilan and Waisel 1973), and possibly it has some role in the related crassulacean acid metabolism (Brownell and Crossland 1974).

5.4.2. Pattern of berry Na^+ accumulation

Sodium is not an essential mineral element, but it could play an important role in terms of grape nutrition (Winkler et al. 1974). According to Rogiers et al. (2006b), Na^+ did not accumulate over time and amounts were quite variable. We have observed that the accumulation of Na^+ in the berry is limited and follows a dynamism similar to that of K^+ accumulation in the whole berry. Na^+ is accumulated throughout the entire period of berry growth (Fig. 3d), and is of the lowest importance in the mineral composition of the berry.

Because the rate of gain in berry size varied throughout development, trends in element concentrations per unit of dry matter ($mg.g^{-1}$ DW) were often very different from the trends in element accumulation per berry (data not shown). The levels of Na^+ varied slightly compared to K^+, Ca^{++} and Mg^{++}. In all cases, the differences between the I and NI treatments were not statistically significant.

5.4.3. Pattern of Na^+ partitioning into berry

During berry growth, Na^+ is differentially distributed in the various berry compartments, i.e. skin, flesh and seeds. Our study showed that vine water status and not the ratio of leaf area/fruit influenced the accumulation of Na^+ in the fruit by reducing or inhibiting the accumulation, depending on the compartment. At véraison, greater accumulation of Na^+ was observed in the flesh in comparison to the skin and seeds (Fig. 4d1, d2, d3). The accumulation of Na^+ in the berry compartments was limited and followed a dynamism similar to that of K^+ accumulation in the skin and flesh. The accumulation of Na^+ in the skin was identical during the lag phase, from the end of the first growth phase (53 daa) to véraison (60 daa), and then the quantity of this element increased during ripening. In seeds the accumulation of Na^+ was variable but remained relatively constant in the post-véraison phase.

6. GRAPE BERRY TRANSLOCATION OF MINERAL NUTRIENTS

The grape berry is supplied through the berry stem or pedicel by a vascular system composed of xylem and phloem elements. The xylem is responsible for transporting water, minerals, growth regulators, and nutrients from the root system to the vine. The berry is also supplied by the phloem, which is the vas-

culature involved in photosynthate (sucrose) transport from the leaves to other plant parts. It has reduced function with regard to berry nutrient transport early in berry development, but becomes the primary route of entry after véraison.

The translocation of solutes in the grape berry is not well understood (Ollat and Gaudillère 1996). Mineral nutrients are transported by the xylem and phloem vascular systems. Potassium, magnesium and sodium are phloem-mobile, whereas calcium is phloem-immobile in the vine, although xylem and phloem transport of elements is largely dependent on the type of plant (Welch 1986, Welch and Rengel 1999).

Nutrients that have low phloem mobility may also be transported by the xylem. Based on the hypothesis that K^+ is transported by xylem and phloem (Mengel 1976) and that Ca^{++} is transported only by the xylem (Hanger 1979), changes in the K/Ca ratio in berries have been used as an indicator of changes in the relative berry K^+ influx *via* xylem and phloem (Hrazdina et al. 1984, Ollat and Gaudillère, 1996, Rogiers et al. 2000, 2001).

Studies carried out on the water and mineral supply to the grape berry showed that before véraison, the fruit is supplied by the peripheral and central vascular system extending from the pedicel (Lang and Thorpe 1989, Greenspan et al. 1994, 1996). Several studies have shown that from véraison onwards there is an interruption in the function of the berry xylem (Findlay et al. 1987, Düring et al., 1987, Creasy et al. 1993, Rogiers et al. 2001) and therefore during ripening the fruit is mainly supplied *via* phloem transport (Ollat et al. 2002). The vascular system and the parameters involved in its transporting of mineral elements is therefore an interesting indicator in conductance studies pre- and post-véraison (Rogiers et al. 2006a, b). This raises the question regarding the influence of water supply on plant mineral supply. Root absorption depends on soil water levels, soil type, the availability of mineral elements in the soil, and the operation of the root system. Moreover, once a mineral element is absorbed, it is distributed according to the plant's needs (sink-source relationships). Several studies have reported a relationship between cultural practices, i.e. tilling and planting of cover-crops, and grape composition (Schaller 1999, Klein et al. 2000, Mpelasoka et al. 2003). On the other hand, there are few reports on the effects of water supply on fruit mineral supply (Dundon and Smart 1984, Esteban et al. 1999).

This study was carried out under controlled conditions in terms of total leaf area, primary shoot length and grape load (1 bunch per primary shoot). It was demonstrated that vine water status is more important compared to the ratio of leaf area/fruit regarding the accumulation of mineral nutrients into berry. The question of availability of these cations in the soil and the relationship between their uptake by the roots and distribution in the different plant organs (sink-source competition) warrants further studies. At any case, these results now

need to confirm a continuing accumulation of Ca^{++} in whole berry during ripening under favourable water conditions. Such steady accumulation by berry suggests a continuous influx of Ca^{+} from the parent grapevine into the enlarging berry, suggesting that partial functioning of the xylem post-véraison is possible. In both, the irrigated and non-irrigated vines, the quantity of Ca^{++} in the skin increased during ripening. This could suggest Ca^{++} transport from other compartments especially from the flesh to the skin.

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4

WATER TRANSPORT & AQUAPORINS IN GRAPEVINE

S.D. Tyerman[1], R.K. Vandeleur[1], M.C. Shelden[1,3], J. Tilbrook[1], G. Mayo[2], M. Gilliham[1] & B.N. Kaiser[1]

[1]School of Agriculture Food and Wine, University of Adelaide, Waite Campus, PMB 1 Glen Osmond, South Australia 5064, AUSTRALIA

[2]Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, University of Adelaide, Waite Campus, Urrbrae, SA 5064, AUSTRALIA

[3]Present Address: Australian Centre for Plant Functional Genomics, School of Botany, University of Melbourne, Parkville, Victoria, Australia 3010, AUSTRALIA

1. INTRODUCTION

Water use and yield in plants are positively linked, and grapevines are no exception. Increased demand for water resources has focused interest on vine physiological factors that may determine how water use affects yield and quality. Yield (*Y*) depends on transpiration efficiency (*W*), total amount of water incident on vines (*I*), transpiration rate (*T*), and harvest index (*HI*, proportion of total dry matter removed as harvested product): $Y = I \times T/I \times W \times HI$ (Gibberd et al. 2001). Crop water use efficiency ($WUE = Y/I$) can be improved by an increase in one or other of three factors; *T/I*, *W* or *HI*. Yield may not be the primary goal for the production of quality wine grapes, but in circumstances where grape quality seems to be linked to yield and thus the relationship between quality and water use is likely to have the same terms as above but with additional non-linear components. We introduce the topic of this chapter through this discussion of *WUE*, because each of the parameters apart from *I* that determine *WUE* are dependent on the regulation of water flow across cellular membranes. Embedded in these membranes exist protein-channels permeable to water called aquaporins. As such, aquaporins control the rate of water transport through grapevines and therefore warrant special attention in the molecular biology and biotechnology of grapevines.

Since the discovery of aquaporins in plants in 1993 (Maurel et al. 1993), research on aquaporins has expanded rapidly and we now know that the aq-

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uaporins belong to a general class of proteins called Major Intrinsic Proteins (MIPs). MIPs constitute a large gene family in plants and a variety of functions have been proposed in different organs. Since the review by Delrot et al. (2001) in the first edition of this book, there have been some exciting discoveries on aquaporins, and the area has been widely reviewed (Tyerman et al. 2002, Vandeleur et al. 2005, Kaldenhoff 2006, Bramley et al. 2007a, Katsuhara et al. 2008, Maurel et al. 2008). We have many clues about the roles of aquaporins in grapevines based on general features discovered in other plants. There have also been several specific studies on grapevine aquaporins, that we review here, in context with the relevant physiological processes. In this chapter we have therefore concentrated more on the roles of aquaporins rather than general aspects of vine water relations. However, we examine similar topics to Delrot et al. (2001), which should be used for reference to older, but still relevant literature on grapevine water relations.

The three factors that determine *WUE* conveniently separate three important physiological processes in water relations of vines that we use to discuss the roles of aquaporins:

1) *Transpiration relative to incident water (T/I)* is linked to root and shoot capacity to transport water and root distribution in the soil, as well as vineyard management practises to minimize leaching (within the constraints of water quality) and to minimize soil evaporation. The water-transport capacity of roots and shoots is linked to aquaporin activity either directly, for example water transport across the root (Lovisolo et al. 2008, Vandeleur et al. 2008), or indirectly, for example likely aquaporin involvement in recovery from xylem embolism (Lovisolo and Schubert 2006).

2) *Transpiration efficiency (W)*, the ratio of carbon fixed to water transpired depends on the regulation of stomata to maximize CO_2 uptake and minimize water loss. Internal conductance to CO_2 is also important, accounting for a substantial proportion of the total conductance to the sites of fixation (Flexas et al. 2008, Warren 2008) and aquaporins have been implicated in the transport of CO_2 across membranes to the sites of fixation (Uehlein et al. 2003, Flexas et al. 2006, Miyazawa et al. 2008). Leaf hydraulic conductance may also be linked to *W* through regulation of water transport by aquaporins and effects on mesophyll cell water status (Zwieniecki et al. 2007).

3) *Harvest index (HI)* is determined by the translocation of sugars to berries *via* the phloem and the sink strength of the berry clusters (Hunter and Ruffner 2001). Like *T/I* this will depend on vine management practices aimed at achieving vine balance (Howell 2001). However, the fundamental process of mass flow of water and sugar down a pressure gradient through phloem sieve cells is driven by water flow across membranes in response to osmotic gradients. These gradients need to be sustained so that the sugar concentration in the

berry, equivalent to -5 MPa of osmotic potential, can be developed (Tilbrook and Tyerman 2008). How water balance is maintained within the berry is highly relevant during the different stages of ripening, and aquaporins have been implicated in various stages of berry ripening (Picaud et al. 2003, Tyerman et al. 2004, Fouquet et al. 2008).

2. CAPACITY OF ROOTS AND SHOOTS TO TRANSPORT WATER

According to the cohesion tension theory for ascent of sap in plants the liquid water column from roots to leaf apoplast is a continuous unbroken chain of water molecules (Steudle 2001). The flux of water ($m^3\ s^{-1}$) through any particular component of the continuum within the plant is equal to the product of the conductance (L, units $m^3\ s^{-1}\ MPa^{-1}$) and the gradient in water potential ($\Delta\Psi$, units MPa). However, water potential gradients consisting of osmotic components can only be realised as a flow if the gradients occur across semi-permeable membranes (such as across leaf or root cells).

2.1. Conductance and conductivities

Conductance is dependent on the length of the flow-path and to strictly obtain *conductivity*, conductance is multiplied by the path length (Sperry et al. 2002). Conductance or conductivity may be normalized to various dimensions of the particular organ or system relevant to the flow-path, thus conductance of the root system may be normalized to root surface area, root length or root dry weight (Tyree et al. 2003). When water transport across a surface is considered (a cell or a root) conductance is often normalized to surface area to give what is also termed hydraulic conductivity (L_p, $m\ s^{-1}\ MPa^{-1}$). Although it can provide a useful measure, it has been argued that standardizing the measurements of hydraulic conductivity by total root surface area may not be appropriate as water uptake is confined to local regions of the root (Zwieniecki et al. 2002). Conductivity of a stem segment or petiole (units $m^4\ s^{-1}\ MPa^{-1}$) can be normalized to xylem cross sectional area of a stem segment. Leaf hydraulic conductance is generally measured as the flux ($mmol\ s^{-1}$) divided by $\Delta\Psi$ and leaf surface area to give units of $mmol\ m^{-2}\ s^{-1}\ MPa^{-1}$ (Sack and Holbrook 2006).

Of the total liquid pathway in the plant, root conductance is often the lowest (Tsuda and Tyree 2000). In grapevines, the petiole conductance may be low and is recognized sometimes as a large (0.4 MPa) difference between leaf and stem water potential at high transpiration rates (Williams and Trout 2005). Grapevine leaves have leaf hydraulic conductance of about 8 $mmol\ m^{-2}\ s^{-1}$

MPa^{-1}, which decreases with decreasing water potential (Schultz 2003) and is just below the average of a range of species and is similar to other temperate woody angiosperms (Sack and Holbrook 2006). It has emerged that conductances of the liquid pathway are not constant even in the short term (Tsuda and Tyree 2000, Cochard et al. 2007, Vandeleur et al. 2008). Also these changes in conductance, particularly in the root, can have large impacts on water potential gradients in the plant (Tsuda and Tyree 2000). Conductance regulation (stomatal, root and leaf internal) with a decreasing soil water availability conceivably should allow a plant to extract the maximum amount of water from the soil without breaching hydraulic limits causing cavitation and embolism (Sperry et al. 2002).

2.2. Soil-to-root conductance

Soil texture, moisture and root density influence soil-root conductance (Gardner 1960). The majority of grapevine roots occur in the top meter of soil, with fine lateral roots concentrated between 100-600 mm from the surface (Richards 1983). Soil physical properties, in particular soil moisture which influences aeration and mechanical resistance, can impact on the distribution of roots (Richards 1983). Grapevine root density is low (0.35-0.36 mm mL^{-1}) compared to other plant species (Mohr 1996, Schreiner 2005). However, greater colonization of grapevine roots by arbuscular mycorrhizal fungi compared to other fruit species has been observed in some circumstances (Schreiner 2005), which would improve the ability of the roots to explore the soil for moisture and nutrients and improves the drought tolerance of grapevines (Nikolaou et al. 2003). The shoot-to-root ratio of grapevines, as for other species, is influenced by the availability of resources, such as light or nitrogen supply (Grechi et al. 2007). Grapevines are able to adjust their shoot-to-root ratio to address an imbalance due to a limiting resource, for example if nitrogen supply is limited there is a greater allocation of biomass to the roots. In studies on other species, altered expression of aquaporins in roots can change the shoot-to-root ratio (Kaldenhoff et al. 1998), presumably to compensate for altered root hydraulic conductivity.

Root growth in grapevines varies by genotype x environment (Bauerle et al. 2008) and is dependent on carbohydrate supply (Comas et al. 2005). Fine lateral roots, which may be important in water absorption, show rapid decay in metabolic capacity and nitrate uptake with age (Volder et al. 2005). Metabolic capacity and nitrate transport is closely associated with root hydraulic conductivity and probable activity of aquaporins (Gloser et al. 2007, Gorska et al. 2008).

2.3. Water transport across the root

Water moves radially into roots through the epidermis, exodermis, cortex, endodermis, the pericycle, stele parenchyma and finally into the xylem vessels. The radial conductance of roots is about two orders of magnitude lower than the axial conductance that is largely determined by the dimensions, and the number of xylem vessels (Frensch and Steudle 1989, Bramley 2006, Bramley et al. 2007b). In maize roots the xylem was found to have less resistance to water movement compared to the radial pathway (Frensch and Steudle 1989). Accordingly, in discussions of root water transport it is reasonable to assume that the radial flow-path is rate limiting.

The composite transport model has been used to describe the flow of water through roots and to help explain why hydrostatic gradients may result in higher root hydraulic conductivity (L_p) than for osmotic gradients (Steudle 2000). However, higher root L_p for hydrostatic than for osmotic gradients is not uniformly observed (Bramley et al. 2007b). The composite transport model comprises apoplastic, symplastic and transcellular flow-paths operating in parallel. The apoplastic flow-path is outside of the cells' plasma membrane, the symplastic flow-path is through the cytoplasm of cells connected by plasmodesmata, and the transcellular flow-path is across cell membranes (Fig. 1). The combination of symplastic and transcellular flow-paths is known as the cell-to-cell pathway. Aquaporins would be involved in regulating the movement of water in the transcellular pathway (Tyerman et al. 1999).

The movement of water through the apoplast is driven only by hydrostatic gradients, while across a membrane-delimited (transcellular) pathway both hydrostatic and osmotic gradients are involved. When plants transpire the hydrostatic gradient dominates due to the tensions developed in the xylem. Water moves *via* both the apoplastic and the cell-to-cell pathway driven by hydrostatic gradients, the proportion depending on the relative hydraulic conductances of the two pathways. When transpiration rate is slow, during the night or under water-limiting conditions, osmotic flow may dominate, because without large hydrostatic-driven water flows ions in the stele are not diluted, creating an osmotic gradient. It is not correct to state that flow under normal transpiration is predominantly in the apoplast, because this depends on apoplastic barriers in the root and hydraulic conductance of the parallel cell-to-cell pathway. In grapevines the cell-to-cell pathway and aquaporins can account for well over 40% of the flow across roots under transpiring conditions and is cultivar dependent (Lovisolo et al. 2008).

The flow-path taken by water is influenced by root anatomy. The apoplastic pathway can be inhibited by the presence of Casparian bands, which are deposits of suberin or lignin in the cell wall (Zeier and Schreiber 1997).

Casparian bands occur in radial and transverse walls of the endodermis and exodermis (Steudle and Peterson 1998). Suberin lamellae may also occur on the tangential walls to further inhibit apoplastic flow. Grapevines show strong suberization of both the exodermis and endodermis, with specialized passage cells (not suberised) evident opposite xylem poles and in the exodermis (Vandeleur et al. 2008, Fig. 1). Suberin lamellae can also restrict movement of water along the transcellular pathway. The formation of these barriers to water movement is often associated with the imposition of stress such as water deficits and aging of the plant (Steudle and Meshcheryakov 1996, Vandeleur et al. 2008). Suberized layers may assist in reducing water loss to soil during water deficits.

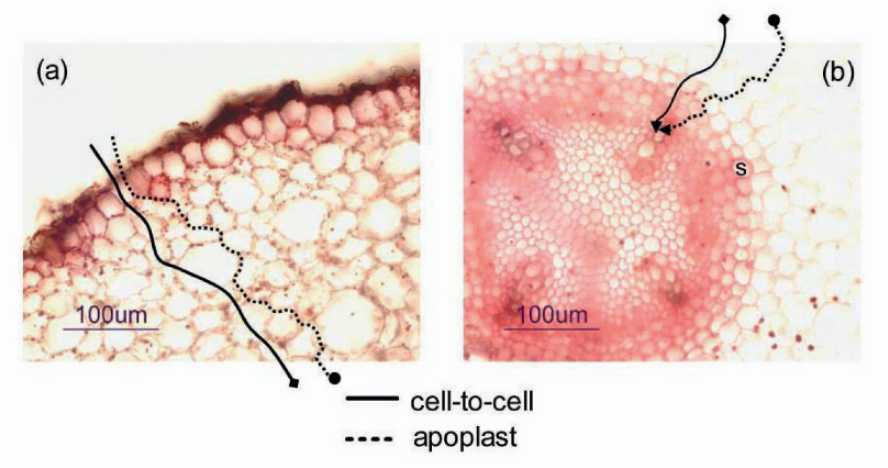

Fig. 1. The apoplastic and cell-to-cell pathways of radial water movement to the xylem in a root. The apoplastic pathway is around the protoplasts. The cell-to-cell path incorporates the symplastic path *via* plasmodesmata and the transcellular path consisting of two plasma membranes crossed per cell layer. In transverse sections of a Grenache root 25 mm from the root tip, stained with Sudan Red 7B for 2 h, suberin lamellae are shown. (a) The outer cortex with exodermis and passage cells (where flow-paths are shown entering). (b) Stele and endodermis with some cells in the endodermis showing suberin lamellae (s).

A large number of cortical cell layers have been observed in grapevine roots (Mapfumo et al. 1994). The large diameter of the cortex would pose a considerable resistance to water flow, compared to smaller diameters observed in other plants, such as soybeans (Rieger and Litvin 1999). Hydraulic conductivity declines with root age which is likely due to suberization and loss of the cortex reducing surface area available for water uptake (Wells and Eissenstat 2002). Root browning occurs in grape roots which is associated with cortical pigmentation followed by cortical cell death, while the stele is still functioning (Comas et al. 2000). It is unclear to what extent hydraulic conductivity is re-

duced in older, woody roots of grapevine.

Grapevine rootstocks and cultivars are likely to differ in root hydraulic conductance but this is complicated by a high variability related to transpiration rates (see below). The hydraulic conductance of two *Vitis vinifera* cultivars, Chardonnay and Grenache, and two rootstocks 101-14 (*V. riparia* × *V. rupestris*) and 1103 Paulsen (*V. berlandieri* × *V. rupestris*), were examined (Vandeleur 2007). The varieties varied significantly in plant size and root hydraulic conductivity normalized to leaf area (L_l) (P<0.05; Table 1). Interestingly, there was no significant difference in root hydraulic conductance normalized to root dry weight (L_o) between the varieties, but there was a slight difference (P<0.1) when hydraulic conductance was normalized to root surface area (L_p). The general trend was the same as when normalized to leaf area. Chardonnay had the highest conductance followed by 1103 Paulsen with Grenache and 101-14 having similar lower values. However, when based on root dry weight the four varieties had similar L_o values, mostly due to the large difference in root surface area to dry weight ratios. Grenache and 101-14 had significantly larger root surface area to dry weight ratios than Chardonnay and 1103 Paulsen. The values of L_p in grapevines, and woody perennials in general, are much lower than for herbaceous species, presumably due to the greater degree of suberization of endodermis and exodermis in woody species (Steudle and Heydt 1997).

Table 1. Varietal differences in hydraulic conductance normalized to root mass, root surface area and leaf area (L_o, L_p, and L_l) of potted vines grown in a glasshouse.

Variety	$L_o \times 10^{-6}$ ($kg.s^{-1}.MPa^{-1}.g^{-1}$)	$L_p \times 10^{-8}$ ($m.s^{-1}.MPa^{-1}$)	$L_l \times 10^{-5}$ ($kg.s^{-1}.MPa^{-1}.m^{-2}$)
Chardonnay	0.51 ± 0.07^{a}	1.81 ± 0.24^{a}	2.08 ± 0.32^{a}
Grenache	0.45 ± 0.06^{a}	0.99 ± 0.07^{b}	0.63 ± 0.11^{b}
101-14	0.46 ± 0.15^{a}	1.10 ± 0.34^{b}	0.64 ± 0.20^{b}
1103 Paulsen	0.45 ± 0.08^{a}	1.32 ± 0.14^{ab}	1.23 ± 0.25^{b}

We have recently investigated the physiological, molecular and anatomical characteristics of root water transport of two cultivars representing isohydric (Grenache) and anisohydric (Chardonnay) behaviours (Vandeleur et al. 2008). Both cultivars showed diurnal changes in root L_o of about two–fold from predawn to midday (Fig. 2A). There was a positive correlation between L_o and transpiration (Fig. 2B) that was also evident for rootstocks. There was a similar reduced predawn L_o under water stress that was attributed mainly to development of apoplastic barriers. However, Grenache showed a much larger reduction in L_o at midday (Fig. 2A). Hydraulic conductivity of root cortex cells doubled in Chardonnay but remained unchanged in Grenache. These changes in root hydraulic behaviour correlated with changes in expression of *VvPIP1;1*,

which interacted with *VvPIP2;2* to induce 3-fold higher water permeability when expressed in *Xenopus* oocytes. These two aquaporins are co-localized in the root for both cultivars and seemed to account for the contrasting behaviour of the roots that reflected the isohydric and anisohydric behaviour of the shoots (Vandeleur et al. 2008).

Hydraulic redistribution has been observed in grapevines with water absorbed in one section of the root redistributed throughout stems and roots *via* flow reversal (Smart et al. 2005). This water may be used to rehydrate tissues during water stress and for refilling of xylem vessels during the night when transpirational demand is low. Redistribution under partial root-zone drying conditions seems to involve changes in conductance of the wet and dry side of the root system (Vandeleur 2007).

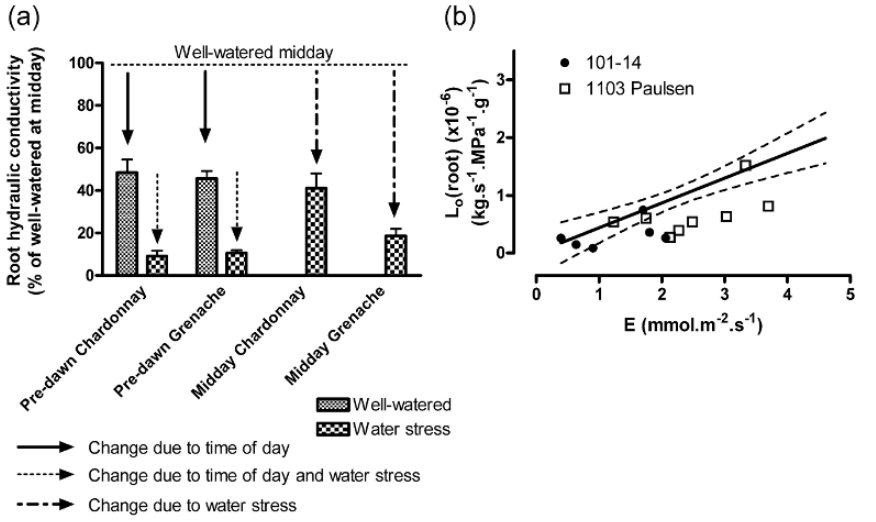

Fig. 2. Root system hydraulic conductance (normalized to dry weight) is highly variable and changes diurnally. (a) Diurnal variation in hydraulic conductance given as a percentage of the maximum measured at midday under well-watered conditions for Grenache and Chardonnay. Grenache and Chardonnay show similar reductions at predawn under well-watered and water stress conditions. Grenache shows a larger reduction in conductivity under water stress at midday. (b) For both Grenache and Chardonnay there is a significant correlation between root conductance mormalized to root dry weight and transpiration measured at the leaf level (solid line ± 95% confidence limits is combined regression from Vandeleur et al. (2008) without data points shown). The rootstocks 101-14 and 1103 Paulsen lie along the same relationship.

2.4. Xylem transport and embolism

Axial transport of water in the plant is facilitated by tracheids and vessel members of the xylem. Both components lack a protoplast when fully mature

and generally lack the end walls between adjacent cells to reduce the resistance to water flow. The diameter of the xylem vessels affects the conductivity according to Poiseuille's law (Tyree and Ewers 1991). The large vessel diameter of grapevines results in high xylem conductivity (Scholander et al. 1955, Esau 1965). However, under water stress, water flow can be interrupted by the presence of embolisms within the xylem (Tyree and Sperry 1989), which is normally measured as a percentage loss of conductivity (PLC).

There are large differences between species in vulnerability to embolism and differences have been observed between cultivars of grapevine (Schultz 2003, Alsina et al. 2007). Out of eight grapevine cultivars, Chardonnay and Sauvignon Blanc had the least vulnerable xylem while Parellada, Tempranillo and Grenache were the most vulnerable. Grenache was more vulnerable to embolism compared to Shiraz which relates to its larger conducting vessels, and this was correlated to more isohydric behaviour in Grenache compared to Shiraz (Schultz 2003). Refilling of embolized vessels occurs in grapevines over diurnal time courses (Holbrook et al. 2001). Imposition of water stress to leaf water potentials of approximately -1.4 MPa caused substantial PLC (about 80%) in petioles and root segments, but lower values in the apical shoot portions (50%) (Lovisolo et al. 2008).

Refilling requires functional plant metabolism or aquaporin activity in roots or shoots, as judged by mercury inhibition of recovery of shoot specific conductivity after drought (Lovisolo and Schubert 2006). Rehydration after drought allows full recovery of leaf water potential, but slower recovery of xylem embolisms (Lovisolo et al. 2008). Transpiration recovery is delayed after rehydration and is correlated to increased shoot abscisic acid (ABA) concentration. This led Lovisolo et al. (2008) to suggest that reduced transpiration, in response to ABA delivered from the roots, is required for embolism repair. Aquaporins have been implicated in refilling of embolized vessels (Sakr et al. 2003, Lovisolo and Schubert 2006) and it is clear that both PIP1 and PIP2 aquaporins are very closely associated with vessel elements in grapevine roots (Vandeleur et al. 2008).

Tyloses can seal damaged or embolized vessels and this occurs below pruning cuts. The stimulation for tylose formation is not embolism but wound induced production of ethylene at the cut surface (Sun et al. 2007).

2.5. Leaf conductances and the role of ABA

Using split root grapevines it has been demonstrated that by drying half the root system there is an increase in ABA concentration in the roots exposed to the drying soil, which is associated with a reduction in stomatal conductance (Dry et al. 2000b). The synthesis of ABA in the leaves only occurs when the

leaf turgor approaches zero (Hartung et al. 2002). Therefore, for stomatal closure to occur before any changes in leaf water potential, it would appear that ABA was transported from the roots *via* the xylem. Gradients of ABA in xylem sap and in mature leaves along the shoot of grapevine inversely correlated with stomatal conductance in unstressed plants (Soar et al. 2004). They suggested that the gradients are due to a combination of ABA synthesis in the leaves, transport from the roots and catabolism. Examining expression of *Vvnced1* and *VvZep* in the leaves and roots demonstrated that ABA in the xylem sap could be sourced from the leaves (Soar et al. 2006). It is also possible that there is a hydraulic signal regulating stomatal conductance (Tardieu and Simonneau 1998). This is supported by the work of Christmann et al. (2007), who suggested that ABA synthesised in the shoot of *Arabidopsis* caused a reduction in stomatal conductance, while the root-to-shoot signal was hydraulic. In tomato, soil heterogeneity during deficit irrigation alters ABA signalling to the shoots, and root xylem content of ABA explained the variation in leaf xylem ABA (Dodd 2007). However, more ABA came from roots in dry soil than would be predicted.

Drying soil also increased pH of xylem sap in tomato (Wilkinson et al. 1998), which was associated with closure of stomata and reduced leaf growth (Sauter et al. 2001). Increase in pH caused ABA concentration in the apoplast of *Commelina* to increase possibly due to reduced sequestration into the symplast (Wilkinson and Davies 1997). As ABA is a weak-acid the reduction in the pH gradient between the apoplast and symplast is hypothesised to reduce the sequestration of ABA into the symplast; according to the Henderson-Hasselbach equation and anion-trap hypothesis the protonated form of ABA will move into the most alkaline compartment and dissociate where the impermeable anion becomes trapped. ABA levels were inversely correlated to stomatal conductance only during mid ripening in two cultivars of grapevine on rootstocks and this was linked to rising sap pH (Rodrigues et al. 2008).

A reduction in cytokinins (zeatin and zeatin riboside) in the shoot of grapevines may also be associated with the closure of stomata during partial root-zone drying (PRD) (Stoll et al. 2000); this was reinforced by the reversal of effects associated with drying roots by application of benzyl-adenine to the shoots. Recently hydraulic signals linked to ABA have been implicated as playing a role in stomatal responses of grapevines (Rodrigues et al. 2008) and other plants (Christmann et al. 2007) to soil water status. Other potential signalling molecules occur in xylem sap including peptides and malate, which may be important in communicating root conditions to the shoot and stomata (Schachtman and Goodger 2008).

Grapevine cultivars vary in their ability to regulate stomatal aperture. Some cultivars are considered to be nearly isohydric (e.g. Grenache) while other cultivars, such as Shiraz, are nearly anisohydric (Schultz 2003, Soar et al.

2006). Isohydric varieties have a greater control over stomatal aperture resulting in minimal fluctuations in leaf water potential in response to changes in soil water content. The differences between Shiraz and Grenache were related to their ABA physiology, with higher ABA levels in Grenache xylem sap (Soar et al. 2006).

Mesophyll conductance to CO_2 in tobacco and soybean was also reduced by ABA over a period of a few days (Flexas et al. 2006, Flexas et al. 2008) and rapid decreases were observed in grapevines in response to increased CO_2 concentration (During 2003). Comparison of micropropagated vines *in vitro* with acclimatized plants showed that mesophyll conductance increased substantially during acclimatization (Fila et al. 2006). Mesophyll conductance has been linked to aquaporin activity in some plants (Flexas et al. 2006, Katsuhara and Hanba 2008), and it has been proposed that CO_2 may permeate the aquaporin pore (Uehlein et al. 2003, 2008)

3. WATER MANAGEMENT AND GRAPE QUALITY

Grapevine vigour can be influenced by water management (Dry and Loveys 1999). The technique of applying water to only half the root system at any one time (PRD), has been developed commercially for vineyards (Dry et al. 2001). PRD imposes a mild water stress that will reduce vigour without significant reductions in yield or quality. Because one half of the root system is always well watered, the risk of severe water stress is reduced (Dry and Loveys 1998). Increasing bunch exposure to sunlight through PRD has been shown to enhance the concentration of anthocyanins and phenols in the berry skin (dos Santos et al. 2005). Non-malvidin anthocyanins relative to total anthocyanins was significantly increased as a result of PRD (Bindon et al. 2008). Partial closure of stomata of Shiraz and Chardonnay grapevines occurred in response to drying of one half of a split root system (Dry and Loveys 1999). This reduction in stomatal conductance as a percent of the well-watered control was also observed for the rootstocks Kober 5BB (*Vitis berlandieri*×*Vitis riparia*) and 110 Richter (*Vitis berlandieri*×*Vitis rupestris*) (Dry et al. 2000a).

Partial stomatal closure increases plant water-use efficiency by reducing transpiration to a greater degree than the reduction in carbon dioxide assimilation (During 1992). The reduction in shoot growth rate and gas exchange was not associated with a reduction in leaf water potential (Dry and Loveys 1999, Dry et al. 2000a), but was associated with increased levels of ABA and higher pH in the xylem sap (Stoll et al. 2000). Shoot growth rate and gas exchange recovered once there was no further decrease in soil water content of the dry container (Dry and Loveys 1999, Dry et al. 2000a). This response led to the devel-

opment of a strategy of the drying being alternated from one side of the vine to the other every 3-14 days to reduce the recovery of shoot growth and gas exchange.

Vigour is also controlled by site selection, in particular planting in soils of limited depth to reduce root volume and subsequently size of the root system (Dry and Loveys 1998). The use of a deficit irrigation regime reduces vigour partly due to the decrease in soil volume that is wetted, again reducing root volume (Mitchell and Goodwin 1996).

4. BERRY WATER RELATIONS

There has been intensive research recently on the water relations of the developing grape berry because of the important role that water plays in delivering sugar *via* the phloem, and ultimately determining quality *via* concentration of sugars and flavours. Yield is also largely determined by water content and loss of water from the berry late in ripening can be substantial in some cultivars such as Shiraz (McCarthy 1999, McCarthy and Coombe 1999a).

4.1. Berry water relations during the stages of development

During the first phase of berry development (berry formation), water inflow occurs *via* the phloem and the xylem (Lang and Thorpe 1989, Greenspan et al. 1994, Dreier et al. 2000, Rogiers et al. 2006a,b), and berry transpiration is high (Rogiers et al. 2004). At the end of the lag stage and just after berry softening (defining véraison), there is rapid accumulation of sugar and water *via* phloem import (Phase 2, Fig. 3). Phase 1 incorporates Stage I and Stage II as defined by Coombe (1992). A final phase (Phase 3) is characterised by berry weight loss in some cultivars and the onset of cell death in the mesocarp (Coombe and McCarthy 1997, Sadras and McCarthy 2007, Tilbrook and Tyerman 2008). Cell death associated with berry weight loss may occur before final flavour development (Coombe and McCarthy 1997) and sugar is further concentrated through a combination of decreased volume and continued sugar import. Figure 3 shows these phases of berry development in context with other changes in berry water relations and mesocarp cell vitality.

The berry becomes less hydraulically connected to the vine during Phase 2 (Greenspan et al. 1994, 1996, Tyerman et al. 2004), however this is very much dependent on variety (Tilbrook and Tyerman 2008). From dye uptake studies and calcium ion signatures, xylem hydraulic disconnection was proposed to be due to discontinuity of xylem vessels in the berry (Findlay et al. 1987, Creasy et al. 1993, Rogiers et al. 2001). Recently, it has been shown that

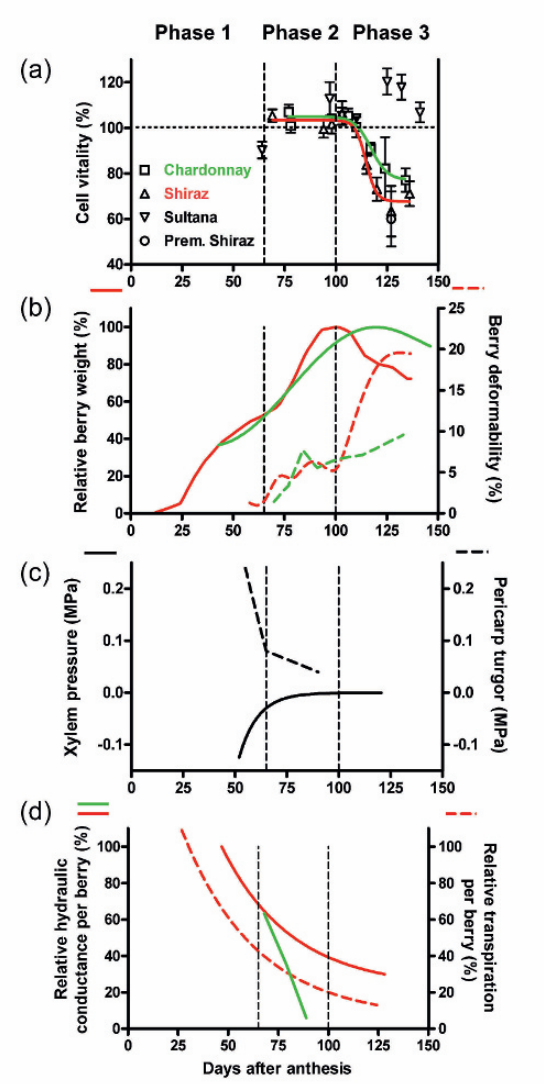

Fig. 3. Summary of the development of grapevine berries with various physiological changes associated with berry water relations. Three phases can be identified based on changes in the rate of change of berry weight. These phases are delineated by a vertical dashed line in each panel. (a) Relative cell vitality as a function of days after anthesis for Chardonnay, Shiraz and Sultana (Thompson Seedless), n=6-7 (Tilbrook and Tyerman 2008). (b) Berry weight and deformability. Shown are fits to data for Shiraz and Chardonnay (Tilbrook, unpublished). (c) Xylem equilibrium pressure and turgor pressure of berry pericarp cells. Shown are fits to data sourced from Tyerman et al. (2004) (xylem pressure) and Thomas et al. (2006) (turgor). (d) Hydraulic conductance (relative to maximum in pre-véraison berries) of the xylem pathway into Shiraz berries (fitted curves from Tyerman et al. 2004, and Tilbrook unpublished), and Shiraz berry transpiration relative to the maximum in pre-véraison berries (Rogiers et al. 2004).

most of the tracheary elements in the peripheral xylem remain intact after véraison (Chatelet et al. 2008a) and that both additional elements and new vascular bundles develop subsequently to véraison (Chatelet et al. 2008b). Our measurements of hydraulic conductance *via* the xylem pathway to the berry showed that this pathway remained functional, though reduced in magnitude depending on variety (Fig. 3, Tyerman et al. 2004). It is also the case that the hydraulic conductance depends on the direction of water flow (Tilbrook and Tyerman, manuscript in preparation). Dye uptake can still occur into the berry provided the appropriate driving force on water flow was sustained (Bondada et al. 2005). Xylem pressure measured *via* the pedicel changed from negative to positive during véraison in two wine grape cultivars, but in a table grape that maintained high hydraulic connection through the xylem, this was not the case (Tyerman et al. 2004, Tilbrook and Tyerman 2008).

Phloem unloading switches from symplastic to apoplastic during véraison (Zhang et al. 2006). This would stimulate flow through the phloem (Patrick 1997) and may explain the transition to positive xylem pressure. An increase in apoplast solute concentration has been observed (Wada et al. 2008) that may explain the reduction in turgor of mesocarp cells (Thomas et al. 2006). Reduction in turgor pressure of the mesocarp at véraison corresponds to increased deformability of the berry (Fig. 3). Degradation of cell membranes, particularly of the large mesocarp cells, does not occur as once thought in the immediate post véraison berry (Lang and Düring 1991, Dreier et al. 1998), because cell vitality measures show continued 100% vitality until after about 100 days post anthesis (Krasnow et al. 2008, Tilbrook and Tyerman 2008). The large negative osmotic potential of the mesocarp cells could only balance negative apoplast pressures and xylem tensions if the membranes remain osmotically competent (Keller et al. 2006).

In the final 2-3 weeks before harvest the fruit becomes very deformable (Fig. 3). It is during this stage, post 90-100 days after anthesis that weight loss can occur in some cultivars (Fig. 3). This has been proposed to be due to a combination of reduced phloem inflow, continued transpiration, and backflow to the vine *via* the xylem (McCarthy and Coombe 1999b, Rogiers et al. 2004, Tyerman et al. 2004). Backflow was directly demonstrated by dye loading of post véraison berries and observing the dye in the xylem of the vine (Keller et al. 2006). We have found that this is also variety-dependent, correlating with xylem hydraulic conductance (Tilbrook and Tyerman, unpublished). Backflow becomes more likely once cell membranes break down in the mesocarp cells because the osmotic potential of these cells can no longer balance the xylem tensions, provided there is still some hydraulic connection *via* the xylem. The cells that surround the vascular bundles are important in this respect and these seem to remain intact for longer (Tilbrook and Tyerman 2008), except in the

brush region in red grape cultivars (Tilbrook and Tyerman, unpublished).

4.2. A hypothesis for berry weight loss

An hypothesis for the mechanism of berry weight loss has been proposed as follows (Tilbrook and Tyerman 2008): The membranes of the pericarp cells begin to lose semi-permeability at the same time that weight loss begins, so the large negative osmotic potential of the berry sap is no longer effective in opposing the xylem tensions developed by leaves. Those cultivars that maintain higher xylem-to-berry hydraulic conductance, such as Shiraz, are therefore more vulnerable to backflow *via* the xylem. This hydraulic conductance may in part be regulated by cells surrounding vascular bundles that express aquaporins (Picaud et al. 2003).

Generally there is reduced expression of aquaporins during ripening (Schlosser et al. 2008). Increased backflow, reduced phloem inflow and continued transpiration would all combine to cause a net loss of water from berries. Cultivars like Chardonnay have very low xylem-to-berry hydraulic conductance (Tyerman et al. 2004), so despite membrane breakdown in the mesocarp, backflow from berries may be restricted. In contrast, berries of Thompson Seedless maintain hydraulic connection with the vine (Tilbrook and Tyerman 2008), but they do not loose weight because all cells remain vital. These differences between cultivars emphasises that caution is needed when attempting to generalise from observations on one variety.

5. AQUAPORINS

Aquaporins are members of the major intrinsic protein (MIP) group of transmembrane channels found ubiquitously in all organisms. The MIP superfamily is a large gene family in plants, *Arabidopsis* has 35 MIP encoding genes (Johanson et al. 2001), 33 have been identified in maize (Chaumont et al. 2001), and 28 in grapevine (Fouquet et al. 2008). Grapevine aquaporins are currently less well characterised than their counterparts in other species. However, as structure and function of this gene family appears to be conserved across different species it will be instructive to summarise key information of different aquaporins to inform future work on grapevine aquaporins.

Plant aquaporins have been divided into four groups based on sequence homology, and for two groups this seems to match their membrane localization: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), and the small basic intrinsic proteins (SIPs) (Johanson et al. 2001, Johanson and Gustavsson 2002). The SIPs are the

smallest subgroup and have been functionally characterised and localized in the endoplasmic reticulum membrane (Maeshima and Ishikawa 2008). The PIPs have been divided into two groups: the PIP1 group often show lower or no water permeability when expressed in *Xenopus* oocytes, in contrast to the PIP2 group that show high water permeability (Chaumont et al. 2000, Moshelion et al. 2002, Fetter et al. 2004). This difference is also the case for grapevine aquaporins.

5.1. Aquaporin structure and selectivity

Aquaporins have a highly conserved structure consisting of 6 transmembrane α-helices (Fig. 4), with the amino and carboxy termini localized on the cytosolic side of the membrane. The pore of AQP1 consists of three hydrophilic nodes that bind four water molecules, with the remainder of the pore being hydrophobic (Sui et al. 2001). The conserved histidine residue in the pore is essential for water specificity. Generally, the 25-30 kD polypeptides form homotetramers (Sui et al. 2001, Tornroth-Horsefield et al. 2006) and each monomer functions as a single water pore (Engel et al. 2000). A number of residues in the protein are conserved throughout the aquaporin family, including the asparagine-proline-alanine (NPA) motif (Fig. 4). The aromatic/ARG filter, a narrow selectivity filter for water, is conserved in all the *Arabidopsis* PIPs while the TIPs and NIPs have a number of different regions (Wallace et al. 2006).

The most divergent regions of the protein are the N and C-termini and the membrane connecting loops. The pores of the channels are believed to be narrow so that the water molecules move through in single file. Mercury generally blocks aquaporins by binding to cysteine residues, and can cause conformational changes (Barone et al. 1997). Mercury may inhibit aquaporins indirectly in living cells *via* general metabolic inhibition (Zhang and Tyerman 1999). Silver and gold compounds have also been shown to strongly inhibit aquaporins (Niemietz and Tyerman 2002).

Some plant MIPs transport other small solutes, such as glycerol and urea, in addition to or alternatively to water. Nodulin 26 (NOD26), is targeted to the symbiosome membrane of nitrogen-fixing nodules, allowing the transport of glycerol and formamide in addition to water (Rivers et al. 1997), and may also be permeable to ammonia (Niemietz and Tyerman 2000). Some members of the TIP subgroup transport ammonia (Jahn et al. 2004). NtAQP1 and NtTIPa transport glycerol, water and urea (Eckert et al. 1999, Gerbeau et al. 1999). Urea transport was also demonstrated for four *Arabidopsis* TIPs (AtTIP1;1, AtTIP1;2, AtTIP2;1 and AtTIP4;1) (Liu et al. 2003) and a maize root PIP (ZmPIP1;5b; Gaspar et al. 2003). Carbon dioxide permeation in leaf mesophyll may be facilitated *via* NtAQP1 (Flexas et al. 2006, Uehlein et al. 2008). Other

important nutrients transported by MIPs in plants include boron (Takano et al. 2006) and silicon (Ma et al. 2006). Some MIPs also appear to transport toxic metalloids, such as arsenite (Bienert et al. 2008). Signalling molecules may also be transported by MIPs, and hydrogen peroxide has been shown to be transported by AtTIP1;1 and AtTIP1;2 in a heterologous yeast system (Bienert et al. 2007).

5.2. Regulation of aquaporins

One mechanism of post-translational regulation of aquaporin activity is reversible phosphorylation (Fig. 4). Phosphorylation of plant MIPs can increase water permeability (Maurel et al. 1995, Johansson et al. 1998, Guenther et al. 2003). In spinach leaves, SoPIP2;1 is de-phosphorylated under drought stress and therefore inactivated (Johansson et al. 1996). De-phosphorylation occurs at two highly conserved serine residues, Ser115 in cytosolic loop B and, Ser274, in the C terminus, by a Ca^{2+} dependent protein kinase (Johansson et al 1996, Johannson et al 1998). A number of residues in loop D of SoPIP2;1 have been identified as being involved in gating of the channel (Tornroth-Horsefield et al. 2006). Structural studies show that in the closed conformation, loop D, which has an additional 4-7 amino acid residues in the PIP subfamily, caps the pore of the aquaporin occluding the pore from the cytosol (Tornroth-Horsefield et al. 2006, Fig. 4).

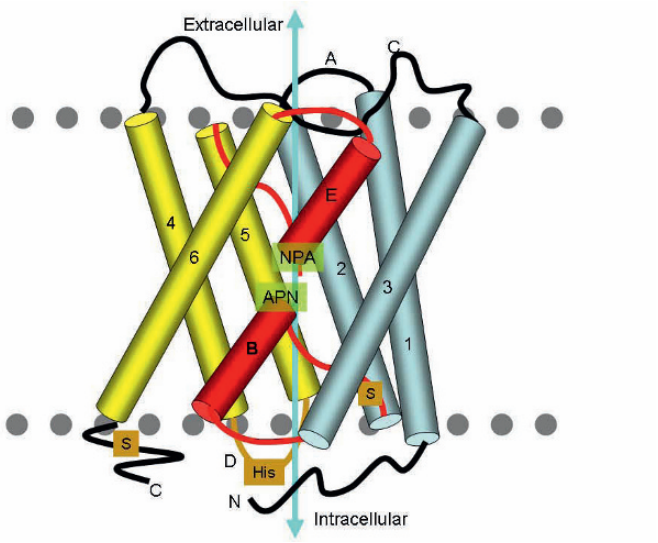

Fig. 4. The structure of a typical plant aquaporin, with six transmembrane helices (1-6) and five connecting loops (A-E). Shown are the highly conserved NPA motifs; histidine (H) residue, involved in cytosolic pH sensing; two phosphorylation sites [serine (S) residues] in the loop B and C-terminal tail of aquaporins of the PIP2 subgroup (from Luu and Maurel, 2005 and Tornroth-Horsefield et al., 2006).

Plasma-membrane water permeability is regulated by cytosolic pCa (free calcium ion concentration) and pH. Measurements on plasma-membrane vesicles from *Beta vulgaris* storage roots has revealed very high water permeabilities (>500 μm s^{-1}), strongly regulated by pCa and pH (Alleva et al. 2006). *Arabidopsis* L_{pcell} was reduced by 35% and 69% in the presence of magnesium and calcium ions, respectively (Gerbeau et al. 2002). Both PIP1 and PIP2 aquaporins have a histidine residue (His 197) that appears to be the pH sensitive residue (Tournaire-Roux et al. 2003) (Fig. 4). This may explain the sudden reduction in root L_p when roots are subject to anoxic stress due to a decrease in cytoplasmic pH (Tournaire-Roux et al. 2003).

There is also evidence for mechano-sensitive gating of aquaporins. Large pressure pulses (greater than 0.1 MPa) decreased the L_{pcell} of maize root cortical cells (Wan et al. 2004). Aquaporin activity, based on L_{pcell} in *Chara*, was also reduced in the presence of high concentrations of osmotic solutes and more strongly with increasing size of these solutes (Ye et al. 2005). The water permeability of the symbiosome membrane containing NOD26 also seems to be gated by osmotic solutes (Vandeleur et al. 2005). Hydroxyl radicals may gate aquaporins directly or indirectly but the mechanism involved is unclear (Henzler et al. 2004, Ye and Steudle 2006).

Regulation of aquaporin activity may also occur by interactions between different aquaporins, either in the membrane or *via* targeting to the membrane (Fetter et al. 2004). The co-expression of ZmPIP1;2, which has low activity, with ZmPIP2;1, ZmPIP2;4 or ZmPIP2;5 increased the osmotic water permeability of *Xenopus* oocytes. This has also been observed for two grapevine aquaporins, VvPIP1;1 and VvPIP2;2 (Vandeleur et al. 2008). In living maize cells it appears that a physical interaction with a PIP2 is required to traffic PIP1 from the endoplasmic reticulum to the plasma membrane (Zelazny et al. 2007). Redistribution of aquaporins *via* endomembrane vesicles is also a means of regulating water/solute permeability and this has been shown to occur in response to osmotic stress (Vera-Estrella et al. 2004).

Transcriptional regulation of aquaporins is also linked to control of water transport *via* changes in channel density in the target membranes. Expression of aquaporins varies diurnally and in response to environmental or developmental influences. Localizations of expression of particular aquaporins can be general or specific for certain cell types and organs. The expression of a PIP1 aquaporin was greater in the endodermis of *Arabidopsis* root than in its cortex (Schaffner 1998). High expression levels of PIPs generally occur in regions of concentrated water flow (Hachez et al. 2006, Vandeleur et al. 2008). The immuno-localization examples in Fig. 5 used an antibody raised to a peptide sequence that will detect all PIP1s in *Vitis*. Cells in the exodermis, cells closely associated with the xylem vessels, and cells in phloem bundles showed strong antibody signals (red

fluorescence) when compared to pre-immune serum controls.

Diurnal fluctuations in the expression of MIPs have been observed (Yamada et al. 1997, Henzler et al. 1999, Lopez et al. 2003, Sakurai et al. 2005) and in some cases these have been correlated with water transport (Henzler et al. 1999, Moshelion et al. 2002, Vandeleur et al. 2008). Changes in expression in leaves during the day have been correlated with leaf water potential (Yamada et al. 1997). The expression of *ZmTIP2-3* in maize began to increase just prior to the light period and was at its greatest after four hours of light (Lopez et al. 2003). Diurnal variation continued for some time in continuous darkness. *Oryza sativa PIP2* genes, *OsPIP1;2* and *OsPIP1;3*, showed diurnal fluctuations in roots, peaking three hours after the onset of light and decreasing to a minimum three hours after the onset of darkness (Sakurai et al. 2005).

Aquaporins have been shown to be up- or down-regulated in response to many environmental factors such as water stress, salinity, anoxia, nutrient depletion, hormones, low temperature and light (reviewed in Bramley et al. 2007a).

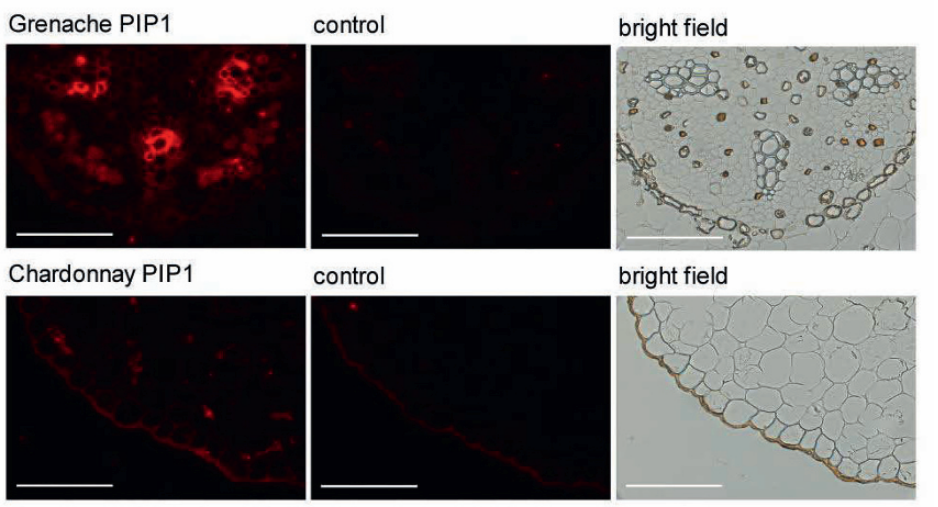

Fig. 5. Transverse sections of a Grenache root (top, showing stele) and Chardonnay root (bottom showing outer cortex and exodermis) showing immunolocalization of VvPIP1 proteins. Control was with pre-immune serum. Scale bar = 100 μm.

5.3. Grapevine aquaporins

In the published literature, ten putative aquaporins with homology to *Arabidopsis* MIPs have been identified in grapevine (Baiges et al. 2001, Picaud et al. 2003). To date, there are 83 MIPs annotated in Genbank, 73 from the wine

grape *Vitis vinifera*, eight from the rootstock Richter-110 (*Vitis berlandieri x Vitis rupestris*) and one from the Chinese Wild Grape (*Vitis pseudoreticulata*). Aquaporins identified from the grapevine *Vitis vinifera* are from a number of cultivars including Syrah, Cabernet Sauvignon, Pinot noir and Nebbiolo.

The first study of grapevine aquaporins, reported the identification of 8 aquaporins, 5 homologous to the PIP subgroup and 3 to the TIP subgroup (Baiges et al. 2001). The cDNAs encoding aquaporins from *Vitis* rootstock Richter-110 were obtained by screening a leaf cDNA library with homologous probes and by using reverse Northerns. Tissue specific differential expression patterns were analysed for each of the putative aquaporins (Baiges et al. 2001). Several aquaporins have been identified from grape berries and expression patterns during development vary for the individual isoforms (Picaud et al. 2003, Fouquet et al. 2008).

We have constructed a *Vitis vinifera* cv Cabernet Sauvignon cDNA library from root and shoot tissues (Shelden 2007). The library was screened for members of the PIP and TIP subfamilies with homologous probes. The library screen resulted in the identification of 13 aquaporin cDNAs 11 of which are full length sequences. Of the cDNAs identified, five are PIP2 aquaporins, six PIP1 aquaporins and two TIP aquaporins. Recently, two high quality draft genome sequences of grapevine; a homozygous line PN40024, bred from *Vitis vinifera* cv Pinot noir (Jaillon et al. 2007) and a heterozygous grapevine cultivar *Vitis vinifera* cv Pinot noir clone ENTAV 115 (Velasco et al. 2007) have been reported. Our *in silico* analysis of the recently sequenced *Vitis vinifera* clone PN40024 suggests at least 24 full-length *MIP* genes may be present in the grapevine genome, comprising four subfamilies (Shelden et al, unpublished).

5.4. Functional studies of grapevine aquaporins

The full-length *PIP* and *TIP* cDNAs identified from *V. vinifera* cv Cabernet Sauvignon were functionally characterised in a heterologous expression system, *Xenopus* oocytes (Shelden 2007). The osmotic water permeability (Pf) was determined for each aquaporin. The water permeability of *Xenopus* oocytes expressing VvPIP2 aquaporins was high, with values comparable to those obtained for human AQP1 in both this study and in the published literature. The VvTIP aquaporins showed moderate water permeability and the VvPIP1 aquaporins had water permeability comparable to that of the water-injected control. Many other studies have shown PIP1 aquaporins to be impermeable to water when expressed in oocytes, including aquaporins identified from maize (Chaumont et al. 2000).

VvPIP1a, isolated from grape berry, had a moderate increase in water permeability and a large increase in glycerol permeability compared to the wa-

ter injected control (Picaud et al. 2003). The addition of mercury chloride resulted in a decrease in water permeability, and also indicated an interaction with a native oocyte urea transporter. VvPIP1b showed no permeability to water, glycerol and urea when expressed in oocytes. A number of other aquaporins identified in the grapevine, *Vitis* rootstock Richter 110, have not been functionally characterised. Further functional characterisation of grapevine aquaporins in the *Xenopus* oocyte heterologous expression system is required, to provide more comprehensive information about the transport specificities of each of these proteins and the role they play *in planta*.

Given the apparent lack of water channel activity of VvPIP1 proteins when expressed in *Xenopus* oocytes, it is possible that these may mediate transport of another solute, such as glycerol, as has been shown for VvPIP1a (Picaud et al. 2003). Alternatively they may regulate the activity and/or targeting of other PIP2 aquaporins as indicated by the strong positive interaction between VvPIP1;1 and VvPIP2;2 when expressed in *Xenopus* oocytes, and their collocated expression in roots (Vandeleur et al. 2008).

The effect of cytosolic pH on gating of VvPIP2;1 and VvTIP1;1 was examined by expression in *Xenopus* oocytes (Shelden 2007). Reduction of cytosolic pH with Na-acetate resulted in an eleven fold decrease in water permeability of VvPIP2;1, which was not observed for VvTIP1;1. The histidine residue implicated in cytosolic pH sensitivity is conserved throughout the PIP family and is found in all *Vitis vinifera* PIP members identified. Conservation of this histidine residue (His196) in VvPIP2;1, combined with the decrease in water permeability observed by acidifying the cytosol, is evidence that His196 is most likely protonated under these conditions.

5.5. Expression in planta

Many aquaporin isoforms have been shown to have tissue specific expression in different plants, including grapevine. There is higher expression of both PIPs and TIPs in grapevine roots of Richter 110, compared to the shoot tissues (Baiges et al. 2001). *PIP1;1* has been reported to have higher expression in the roots of grapevine while five other *PIP* isoforms had higher expression in the leaves (Galmes et al. 2007). Using a semi-quantitative RT-PCR approach, we have shown that both the *PIP* and *TIP* genes did not exhibit organ specificity in grapevine shoots.

All genes examined were expressed in the tendrils, stem, leaf and petiole, although transcript levels did vary (Shelden 2007). Isoform *VvPIP2;3* was found to have slightly higher expression in the petioles than other shoot tissues. Expression levels in the shoot tissues were highest for the PIP1 isoform, *VvPIP1;4,* that had constitutive expression in all aerial tissues. Due to the high

homology between *VvPIP1;4* and *VvPIP1;2* it is likely that the probe used may detect both of these genes. It is interesting to note that although PIP1 genes were highly expressed in the shoot tissues, when these were expressed in *Xenopus* oocytes, no water permeability was observed. Therefore, it is possible that in the shoot, VvPIP1 proteins may play a role in the movement of other small neutral solutes, such as CO_2, or that post-translational modification and/or interactions with other PIPs may be required *in planta* for the formation of functional water channels. Expression of *VvTIP1;1* was highest in the stem, whilst *VvTIP2;1* was constitutively expressed in all shoot tissues. *VvTIP2;1* (previously *Vitis TIP1*) had highest expression in the root tips and lateral roots (Baiges et al. 2001).

Expression of two *PIP1* genes over the developmental stages of the berry in three different cultivars of grapevine (Chardonnay, Ugni blanc and Pinot Meunier) was examined (Picaud et al. 2003). For all three cultivars *PIP1* expression was lowest pre-véraison. Expression peaked post-véraison in both Chardonnay and Ugni blanc and at harvest time for Pinot Meunier. Combined with the functional data, the authors suggested that PIP1a may be contributing to the water and sugar flux into berries during ripening. Further research into the role of aquaporins in grape berry ripening is required.

In Chardonnay roots, transcripts of *VvPIP1;1 1;2 1;4 2;1 2;2 2;3* and *2;4* were all detectable, but *VvPIP1;1* and *VvPIP2;2* had the highest expression followed by *VvPIP1;4* and *VvPIP2;1* (Vandeleur et al. 2008). Strong expression of *VvPIP1;1* and *VvPIP2;2* was detected by RNA *in-situ* hybridization, in elongating cortical and vascular tissue, and in the root apex of both Grenache and Chardonnay. At 30 mm and 50 mm from the root apex, *VvPIP2;2* expression occurred in vascular tissue near the xylem poles, and in the cortex.

5.6. Aquaporin expression in response to water stress

Aquaporins have been documented to be regulated in response to water stress transcriptionally, post-transcriptionally and by post-translational modifications (see above). In the hope of elucidating the functional role of aquaporins *in planta*, a number of studies have attempted to correlate AQP expression and localization *in planta* with physiological data obtained in response to environmental stimuli (Sakr et al. 2003, Cochard et al. 2007, Secchi et al. 2007). Many studies have examined the response of aquaporin expression to water stress with responses differing markedly between plant species (Bramley et al. 2007a).

The expression profile of aquaporins in response to water stress in roots and the petiole has been found to differ between two grapevine varieties, Chardonnay and Grenache, in response to both water stress and over a diurnal cycle (Shelden 2007). There were two apparent responses in the transcriptional regu-

lation of aquaporins in the petioles. In the first instance, a diurnal response was observed under favourable conditions when the vines were well watered. A separate mode of transcriptional regulation was apparent in response to water stress.

Both these responses differed in the genes targeted and between varieties, indicating that the transcriptional regulation of aquaporins in grapevines is quite complex and is unique between varieties that contrast in their degree of isohydry. In roots, *VvPIP1;1* expression showed very different responses between Grenache and Chardonnay (Vandeleur et al. 2008), where it was up-regulated under drought in Chardonnay, but unchanged in Grenache. Re-hydration caused down-regulation in Chardonnay and up-regulation in Grenache. Different transcriptional responses of aquaporins occur under water stress in different organs in the drought tolerant rootstock Richter 110 (Galmes et al. 2007).

No correlation was observed between hydraulic conductivities and expression levels in this instance, leading the authors to suggest that the aquaporins are regulated to achieve homeostasis. The transcriptional regulation of aquaporins may help to provide a balance in maintaining cellular water balance, to optimize photosynthesis and prevent water loss under conditions of water stress. A proteomic analysis comparing differences between Chardonnay and Cabernet Sauvignon in response to water deficit and salinity found that cultivar differences were the main source of variation in protein expression (Vincent et al. 2007).

6. SUMMARY

Our knowledge of aquaporin structure and function in plants has rapidly advanced in the last few years. They are gated channels that sense a variety of signals important in cell metabolism and signalling. This enables rapid responses to sudden environmental changes. Transcriptional regulation and membrane targeting occurs over diurnal time scales and may also be rapid. Interactions occur between different aquaporin isoforms indicating that function may not be elucidated by functional expression of one isoform individually.

Combined there are many avenues by which aquaporins may be regulated in the plant making interpretation of the large and often rapid changes in hydraulic conductance in roots and leaves more difficult at the molecular level. The range of molecules besides water that are transported has expanded. Evidence for aquaporins being involved in the transport of CO_2 is becoming more substantial (Kaldenhoff et al. 2008), though some doubt still exists about whether aquaporins permeable to CO_2 can make a direct contribution to membrane permeation considering diffusion through unstirred layers and the nor-

mally very high lipid permeability to CO_2 (Missner et al. 2008).

Signalling between root and shoot in both directions is important to balance conductances under differing transpirational demand. Root conductance, which is linked with the expression of a PIP aquaporin in grapevine, is correlated with leaf transpiration indicating that long distance signalling is occurring. This ultimately allows regulation to achieve maximum extraction of water from the soil. Understanding these signals will be as important as is our understanding of the long distance signalling involved in stomatal regulation.

The efficiency of water and sugar transport to the berry and the retention of berry volume seems to be dependent on coordinated timing of multiple anatomical and physiological transitions both at véraison and also later in development. Aquaporins are suggested to be involved in these transitions but stronger evidence of the links is still required. The differences between cultivars in their water relations physiology (root, leaf and berry) present an opportunity to correlate the physiology with differences in gene expression or even single nucleotide polymorphisms within aquaporin genes.

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5

SUGAR TRANSPORT & SUGAR SENSING IN GRAPE

A. Agasse[1], C. Vignault[2], C. Kappel[3], C. Conde[1], H. Gerós[1] & S. Delrot[3]

[1]Centro de Fisiologia Molecular e Biotecnologia de Plantas, Departamento de Biologia, Universidade do Minho, 4710-057 Braga, PORTUGAL

[2] Laboratoire de Physiologie Moléculaire du Transport des Sucres chez les Végétaux, FRE 3091, 40, avenue du Recteur Pineau, bât GON, 86 022 Poitiers Cédex, FRANCE

[3]Université de Bordeaux, ISVV, Institut des Sciences de la Vigne et du Vin, Domaine de la Grande Ferrade, INRA, BP 81, 33883 Villenave d'Ornon Cedex, FRANCE

1. INTRODUCTION

The ripening of grape berries is accompanied by a massive accumulation of soluble sugars, and by the synthesis and accumulation of a wide range of phenolic compounds and aroma precursors. These processes play major roles in the quality of the berries and wine. Sugars are accumulated in the vacuoles of flesh (mesocarp) cells, which account for 65 to 91 % of the fresh weight in a mature berry. Polyphenols accumulate in the skin and seeds, which represent respectively 6 to 20 %, and 2 to 6 % of the berry fresh weight, depending on the cultivars (Galet 1983). Although sugars and polyphenols do not accumulate in the same cells, evidence is growing for some control of polyphenol metabolism by sugars. The expression and activity of sugar transporters mediating sugar accumulation in the berries are also partially controlled by sugars.

During the past few years, molecular approaches have allowed a better understanding of these processes. Furthermore, new sequences encoding so far unidentified factors of these events are raising from the recent release of the complete grape genome (Genoscope data base http://www.cnrs.fr/vitis, Jaillon et al. 2007, Iasma Genomics http://genomics.research.iasma.it, Velasco et al. 2007). These databases, and associated transcriptomic tools, also open the possibility to compare the ripening of a non-climacteric fruit such as grape berry with that of a climacteric fruit such as tomato (*Lycopersicon esculentum*).

After summarizing the physiological basis of sugar accumulation in the

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grape berry, this chapter will give an overview of our present knowledge on the molecular biology of sugar transporters and sugar sensing.

2. PHYSIOLOGICAL BASIS OF SUGAR ACCUMULATION

The ripening grape berry is a strong sink for dry matter transported from current photosynthesis and wood reserves (Coombe 1989). Sucrose derived from leaf photosynthesis is exported *via* the phloem to the berries. From véraison and throughout ripening the berries accumulate roughly equal amounts of glucose and fructose, reaching over 1M of each hexose (Coombe 1987). This implies that phloem transported sucrose is hydrolyzed at some step during its transport from the sieve tube to the vacuole of the mesocarp cell (Fig. 1). The remarkable sink strength of the berry is well illustrated by the fact that its dry mass increases four-fold during a 6 week-period, with little change observed in the dry mass of other plant parts (Conradie 1980).

In situ measurements carried out at different times on the same berry showed that glucose and fructose accumulation begins suddenly on the same day as berry softening begins. It seems that, once begun, sugar accumulation in grapes is undeviating and massive. The limitation to sink strength within individual berries is set by sink activity, not berry size. Indeed, after it is triggered, the accumulation of hexoses is linear with time, although the increase in berry volume proceeds at a variable rate (Coombe 1989).

The fleshy portion of the berry originates from the ovary wall that develops to form the pericarp, mesocarp and endocarp. The developing berry is fed with assimilates transported through the carpellary vascular bundles which are divided into the peripheral and central bundles. A dorsal bundle network extends at the periphery of the fruit, and central vascular bundles are connected to the seeds and irrigate the central flesh. A detailed analysis of the unloading pathway in the ripening berry was conducted in a hybrid grape (*Vitis vinifera* x *Vitis labrusca*; Zhang et al. 2006). A variety of techniques, including electron microscopy, determination of enzymatic activities, and transport studies with carboxy fluorescein (a symplastic tracer) and with companion cell expressed and tagged viral movement proteins, were used. Until véraison, both carboxyfluorescein and the tagged viral movement protein could be released from the functional phloem strands, whereas at the late stage of ripening they remained confined in the strands.

This shift from a symplasmic to an apoplasmic unloading pathway, which occurs at or just prior the onset of ripening, is accompanied by a concomitant increase of the expression and activity of cell wall invertases (cwINV) (Zhang et al. 2006). As a result, apoplastic sugar concentration and osmotic pressure in-

crease, which may enhance sugar uptake *via* stimulation of the proton-pumping ATPase activity (Li and Delrot 1987). Interestingly, a loss of symplastic connections also occurs at the inception of tomato fruit ripening (Ruan and Patrick 1995), with cwINV activity and sugar uptake increasing throughout ripening (Baxter et al. 2005).

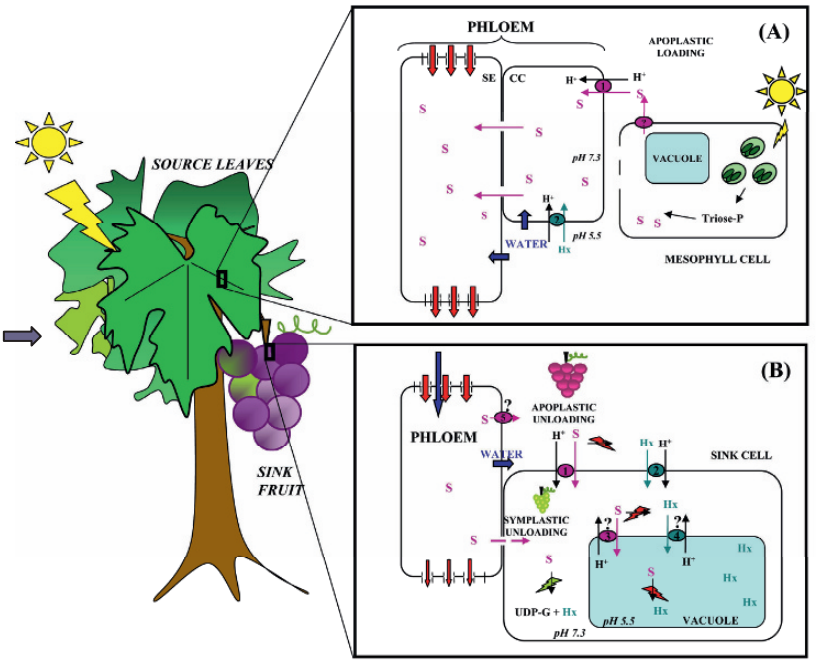

Fig. 1. Simplified scheme of long-distance sugar transport in plants. (A) Pathways for phloem loading: Sucrose (S) is synthesized in mesophyll cells through photosynthesis. S is loaded into the sieve elements/companion cell complex (SE/CC) *via* the apoplast. Apoplastic loading involves the retrieval of S leaking from the mesophyll or the vascular parenchyma (mechanism yet uncertain) and may occur along the phloem path. Hydrostatic pressure drives phloem sap movement toward sink tissue. (B) Pathways of phloem unloading: S enters the receiving cell by the symplastic route before véraison, using plasmodesmata, or the apoplastic pathway after véraison. The latter predominates in the ripening fruit and requires the activity of membrane transporters mediating the transport of S, and of the hexoses (Hx) resulting from S hydrolysis by metabolic enzymes (invertases, sucrose synthases). Hx are accumulated in the vacuole. Water fluxes respond to sugar concentration gradient. (1) S/H^+ symporter; (2) Hx/H^+ symporter; (3) S/H^+ antiporter; (4) Hx/H^+ antiporter; (5) S efflux transporter; invertase; sucrose synthase; water flux.

In grape berries, the analysis of different zones of the skin and flesh showed that the sugar concentrations are heterogeneous in both tissues (Coombe 1987). Concentrations in skin are generally lower than those present in the flesh tissue. Furthermore, there are longitudinal differences in the levels

of glucose and fructose in the outer and central flesh tissues. Hexose concentrations in the flesh increase from low near the brush to high at the stylar end. Sucrose concentration in the flesh is low throughout ripening, but increases in the skin when the berries reach 17 -26° Brix and in the central tissues (including the brush) in overripe berries. Uptake and efflux studies with isolated skin suggested active uptake of D-glucose but not L-glucose. Surprisingly, active uptake of monosaccharides was measured in skin samples from both green unripe and ripe berries, whereas it was evident only in flesh tissues sampled from ripening berries (Coombe and Matile 1980). However, contrarily to what is observed in mesocarp tissues, a high proportion of the sugar absorbed by skin pieces is diffusive, whatever the sampling time.

The limiting steps and the molecular mechanisms leading to final trapping of high concentrations of hexoses in the vacuole of mesocarp cells are still elusive. The compartmentation and enzymatic events leading from sucrose in the sieve tubes to hexoses in mesocarp cell vacuoles involve (a) sucrose efflux from the phloem conducting complex, (b) sugar entry into the flesh cells, and (c) sugar uptake into the vacuoles of these cells. Sucrose may be cleaved in the cell wall prior to its entry into the mesocarp cells, or after compartmentation into the vacuoles. The possibility of sucrose breakdown/resynthesis and final breakdown, often referred to as sucrose futile cycle, should also be considered.

Together with sugar transporters, the sucrose metabolic enzymes invertases (INVs) and sucrose synthases (SuSys) participate in the maintenance of the sucrose gradient needed to sustain mass flow of the phloem sap. Furthermore, as they modulate the pool of available sugars, these enzymes may certainly play important roles in the context of sugar signalling (Delrot 1994, Roitsch et al. 1995, Sherson et al. 2003, Koch 2004). INVs are hydrolases cleaving sucrose into glucose and fructose. In tomato and *Arabidopsis*, INVs are encoded by small gene families with various expression patterns regarding the organ and the environmental and/or metabolic stimuli (Godt and Roitsch 1997, Sherson et al. 2003). These enzymes are either acidic or neutral.

Acidic INVs, such as cwINV and vINV, are localized in the cell wall and in the vacuole, respectively, while the nINV, a neutral form, is present in the cytoplasm. In grape, the cDNA sequence of a cwINV (AY538262) and the promoter region of the gene (EF122148), the complete cDNA sequence of a nINV (*NIN1*, EU016365), as well as 3 incomplete genomic sequences, and 2 vINVs cDNAs (*VvGIN1* AAB47171.1 and *VvGIN2* AAB47172.1) have been cloned (Davies and Robinson 1996, Hayes et al. 2007). Based on protein motif analysis (pfam, Interpro), 10 to 12 encoding putative nINVs and 10 encoding putative acidic INVs genes were found in the grape genome sequence (http://www.genoscope.cns.fr/spip/Vitis-vinifera-whole-genome.html). Although INV activities were considered to be non-limiting (Davies and Robinson, 1996), re-

cent reports by Zhang et al. (2006) and Hayes et al. (2007) clearly indicate that expression and activity of cwINV is induced just prior to véraison.

However, although the co-regulation of cwINV and some monosaccharide transporters in sink tissues has been confirmed in several plant species and organs, including young grape leaves, the same could not be demonstrated in grape berries (Fotopoulos et al. 2003, Weschke et al. 2003, Roitsch and Gonzalez 2004, Baxter et al. 2005, Hayes et al. 2007). Furthermore, cwINV enzyme activity in berry represents only 4% of total INV activity (Rüffner et al. 1990, Davies and Robinson, 1996) and recent microarray results showed a constant level of both *cwINV* and *nINV* mRNAs throughout berry development (Deluc et al. 2007). This suggests that cwINV alone cannot be responsible for the increase of monosaccharide concentration in the ripening berry. Acidic vINV activity, although participating in sink activity and strength, as the major sucrolytic activity in grape berry, occurs too early to trigger by itself hexose accumulation at ripening inception (Rüffner et al. 1990, Davies and Robinson 1996, Patrick 1997, Dreier et al. 1998). *VvGIN1* and *VvGIN2* transcripts and protein levels accumulate before véraison and decrease during fruit ripening (Davies and Robinson, 1996, Sarry et al. 2004, Deluc et al. 2007). However, vINV activity is important to drive the import of sugars along ripening, since its natural reduction in the Steuben grapevine hybrid contributes to an increase of the relative proportion of sucrose in the maturing berry (Takayanagi and Yokotsuka 1997). Likewise, vINV activity in tomato fruit is crucial to determine both the levels and the nature of accumulated sugars in the vacuole (Klann et al. 1993, Ohyama et al. 1995, Nguyen-Quoc and Foyer 2001).

SuSys are cytosolic glycosyl transferases which, in the presence of UDP, convert sucrose into UDP-glucose (UDP-G) and fructose. *In vivo*, SuSys are also able to catalyse the reverse reaction, but with lower efficiency (Geigenberger and Stitt 1993). Unlike INVs, SuSy enzymes are active and transcriptionally upregulated under low oxygen conditions. Interestingly, *SuSys* that respond to low oxygen are also highly expressed under carbohydrate depletion (Zeng et al. 1999, Koch et al. 2000). In addition, SuSy activity is known to be involved in key metabolic processes such as storage, defence and cell wall synthesis since its UDP-G product is implicated in the formation of callose and in the synthesis of several cell-wall polysaccharides (Albrecht and Mustroph 2003).

In tomato, SuSy but not INV, is involved in sugar unloading and metabolism at the beginning of fruit development (Dali et al. 1992, D'Aoust et al. 1999, N'tchobo et al. 1999). Later in maturation, SuSy activity is strongly reduced but sucrose unloading rates, although low, are maintained, and might be driven by both sugar uptake and INV activities (D'Aoust et al. 1999, Nguyen-Quoc and Foyer 2001). In grape, 6 to 9 genes encoding putative SuSys are expected based on recent genome sequencing. However, SuSys activities do not vary much along ripening and are not conclusively correlated with the modifica-

tions on the soluble sugar concentration (Zhang et al. 2006). For instance, the expression of a *SuSy* gene homologue to *CiTSUA* (1609402_at, TC62599, http://www.plexdb.org/modules/PD_browse/experiment_browser.php? plex_name=GrapePLEX) increases gradually along berry development. However, whether the subsequent enzyme is involved in sugar import or cell wall expansion during berry softening is not clear. Hence, it appears that sugar accumulation in berry, and sink organs in general, would rather result from the coordinate action of several mechanisms, involving various transporters and hydrolytic enzymes (Deluc et al. 2007).

The simultaneous events of sucrose (or starch) synthesis and degradation occurring in plants cells are sometimes referred to as 'futile cycle'. Although SuSy is able to catalyze the synthesis of sucrose, in the cytosol this sugar is mainly synthesized by sucrose phosphate synthase (SPS) (Huber and Huber 1996). This holds true both for photosynthetic tissues and for non-photosynthetic storage organs. Sucrose futile cycle implicates the re-synthesis of sucrose from the hexoses present in the cytosol, its entry in the vacuole and its degradation by vINV. A continuous sugar exchange between the cytosol and the vacuole (sucrose influx, hexose efflux) has also been suggested in tomato pericarp cells (Scholes et al. 1996). Sucrose futile cycles would then favour the unloading and storage of sugars into the ripening fruit which becomes symplastically isolated (Nguyen-Quoc and Foyer 2001). In grape berry, an *SPS* gene is preferentially expressed in the pericarp, suggesting a probable participation in the re-synthesis of sucrose following its unloading from the phloem into the fruit (Grimplet et al. 2007).

3. MOLECULAR BIOLOGY OF SUGAR TRANSPORTERS

3.1. Monosaccharide transporters (MSTs)

In higher plants, *AtSTP1* (*Arabidopsis thaliana* Sugar Transporter Protein 1) was the first MST cDNA identified and functionally characterized as a hexose-proton symporter, by complementation of hexose transport null-mutant yeasts (Sauer et al. 1990). Since then, many clones have been identified in various plant species where they belong to multigene families: the *Arabidopsis* genome display 53 homologous sequences encoding putative MSTs, distributed into 7 distinct clusters (Büttner 2007).

They all share a structure of 12 trans-membrane domains with N- and C-cytoplasmic termini characteristic of all sugar transporters belonging to the MFS (Major Facilitators Superfamily, Williams et al. 2000, Delrot et al. 2001). In grape, 59 putative hexose transporters encoding genes have been identified based on protein motif recognition (Samson et al. 2004, Jaillon et al. 2007). Six

full length cDNAs encoding for MST and named *VvHT1* to *6* (*V. vinifera* Hexose Transporter, *VvHT1* AJ001061, *VvHT2* AY663846, *VvHT3* AY538259 and AY854146, *VvHT4* AY538260, *VvHT5* AY538261, *VvHT6* AY861386, DQ017393) were previously cloned from various grape cultivars such as Pinot noir, Ugni blanc, Chardonnay, Cabernet Sauvignon and Syrah (Fillion et al. 1999, Vignault et al. 2005, Hayes et al. 2007).

The predicted peptides share about 60% homology to each other (Büttner and Sauer 2000, Büttner 2007). Interestingly, computer analysis revealed the presence of N-terminal signal peptide for VvHT3 and VvHT4 and a 200 aminoacid cytoplasmic extension in the centre of the VvHT6 protein, between transmembrane helices 6 and 7 (Hayes et al. 2007). Except the latter one, all VvHTs identified so far present high homologies with AtSTPs, *i.e.* functional hexose transporters (Fig. 2). VvHT6 is related to AtTMT2, a member of the TMT (Tonoplast Monosaccharide Transporter) subfamily of MFS transporters. AtTMTs are tonoplastic hexose-proton antiporters and possess a long hydrophilic central loop. These transporters are induced by abiotic stresses such as cold or drought and were suggested to play a role as sensors (Wormit et al. 2006).

A cDNA encoding a putative plastidic glucose transporter (*pGLT* AY608701), sharing high homology with *AtpGLcT* (CAC01856) has also been reported (Terrier et al. 2005, Glissant 2005). Another *pGLT*-like transcript (1608991_at, TC60060) is highly expressed during berry maturation (Deluc et al. 2007). Consistent with the observed increase of starch metabolism during berry development, *Vv*pGLT-like genes would encode plastidic transporters involved in the export of glucose resulting from starch degradation (Weber et al. 2000, Deluc et al. 2007). Eventually, Deluc and co-workers (2007) identified a transcript homologous to a sugar/polyol membrane transporter (*At*PLT5), whose expression increases along berry development (1613408_at, TC66667, http://www.plexdb.org/moules/PD_browse/experiment_browser.php?plex_name=GrapePLEX, Deluc et al. 2007).

Uptake activities of VvHT1, VvHT4 and VvHT5 have been demonstrated by heterologous expression in the *hxt-null* mutant yeast EBY VW 4000 (Wieczorke et al. 1999). All three VvHTs are high affinity, H^+-dependent transporters mediating the uptake of radiolabelled D-[U-^{14}C]glucose according to saturable Michaelis-Menten kinetics. VvHT1 exhibits the highest affinity for glucose (K_m of 70 µM, V_{max} about 14 µmol min^{-1}g FW^{-1}) compared to VvHT4 and VvHT5 (K_m about 150 µM and 100 µM respectively, V_{max} about 5 and 0.15 µmol min^{-1} g FW^{-1}, respectively) and is the only one able to restore the growth of the complemented yeast on glucose. VvHT3 was not able to transport any of the tested radiolabelled sugars in the deficient yeast model (Vignault et al. 2005, Hayes et al. 2007). Up to date, attempts to confirm the transport activity of both VvHT2 and VvHT6 in yeast had little success.

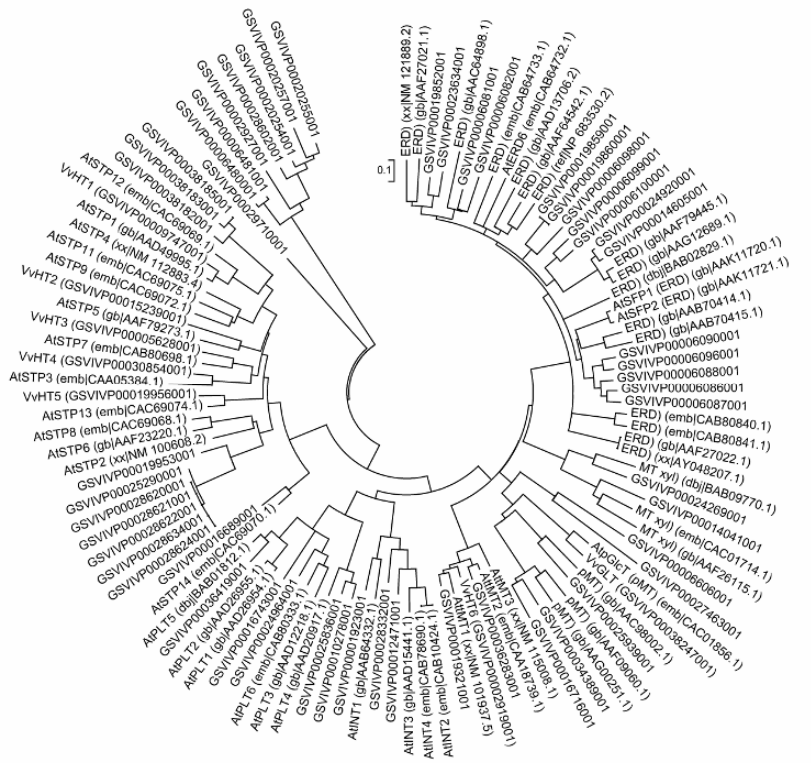

Fig. 2. MST evolutionary relationships of 111 taxa from *A. thaliana* and *V. Vinifera*.The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky and Nei 1992). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar 2000) at a search level of 1. The Neighbor-joining algorithm (Saitou and Nei 1987) was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 430 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007) from initial multiple alignments realised with muscle (Edgar 2004). Disclaimer: Although utmost care has been taken to ensure the correctness of the caption, the caption text is provided "as is" without any warranty of any kind. Authors advise the user to carefully check the caption prior to its use for any purpose and report any errors or problems to the authors immediately (www.megasoftware.net). In no event shall the authors and their employers be liable for any damages, including but not limited to special, consequential, or other damages. Authors specifically disclaim all other warranties expressed or implied, including but not limited to the determination of suitability of this caption text for a specific purpose, use, or application.

VvHT1 displays broad substrate specificity, being able to transport galactose, xylose and glucose analogs such as 3-*O*-methyl glucose. Mannose competes with glucose for the active binding site but is not transported through the membrane. Fructose does not significantly affect glucose uptake rates in the mutant yeast expressing *VvHT1*, whereas it behaves as a competitive inhibitor in grape cell suspensions (Vignault et al. 2005, Conde et al. 2006, Hayes et al. 2007). Furthermore, uptake of radiolabelled D-[U-^{14}C]fructose in grape cells also display Michaelis-Menten kinetics with a V_{max} similar to the one measured for glucose uptake, suggesting that both monosaccharides are transported by the same protein, although the K_m for fructose was much higher (Conde et al. 2006). However, AtSTP1, its closest relative in *A. thaliana*, is unable to transport fructose (Boorer et al. 1994). The involvement of an additional monosaccharide transporter sharing similar characteristics in the grape suspension cells model might explain these results (Conde et al. 2006). VvHT4 transport activity may be restricted to glucose, which constitutes a major difference from its *Arabidopsis* homologue, AtSTP3 displaying low affinity for glucose (Büttner et al. 2000). Conversely, both glucose and fructose are substrates of VvHT5 (Hayes et al. 2007).

The plasma membrane localization expected for these three VvHTs, as well as for the non-functional VvHT3, has been confirmed by immunofluorescence, immunolabelling and C-terminal GFP fusion (Vignault et al. 2005, Hayes et al. 2007). VvHT2 and VvHT6 appear to be expressed in the tonoplast (Vignault et al. unpublished) whereas VvpGLT resembles typical chloroplastic translocators. The involvement of tonoplast sugar transporters is expected because the vacuole is the major compartment of hexose storage in berry flesh during ripening. Furthermore, the recent characterization of the tonoplastic glucose-proton antiporter AtVGT1 (At3g03090) in *A. thaliana* supports the involvement of such mechanisms in other species (Aluri and Büttner, 2007).

The expression of hexose transporters is mainly associated with sink tissues where they import the unloaded sugars from the apoplast. In grape berry, *VvHT1*, *VvHT2* and particularly *VvHT3* are highly expressed compared to the other *VvHT*s, at all developmental stages. *VvHT2* mRNA levels remain constant throughout berry development. However, recent data report that *VvHT2* expression is most specifically associated with véraison (Terrier et al. 2005, Hayes et al. 2007, Vignault et al. unpublished).

Nonetheless, some specific expression patterns can be distinguished: both *VvHT1* transcripts and protein levels (Conde et al. 2006) are much higher at pre-véraison stages, whereas *VvHT5* mRNA accumulation, although weak, is mostly associated with late ripening days. *VvHT3* mRNA levels are sharply reduced at véraison but high at both green and ripening stages (Hayes et al. 2007), whereas *VvHT6* transcripts are highly accumulated at véraison, suggesting that this transporter may be responsible for early import of hexose at the inception of

ripening (Vignault et al. 2005, Terrier et al. 2005, Deluc et al. 2007). *VvHT1* is expressed in the plasma membrane of flesh cells mainly at green stages and provides sugar to cells when energy is required to complete cell division and growth. However, this transporter is barely detectable in plasma membrane fraction of ripening berries and thus cannot be directly responsible for the post-véraison sugar accumulation (Vignault et al. 2005, Conde et al. 2006).

Monosaccharide import into berries during ripening would be mainly due to the VvHT2 and VvHT3 transporters although their sugar transport activity has not yet been experimentally demonstrated. To a lesser extent, *VvHT4*, although weakly expressed, and *VvHT5* could also be involved (Hayes et al. 2007). In spite of the high number of putative MST genes in the grape genome, no other VvHT was identified in grape berries, suggesting that the most important transporters for sugar accumulation in this organ have already been cloned. In addition to their expression profiles, which give some clues about their physiological role, their tissue and cell specific expression needs to be determined. The diversity of hexose transporter genes expressed along berry development is consistent with the shift from a symplastic to an apoplastic phloem unloading pathway that occurs prior to véraison, the later being the dominant mechanism of sugar import into cells at ripening stages (Bondada et al. 2005, Zhang et al. 2006).

In both young and mature leaves, *VvHT1* and *VvHT3* are the most abundant *VvHTs* transcripts. Expression of *VvHT1*, *VvHT3* and *VvHT5* is enhanced with the transition from sink to source activity. Conversely, *VvHT2* transcript levels, constantly weak, seem to be preferentially associated with sink activity (Hayes et al. 2007). *In situ* hybridization showed that *VvHT1* transcripts are abundant in the phloem region of the conducting bundles of the leaf, petiole and berry (Vignault et al. 2005). Although the phloem sap mainly contains sucrose, minor amounts of monosaccharides are also detected, and VvHT1 could participate in the retrieval of hexose leaking from the conducting complex. Its high affinity and its particular location support that hypothesis.

3.2. Sucrose transporters (DSTs)

In plants, SoSUT1 (*Spinacia oleracea* Sucrose Transporter 1) was the first disaccharide transporter (DST) functionally characterized in a yeast mutant deleted for invertase and expressing sucrose synthase (Riesmeier et al. 1992). DSTs genes, which belong to small multigenic families with 9 members in *Arabidopsis* and 4 in tomato for instance, encode for a 55 kD polypeptide (*Arabidopsis* Genome Initiative 2000, Delrot et al. 2001, Sauer et al. 2004, Hackel et al. 2006). Plant DSTs can be clustered into 4 groups regarding their protein sequence homologies: group 1 and group 2 are exclusively composed by

monocots and dicots transporter proteins, respectively, whereas groups 3 and 4 are mixed. Group 3 clusters proteins possess longer N-termini and central cytoplasmic loops whereas group 4 sequences display shorter C-termini (Barth et al. 2003).

Group 3 transporters have been reported to localize all along the sieve element and have been proposed to sense the sucrose flux through the plasma membrane (Schulze et al. 2000). Group 4 sucrose transporters are low affinity high capacity transporters (LAHC) localized in the membranes of minor veins of source leaves (Weise et al. 2000). However, their subcellular localization is controversial since, although correctly directed to the yeast plasma membrane, some of these proteins may be specific to the tonoplast in plants (Endler et al. 2006). Up to date, three *DST* cDNAs have been cloned from Shiraz and Cabernet Sauvignon (*VvSUC11*; AF021808, also identified as *VvSUT1* AF182445; *VvSUC12* AF021809; *VvSUC27*, AF021810) and characterised as proton-dependent sucrose transporters, whereas 9 DSTs sequences are present in *Arabidopsis* genome (Sauer et al. 2004). VvSUC11 and VvSUC12 are intermediate affinity sucrose transporters (K_m, 0.9 mM and 1.4 mM, respectively) (Ageorges et al. 2000, Manning et al. 2001), whereas VvSUC27 has a K_m of about 10 mM and is thus a low affinity sucrose transporter (Zhang et al. 2008). The grape genome sequence recently released (Jaillon et al. 2007, Velasco et al. 2007) suggests that sucrose transporter genes would constitute a small multigenic family of 4 members in this species (Fig. 3).

VvSUC11 is expressed in flowers and fruits whereas *VvSUC12* expression is restricted to berries and young leaves. In addition, *VvSUC11* is expressed in both young and expanded leaves. *VvSUC27* expression is closely related to sink activity since its transcripts are strongly accumulated in flowers and unripe berries, roots and tendrils but poorly present in mature leaves (Davies et al. 1999). *VvSUC27* expression is associated with the early stages of berry development, *VvSUC11* and *VvSUC12* transcription concomitantly increases with post-véraison sugar accumulation, which suggests a direct pathway for sucrose acquisition by berry cells (Davies et al. 1999). However, information regarding sucrose uptake in berry along ripening is scarce. Sucrose uptake activity has been demonstrated in berry slices (Conde et al. unpublished results) but further investigation, such as sucrose transporters localization in berry flesh is needed (Hayes et al. 2007).

Indeed, even if most of the sucrose transporters yet characterised are responsible for the loading of phloem conducting cells, the involvement of sucrose carriers into the uptake by the storage cells of sugars unloaded at the end of the phloem path has been described [*e.g.* DcSUT2 in carrot (Shakya and Sturm, 1998), StSUT1 in potato (Kühn et al. 1997), CiSTU2 in Citrus (Li et al. 2003), AtSUC3 in *A. thaliana* (Meyer et al. 2004) and LeSUT1 in tomato (Hackel et al. 2006)]. The presence of SUT transporters in sucrose releasing

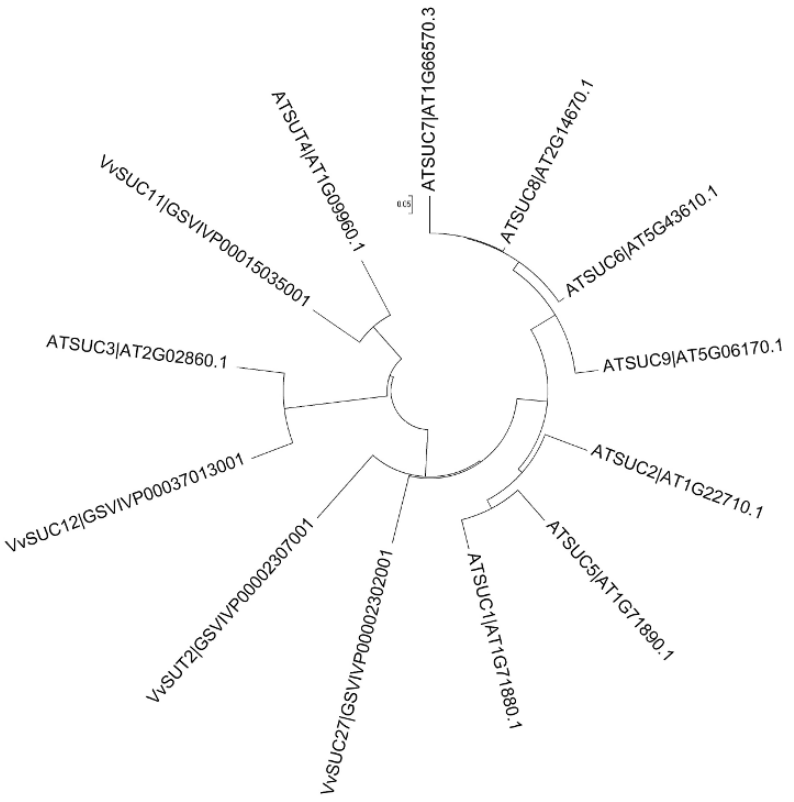

Fig. 3. DST evolutionary relationships of 13 taxa from *A. thaliana* and *V. vinifera.* For a legend, see Fig. 2.

tissues of cereals (Bagnall et al. 2000, Aoki et al. 2006) and the demonstration that AtSUC2 loss-of-function and StSUT1 sink-specific antisense repression affect phloem unloading in *Arabidopsis* and potato, respectively (Gottwald et al. 2000, Kühn et al. 2003) suggest that some of the SUT proteins can be directly involved in phloem unloading and thus may function as reversible transporters. Indeed, Carpaneto and co-workers expressed *ZmSUT1* (orthologue of AtSUC2) in *Xenopus* oocytes and succeeded in inversing its transport mode (Carpaneto et al. 2005).

The involvement of a classical sucrose/proton symporter in the proton-independent sucrose efflux activity observed in potato plasma membrane vesicles has also been suggested. This protein would then act in a different way under certain conditions, as it has been reported for the CkHUP1 glucose transporter (Komor and Tanner 1974, Kühn et al. 1997). Since sucrose unloading occurs down the sucrose gradient, sucrose efflux involves biochemical mechanisms that differ from those driving its uptake into sucrose accumulating tissues and the functional asymmetry of these transporters would be driven by specific

membrane potential and gradients (Carpaneto et al. 2005). Furthermore, the roles of a putative sucrose/proton antiporter and of a non-selective channel has also been suggested in seed coats of broad bean and pea, respectively (De Jong et al. 1996, Walker et al. 1995, 2000). Eventually, the participation of DSTs as retrievers of unloaded sucrose into the apoplasm, or as direct modulators of sucrose concentration and sink strength is well demonstrated in sink tissues (Lalonde et al. 2003).

4. SUGAR SENSING AND REGULATION OF TRANSPORTERS

Because sugar producing and sugar consuming and/or storage organs are spatially separated, plants have developed ways to understand their "sugar status" in order to adapt carbohydrate synthesis and transport. In a manner resembling the well-known glycaemia regulation in mammals, plants sense their sugar levels, and modulate them according to the general "feast and famine" pattern of responses: (a) carbohydrate excess favours the expression of enzymes connected with biosynthesis and storage of reserves and (b) represses transcripts encoding enzymes involved in photosynthesis and reserve mobilisation (Koch 1996).

Additionally, sugars provide signals able to control many aspects of seed and plant development. The mechanisms of sugar perception and the subsequent transduction pathways regulated are referred to as sugar sensing and signalling, respectively (Smeekens 2000). These terms, however, qualify for a complex scheme regarding (a) the diversity of perception mechanisms and levels involved, (b) the molecular pathways induced, (c) the multilevel control of gene and protein expressed and, (d) the interactions with pathways mediating the transduction of hormonal and environmental stimuli. The study of the molecular mechanisms of sugar sensing and signalling pathways in plants takes advantage of the well-known pathways described in yeasts (Rolland et al. 2006, Santangelo 2006). The following paragraphs give an overview of the sugar control over sugar transport and polyphenol metabolism in grapevine.

4.1. Sugar regulation of sugar transport

A fine way to control resource distribution among tissues and organs is to regulate their transport throughout the plant (Koch 1996). The mechanisms by which sugars regulate their own transport proteins have been extensively studied in yeast and mammals but much less in plants, even if many examples now provide evidence for a fine and complex action of sugars (*e.g.* BvSUT1,

Vaughn et al. 2002). Furthermore, altered concentrations of the same sugar can dually control the same target (*e.g*. VfSUT1, Weber et al. 1997) and/or differentially affect the expression of the plasma membrane transporters, from gene transcription to correct targeting and/or activity of the protein itself (for review see Rolland et al. 2006).

In grape suspension cultured cells, *VvHT1* expression is tightly controlled by sugars: (a) high glucose concentration (150 mM) represses *VvHT1* transcription through a pathway mediated by the hexokinase (HXK) sensor, a cytosolic enzyme that also independently catalyses the first step of glycolysis, (b) physiological concentrations (58 mM) are essential to maintain basal levels of *VvHT1* transcription (Atanassova et al. 2003), and (c) high glucose concentration decreases glucose uptake activity and this inhibition is at least in part due to the decrease of VvHT1 protein amount in the plasma membrane *via* an HXK-independent pathway (Conde et al. 2006). The dual transcriptional regulation of *VvHT1* by glucose levels is similar to the control of *HXT2* and *KHT2* expression described in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, respectively (Wendell and Bisson 1994, Milkowski et al. 2001).Indeed, the expression of the high affinity glucose transporters HXT6 and HXT7 in *S. cerevisiae* is induced by very low external sugar concentrations (< 50 mg/L) and repressed by higher concentrations, whereas the low affinity transporter HXT1 is transcriptionally upregulated by glucose concentrations up to 100 mg/L. HXT2 and HXT4, are expressed at glucose concentrations up to 100 mg/L but repressed by higher glucose concentrations. HXT3 expression does not depend on glucose stimulation (Boles and Hollenberg 1997, Rolland et al. 2002, Klockow et al. 2008). In grape, sugars also control the expression of the other *VvHTs*, although their responses are usually weaker (Fig. 4). In suspension cells, *VvHT2*, *VvHT3* and *VvHT6* transcripts transiently accumulate under sugar starvation. Furthermore, whereas *VvHT3* is repressed by high glucose concentration, *VvHT6* expression is strongly induced by high glucose levels (Conde et al. 2006). This recalls the strong expression of *VvHT6* in the fruit at the véraison stage, a step characterized by the sudden and sharp import of sugar into the berry (Terrier et al. 2005, Vignault et al. 2005, Hayes et al. 2007). *VvHT4* and *VvHT5* do not respond to any sugar treatment in grape suspension cells, and appear therefore insensitive to sugar regulation in this experimental system (Conde et al. 2006).

Sugar (sucrose or glucose) induction of *VvHT1* transcription may be mediated by a putative plasma membrane sugar sensor. Indeed, palatinose, a non-metabolizable and non-transportable sucrose analogue, upregulates *VvHT1* in the same way as sucrose (Atanassova et al. 2003). Membrane sugar sensors are well known in yeasts, and to a lesser extent in mammals (GLUT-2 and SGLT3, Guillemain et al. 2000, Diez-Sampedro et al. 2003). They have been also identi-

fied in filamentous fungi (Madi et al. 1997). In *S. cerevisiae*, the modified glucose facilitators SNF3 and RGT2 are unable to transport sugar but possess a C-terminal specific extension giving them the ability to sense glucose as a signal (Coons et al. 1997, Özcan et al. 1998, Rolland et al. 2002).

Furthermore, each sensor displays different glucose affinity and thus would initiate adapted responses. SNF3, as a high affinity glucose sensor, is required for the transcription of high affinity transporters such as HXT2 and HXT4, at low glucose concentration and its own transcription is repressed by high glucose concentrations. Conversely, RGT2 is constitutively expressed and integrate high sugar levels as inducers of the expression of low sugar affinity facilitators such as *HXT1*. The importance of both sensors is underlined by the fact that the double mutant *snf3rgt2* is unable to maintain any HXT in its plasma membrane (Özcan et al. 1998, Rolland et al. 2002).

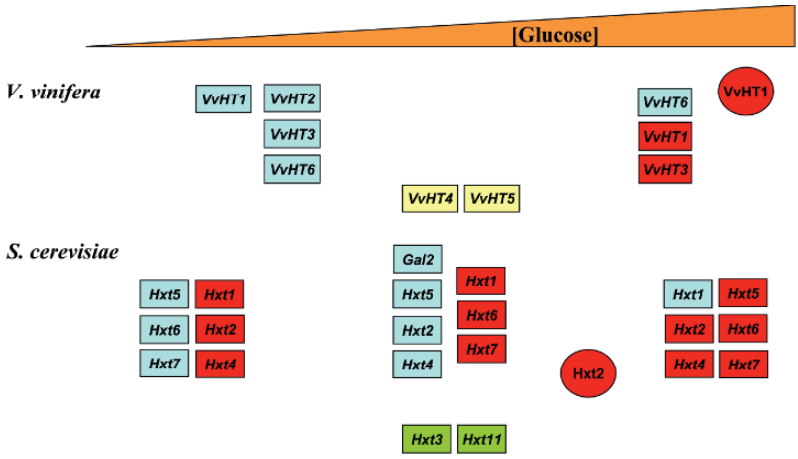

Fig. 4. Expression patterns of glucose transporters in *V. vinifera* and *S. cerevisiae* according to glucose availability. Glucose levels affect both gene (rectangles) expression and protein (circle) amounts. Some transporters are induced (blue) and/or repressed (red) by different levels of glucose whereas others are constitutively expressed (green) or not regulated by sugar concentrations (yellow).

The occurrence of similar sensors, suggested by the effects of poorly permeant sugar analogues, cannot be ruled out in plants. A role as sugar sensors was proposed for SUT2/SUC3-type transporters. Indeed, the tomato protein LeSUT2 was thought to be a sensor because (a) its structure, resembles the yeast SNF3 and RGT2 proteins (*i.e.* unusual protein extensions), (b) its transport inability once expressed in baker's yeasts, and (c) its colocalisation with the functional transporters LeSUT1 and LeSUT4 in sieve elements (Barker et al. 2000). The poor efficiency of AtSUT2 (orthologue of LeSUT2) led to modify the putative role of the SUT2/SUC3-type transporters from orthodox sensors to flux

sensors, measuring the transport rates of sucrose across the membrane (Meyer et al. 2000, Schulze et al. 2000). The capacity of LeSUT2 to interact with other transporters was suggested to be a putative mechanism to modulate sucrose import into the phloem (Reinders et al. 2002). However, none of these arguments are sufficient to confer such a specific role to SUT2/SUC3-type transporters: (a) although no specific function could be attributed to the amino acid extension of these proteins, it did not interfere with the transport function, (b) the poor efficiency of the transport in baker's yeasts depends on the correct expression of the heterogously expressed protein, (c) the colocalization is not a general feature of these transporters, as demonstrated in *Plantago major*, where PmSUC3 and PmSUC2 are expressed in different cell types and their respective genes at different developemental stages (Barth et al. 2003, Eckardt 2003).

Likewise, the role of some AtTMT monosaccharide transporters as putative glucose sensors has been suggested but still remains elusive (Wormit et al. 2006). In yeast and mammals, other proteins than sugar transporter-derived sensors are able to detect sugars as signals and initiate responses. Such membrane proteins are members of the G protein coupled-receptors (GPCR) family (Rolland et al. 2006, Le Gall et al. 2007). In plants, the sensor AtRGS1 mediates HXK-independent glucose signalling and plays a role in *A. thaliana* development (Chen and Jones 2004, Johnston et al. 2007). AtRGS1 acts as a glucose sensor and regulates the GTPase activity of AtGPA1, a GTP-binding protein. Indeed, AtRGS1 is a plasma membrane receptor that interacts with AtGPA1 upon glucose binding to hydrolyse GTP and control of cell proliferation. Interestingly, the AtRGS1 protein is also involved in ABA signalling in seed germination (Chen et al. 2006). A homologue of AtRGS1 was identified in the grape genome (CAO46706.1) by conserved domain homologies.

The hexose content is considered as a regulator of cell division and expansion whereas the sucrose concentration is related to maturation and differentiation processes (Wobus et al. 1999). Controlling the concentrations of these sugars may then be a way to direct tissue differentiation. Sucrose metabolizing enzymes, because they modulate these pools, are natural candidates to be involved in sugar sensing mechanisms. It is therefore not surprising that the genes encoding for these proteins are under sugar control (Roitsch et al. 2003, Koch 2004). For instance, the maize *vINV* gene, *Ivr1* is repressed by sugar but transcriptionally enhanced by sugar depletion. However, its homologue *Ivr*2 is induced by addition of sugar. Similar patterns have been shown for the *SuSy* genes, *Sh1* and *SUS2* (Xu et al. 1996). Interestingly, sugar-mediated regulation of *Ivr2* expression may occur in water stressed leaves (Kim et al. 2000, Trouverie et al. 2004).

The complex response of *VvHT1* to sugar signals correlates with the presence in its promoter of 4 putative *cis*-elements previously found in the promot-

ers regions of sugar-modulated genes (Fillion et al. 1999, Delrot et al. 2000, Atanassova et al. 2003): (a) the positive sugar-responsive *cis*-elements *SURE1* first identified in the promoter of the potato storage proteins patatin (Grierson et al. 1994), and *Sucrose box 3*, identified in the promoter of petunia chalcone synthase (*CHS*) gene (Tsukaya et al. 1991), and (b) the motifs *AMYBOX2* (*TATCCA*) and *AMYBOX1* present in the promoter of the rice α-amylase (Huang et al. 1990, Lu et al. 1998, Toyofuku et al. 1998), which are responsible for inducing expression upon sugar starvation.

These different *cis*-elements were able to drive the expression of the GUS reporter gene in response to physiological concentrations of sucrose and glucose in tobacco BY-2 suspension cells (Leterrier et al. 2003). Other sugar responsive elements have been identified, as *SP8*, present in the promoters of sporamine and α-amylase genes in sweet potato (Ishiguro and Nakamura 1994), and *ACT* accessory factor, important for sucrose repression of the RUBISCO small subunit *Rcbs2* gene (Urwin and Jenkins 1997), and the coupling elements *CE*, involved in ABA control of gene expression (Niu et al. 2002).

An interesting study reported the diversity of promoter motives involved in sugar sensitivity in *Arabidopsis* (Li et al. 2006). However, these sugar responsive *cis*-regulating elements are not systematically encountered in all sugar regulated genes, and, to be functional, some require the presence of other regulatory sequences in the promoter. For instance the *AMYBOX2* sequence is often associated with the *G*- and *GC*-box motives in a sugar response sequence (*SRS*) responsive to sugar depletion in cereals (Lu et al. 1998, 2002, Toyofuku et al. 1998). Eventually, some of these elements are related to both sugar and phytohormones responses (Rook et al. 2006, Li et al. 2006). Transcription factors able to act through these sequences have been identified. They are *MYB* factors acting differentially on the *AMYBOX2* sequence in rice (Lu et al. 2002, Chen et al. 2006), WRKY proteins, able to recognize the *SURE* elements in barley (Sun et al. 2003) or bZIP proteins binding to *G*-box sequences (Lee et al. 2003). In grape, the ASR protein VvMSA induces the *VvHT1* promoter activity through the particular overlapping motifs *SURE1* and *Sucrose box 3*, and the action of a MADS-box transcription factor on the same sequence is suggested (Çakir et al. 2003, Agasse et al. 2007).

As already mentioned, VvHT1 is down-regulated by glucose at the protein level. However, whether the translational process or the turn-over is affected is still unclear. Furthermore, a concomitant effect of a putative decrease in *VvHT1* mRNA stability cannot be ruled out (Conde et al. 2006). Several examples of post-transcriptional control by sugars have been reported: (a) sugars modulate the stability of the *α-AMY3* and *Incw1* mRNAs, acting through their *3′UTR* sequences, in rice and maize respectively (Chan and Yu, 1998, Cheng et al. 1999); (b) sucrose impairs the translation of *bZIP* transcription factors through a conserved upstream *5′UTR* open reading frame (Rook et al. 1998,

Wiese et al. 2004, 2005); (c) glucose enhances the degradation of the *ETHYLENE-INSENSITIVE3* (*EIN3*) transcription factor, a component in ethylene signalling, *via* an HXK-dependent pathway (Yanagisawa et al. 2003).

Information about intermediates of sugar signalling pathways implicated in the control of sugar transport during grape maturation is scarce. Kinases, phosphorylases and calcium fluxes, widely involved as intermediate steps in signalling, may be part of sugar transport control pathways as suggested by the regulation of BvSUT1, whose activity and expression are both affected by phosphorylation (Roblin et al. 1998, Ransom-Hodgkins et al. 2003). Sugar feeding to autotrophically grown *Arabidopsis* roots induced an increase of cytosolic Ca^{2+} concentration in the whole plant (Furuichi et al. 2001).

Furthermore, a Ca^{2+}-dependent, calmodulin-independent protein kinase (CDPK) activity as well as a mitogen-activated protein kinase (MAPK)-like activity have been characterized in the developing mesocarp of grape berry (Shen et al. 2004). A Ca^{2+}-dependent protein kinase (ACPK1) whose activity is stimulated by ABA, is expressed at the plasma membrane and chloroplast/plastid membranes of berry cells in a developmental manner (Yu et al. 2006). SnRK protein kinases, identified in plants and related to the yeast SNF1 protein, a kinase involved in sugar signalling pathways, also mediate sugar signal responses (Rolland et al. 2006). Further studies, using pharmacological abolishers of phosphorylation events or Ca^{2+} fluxes for instance (okadaic acid, staurosporine, lanthane chloride) would provide important clues about their involvement in sugar-transport regulation.

Recently, the hypothesis of a favoured localization of plant sugar transporters in a specific lipidic environment, similar to the mammalian and yeast well documented lipid rafts, has been postulated. Indeed, the *Chlorella* HUP1 does not show a homogeneous distribution in the plasma membrane. A patchy distribution pattern is also observed once expressed in *S. cerevisiae* as a HUP1-GFP fusion protein and its purification from yeast membrane indicates its favoured localization with sterols and sphingolipids. Furthermore, the localization of HUP1 within the raft cluster increases the catalytic activity of the transporter in yeast (Grossmann et al. 2006). The existence of raft domains in plant plasma membranes is suggested by detergent solubility experiments (Peskan et al. 2000). In mammals and yeasts rafts may play important roles in protein trafficking, and allow protein interaction. Raft may also maintain apart proteins until a signal promotes their interaction (Opekarova and Tanner 2003). A possible involvement of raft domains in sugar signalling would deserve attention.

4.2. Sugars and production of phenolic compounds

Acquisition of the red/blue color of grape berries in red varieties along

ripening is a visual indicator of the biochemical processes occurring in grape. This reflects more precisely the accumulation of anthocyanins pigments in the vacuoles of skin cells, which does not occur in the white grape varieties. Interestingly, anthocyanins precursors and intermediates are ubiquitously synthesized within the fruit while the final pigments accumulate only in the epidermal tissue of the berries. Recently, the role of a putative anthocyanin carrier has been evidenced that would explain such phenomenon (Braidot et al. 2008). Sucrose-induced production of anthocyanins has been demonstrated in various species, such as petunia (Tsukaya et al. 1991, Weiss 2000), grape (Larronde et al. 1998, Vitrac et al. 2000), and radish (Hara et al. 2004). Expression of *CHS* is upregulated by sucrose treatment, both in petunia and *Arabidopsis* (Tsukaya et al. 1991, Ohto et al. 2001).

In *V. vinifera*, the enhanced expression of various genes of the anthocyanin biosynthetic pathway in the berry skin may be correlated with the concomitant accumulation of sugars in the flesh (Boss et al. 1996). In *V. vinifera* cell suspensions, sucrose treatment promotes anthocyanin synthesis (Larronde et al. 1998). Indeed, sucrose upregulates the expression of dihydroflavonol reductase (DFR) and anthocyanin synthase/leucoanthocyanidin dioxygenase (ANS/ LDOX). This upregulation and the accumulation of anthocyanins are sucrose specific in *Arabidopsis*. However, in grape both glucose and sucrose are able to enhance DFR and ANS expression which suggests different sugar-sensing mechanisms (Gollop et al. 2001, 2002).

In grape, the sucrose signal is transmitted *via* a pathway involving variation of Ca^{2+} concentrations and the action of kinases and phosphatases (Vitrac et al. 2000). Interestingly, in *Arabidopsis*, the sucrose transporter SUC2 is involved in anthocyanin biosynthesis: the *pho3* mutation impairs the expression of the correct SUC2 protein, resulting in an impaired phloem loading and thus a concomitant accumulation of sugars in leaves. As a result, high levels of anthocyanins are accumulated because of the specific action of sucrose on the expression of anthocyanin biosynthesis-related genes as well as on the levels of transcription factors such as *AtMYB5*, also known as *PRODUCTION OF ANTHOCYANIN 1* (*PAP1*), which is sucrose-inducible (Lloyd and Zakhleniuk 2004, Teng et al. 2005, Solfanelli et al. 2006). Furthermore, recent studies reveal the role of the transporter AtSUC1 in the sucrose-dependent signalling leading to accumulation of anthocyanins in seedlings. AtSUC1 mediates sucrose uptake into germinating pollen and its expression in roots is sucrose-inducible (Stadler et al. 1999, Johnson et al. 2004, Sivitz et al. 2008). Its activity would also trigger a sucrose-dependent transduction pathway leading to anthocyanin accumulation in cotyledons (Sivitz et al. 2008).

Interestingly, sucrose also accelerates the accumulation of carotenoids in the non-climacteric citrus fruit (Iglesias et al. 2001). Carotenoids are accumulated in chloroplasts-derived plastids, the chromoplasts, through the general iso-

prenoid biosynthetic pathway which is upregulated along fruit maturation (for review, see Cunningham and Gantt 1998). Like polyphenols, carotenoids are antioxidant molecules recommended in human nutrition and may protect against cancer and eye degenerative disease (Jonhson 2002).

Tomato fruit accumulates high levels of carotenoids (mainly lycopene) as it ripens, contributing to its characteristic red and orange color. Light and hormones are involved in the control of carotenoid biosynthesis (Giovannoni 2004); the *Never ripe* mutant (*Nr*), affected in ethylene sensitivity accumulates low levels of lycopene (Lanahan et al. 1994). Similarly, this climacteric-associated hormone also enhances anthocyanins production in grape berry (El-Kereamy et al. 2003). Sucrose promotes specifically lycopene and phytoene accumulation in tomato without affecting other carotenoids. Accordingly, sucrose enhances the accumulation of the phytoene synthase (*PSY1*) mRNAs, without acting on the expression of other carotenoid biosynthesis pathway-related genes (Télef et al. 2006).

4.3. Sugar, hormonal and environmental signalling cross-talk

Sugar signalling often inter-connects with hormone-induced pathways to modulate gene expression and physiological processes along plant life. In *Arabidopsis*, interactions between sugar and ABA signalling have been mostly studied during early seedling development through the characterization of mutants. Many of these mutations affecting sugar signalling are allelic with components of ABA synthesis or ABA transduction pathway (Leon and Sheen 2003, Rook and Bevan 2003, Gibson 2004). Glucose induces a specific accumulation of ABA during this stage of plant life, an essential increase for the plant to potentiate the efficiency of sugar signals (Rolland et al. 2006). In grape, ABA and sugars, both accumulating during berry ripening, affect important physiological processes in a coordinated manner, such as anthocyanin production and hexose transport. Indeed, in spite of some contradictory reports, it appears that sucrose and ABA act positively on polyphenols and anthocyanins accumulation (Pirie and Mullins 1976, Larronde et al. 1998, Vitrac et al. 2000, Hiratsuka et al. 2001).

A cross-talk between ABA and glucose metabolism is also suggested to enhance phenolics production (Weiss 2000). Furthermore, ABA induces the expression of *VvHT1* (Atanassova et al. 2003) and acts synergistically with sugars (sucrose and glucose at physiological concentrations) to transiently induce *VvHT1* transcription. In fact, *VvHT1* response to both signals involves the presence in its promoter of the *cis*-element *S3S1*, a specific overlapping configuration of the two sugar response *cis*-elements *Sucrose box 3* and *SURE1*. *S3S1* constitutes a target for the fixation and the action of the VvMSA (*V. vinifera*

Maturation, Stress Abscisic acid-induced, also known as VvASR, *Vitis vinifera* Abscisic acid, Stress, Ripening-induced, AF281656) protein. *VvMSA* gene expression is strongly upregulated by the combined effect of sugars and ABA and VvMSA enhances *VvHT1* promoter activity (Çakir et al. 2003).

ASRs are globular and highly hydrophilic proteins identified in many plant species but absent from *A. thaliana* and some other members of the *Brassicaceae* family (Carrari et al. 2004, Shkolnik and Bar-Zvi 2008). They are involved in abiotic stress (water deficit, salt stress) responses, senescence, pollen maturation and fruit ripening and they respond to ABA, a phytohormone involved in water deficit stresses (Iusem et al. 1993, Kalifa et al. 2004b, Yang et al. 2005), but the different homologues so far identified have different expression patterns, which are organ-specific (Maskin et al. 2001, Frankel et al. 2006). They are small enough to enter the nuclear pore and some, but not all so far identified ASRs, possess a C-terminal nuclear localization signal sequence. However, this signal is neither a sufficient nor exclusive condition for the targeting of the protein to the nucleus. Indeed, regardless the presence of a nuclear targeting signal, most ASR proteins are localized in the cytosol and the nucleus, and some are able to bind to DNA (Carrari et al. 2004). Their role as transcription regulators was first evidenced in grape (Çakir et al. 2003) and confirmed in tomato (Kalifa et al. 2004a, Rom et al. 2006).

Indeed, the tomato ASR1 (SIASR1) is a DNA binding zinc-dependent protein that preferentially binds to the $C_{2\text{-}3}$(C/G)A sequence (Kalifa et al. 2004a). The potato ci21A/Asr1 plays also a role in the control of hexose transport in heterotrophic organs, since when overexpressed, the tubers display reduced levels of plasma membrane *HT* mRNAs and consequently lower glucose uptake rates (Schneider et al. 1997, Frankel et al. 2007).

Recent data indicate that SIASR1 competes with *ABI4* transcription factor for binding the promoter *CE1 cis*-acting element, and that the overexpression of *SIASR1* in *Arabidopsis* results in an *abi4* phenotype (Shkolnik and Bar-Zvi 2008). *ABI4* transcription factors are involved in ABA sensitivity in *Arabidopsis* seedlings and many *abi4* allelic mutations were discovered while screening for mutants impaired in sugar and salt signalling, suggesting that the ABI4 protein is a cross-road between these pathways (Finkelstein et al. 1998, Quesada et al. 2000, Niu et al. 2002, Barrero et al. 2006). Indeed, a common feature for all ASR proteins is their response to ABA.

In grape suspension cultured cells, *VvASR* expression is strongly enhanced by the addition of ABA. In ripening berries, ABA and sugars accumulate simultaneously after véraison and it is tempting to associate the high expression of *VvASR* at this time with the presence of both compounds. One should keep in mind however, that the expression patterns displayed by *VvASR* and *VvHT1* in grape berry along ripening are strictly opposite to each other, which, together with the role of VvASR on *VvHT1*'s transcription, can consti-

tute a brain-teasing problem. Interestingly, the potato ASR *ci21A/Asr1* (orthologue of tomato *Asr1*) expression is also negatively correlated with *HT* mRNAs accumulated along fruit maturation (Frankel et al. 2007).

However, it is important to recall that positive effect of VvASR on *VvHT1* transcription was demonstrated in tobacco, a heterologous system where the protein may not have been in contact with its natural partners. Furthermore, in the light of recent results (Shkolnik and Bar-Zvi 2008), it is possible that *VvASR*, as *SlASR1*, is able to compete with other transcription factors for preferred promoter binding sites. Eventually, additional roles, such as a modulation of DNA topology at the image of non-histone chromosomal proteins in response to water and salt stresses, as well as a protective role against water loss cannot be ruled out. ASRs are members of hydrophilins proteins, and enhance water retention once over-expressed, due to their highly hydrophilic nature (Gilad et al. 1997, Koag et al. 2003, Carrari et al. 2004, Kalifa et al. 2004b, Yang et al. 2005, Frankel et al. 2007, Maskin et al. 2007).

Additional connections with the sugar signalling pathways are demonstrated mostly in *Arabidopsis* with hormones, such as auxins and gibberellins (GAs), cytokinins, ethylene and brassinosteroids. The connection occurs either through the allelic mutation of a gene or the involvement of common *cis*-acting elements: the *AMYBOX2* (*TATCCA*) sequence is also involved in GAs sensitivity of the *α-Amy3* gene encoding for an amylase in rice (Gubler and Jacobsen 1992). The *DRE*-related element (Drought Response Element) conferred glucose-, ABA- and water stress response (Seki et al. 2007). Recent data reported by Li and co-workers (2006) propose a detailed study of *cis*-acting elements that confer gene response to glucose and ABA or light signalling as well as transcription factors involved in *A. thaliana.* Indeed, their microarray studies demonstrate the close connection between these pathways at the levels of promoter, *trans*-acting elements and gene expression patterns.

In *A. thaliana*, the *hls1* mutant is affected in both sugar and auxin signalling (Ohto et al. 2006). In grape berry and tomato fruit, an early treatment with auxin delays the accumulation of both sugars and secondary metabolites (Cohen 1996, Davies et al. 1997). Besides its positive effect on anthocyanin production, ethylene induces the expression of the sucrose transporters *VvSUC11* and *VvSUC12* and sugar accumulation in grape (Chervin et al. 2006). Furthermore, this climacteric-associated hormone upregulates the alcohol dehydrogenase gene, allowing the further development of aroma and participates in the control of berry acidity (Chervin et al. 2004, Tesnière et al. 2004). Some *A. thaliana* mutants affected in the perception (Zhou et al. 1998) or the transduction of ethylene, are hypersensitive to sugars, pointing to the existence of negative interactions between both signal transduction pathways (Gibson et al. 2001, Leclercq et al. 2002, Yanagisawa et al. 2003).

In tomato, the *ripening-inhibitor* (*rin*) mutation impairs the typical ripening-associated increase in ethylene production that occurs during climacteric fruit maturation and the fruit does not ripen. The mutation affects a gene encoding for a MADS-box transcription factor. Such proteins are widespread in all eukaryotic kingdoms and involved in growth and developmental processes. In plants, they were first characterized in the context of floral initiation and meristem differentiation, and recently implicated in developmental, non-hormonal control of fruit ripening (Vrebalov et al. 2002, Giovannoni 2004). In grape, although many genes encoding MADS-box proteins are expressed in flower, some are found in the fruit: *VvMADS5* is expressed during early berry development while *VvMADS1* and *VvMADS4* expression is strong throughout development (Boss et al. 2001, 2002, 2003, Chatelet et al. 2007). However, there is no information available about its putative involvement in ethylene signalling.

Processes like the accumulation of sugars, the metabolism of organic acids, the synthesis of aroma compounds and the accumulation of anthoyanins in the skin of grape appear to be controlled by brassinosteroids (BRs; Symons et al. 2006). The role of these hormones in both climacteric and non-climacteric fruits ripening processes is also evidenced. Indeed, application of these compounds promotes tomato fruit and grape berry ripening, increases sugar levels and decreases acid contents in both species. They also enhance the accumulation of lycopene in the pericarp of tomato fruit and of anthocyanins in grape berry (McMorris 1997, Vidya Vardhini and Rao 2002, Symons et al. 2006, Pilati et al. 2007). In tomato however, their effect on fruit ripening could be indirect, through the increase of ethylene levels (Vidya Vardhini and Rao 2002). In grape, there is a dramatic increase of some BRs at the onset of ripening (Symons et al. 2006).

The characterization of pleiotropic mutations also provides clues regarding the complexity of inter-connections of sugars, hormonal and environmental transduction pathways. Besides being involved in sugar signalling, the *prl1* (Németh et al. 1998), *gin2* (Moore et al. 2003), *ctr1* (Gibson et al. 2001, Leclercq et al. 2002) and *bls1* (Laxmi et al. 2004) mutations are also implicated in hormonal and environmental responses. The discovery that a SNF1-Related kinase complex is also differentially regulated by ABA and GAs provides strong evidence for a link between hormonal and sugar-sensing pathways controlling seed development, dormancy, and germination in tomato (Bradford et al. 2003).

5. PERSPECTIVES

The characterization of mutants provided considerable information allowing the understanding of major developmental and physiological processes by

functional genomics in *A. thaliana*. Molecular and genetic understanding of fleshy fruits maturation also made significant progress thanks to tomato mutants affected in fruit set and maturation. However, information regarding non-climacteric fruits is still scarce.

The characterization of natural and artificial mutants affected in berry development and/or ripening would provide an important tool for studying the molecular processes involved. For instance, the natural mutant Pinot Meunier *Vvgai* results in a dwarf and a short-cycling phenotype. Due to its short-cycling and its reduced size, this mutant, which is not affected in berry growth, is a promising tool to accelerate transgenic approaches on genes controlling berry development (Boss and Thomas 2002).

The grape mutant *fleshless berry* (*flb*) is affected in many aspects and constitutes an interesting model. Indeed, this loss-of-function mutation alters flesh development without impairing the production of normal number and fully developed seeds. The final volume of these berries is reduced to 10 % of control values, whereas softening of the pseudo-flesh and colour change are not affected. Malate accumulation in the mutant is reduced at green stage compared to the wild-type; sucrose contribution to the total sugar of the berry is increased in the ripe mutant whereas hexoses are mostly accumulated in the wild-type (Fernandez et al. 2006). The mutation promotes the expression of several genes related to ripening and/or to stress and impairs the expression of several regulatory genes. The *fleshless berry* mutant appears then a good model to identify genes putatively involved in the early development of grapevine fruit (Fernandez et al. 2007).

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ALCOHOL DEHYDROGENASE GENES & PROTEINS IN GRAPEVINE

C. Tesniere & P. Abbal

INRA, UMR SPO 2 Place Viala, F-34060, Montpellier Cedex 1, FRANCE

1. INTRODUCTION

Alcohol dehydrogenase (ADH; alcohol: NAD oxidoreductase; EC 1.1.1.1) is a dimeric-zinc enzyme which catalyses the inter-conversion of acetaldehyde to ethanol, using NAD/NADH as a cofactor. This is the terminal step of glycolysis, leading to fermentative metabolism in anaerobic conditions. In this context, the evolution of this enzyme's activity, and gene expression have been widely investigated in response to anaerobiosis in the plant kingdom, especially in organs such as roots, tubers, seeds and fruit (Kadowaki et al. 1988, Matton et al. 1990, Sun Chen and Chase 1993, Millar et al. 1994, Ke et al. 1994, Chung and Ferl 1999). ADH activity is generally low under normal oxygen tension, but strongly increases when the absence of oxygen leads to ethanolic fermentation. It appears to play an important role in the plant response to anaerobiosis, as ADH induction was generally detected in organs adapted to this stress. In maize, Gerlach et al. (1983) showed that ADH is fundamental to anaerobic survival for ADH null mutants. Paul and Ferl (1991) described the enhanced transcription of two genes responsible for an increased ADH synthesis. During oxygen deprivation, the assumed role of ADH is to reduce toxic acetaldehyde to ethanol and to recycle NADH to NAD, thereby allowing anaerobic glycolysis to be used as an energy-generating pathway. It could also be involved in cytoplasmic homeostasis, as the level of ADH activity is tightly regulated by pH, and as uncontrolled cytoplasmic acidosis is lethal (Roberts et al. 1984). ADH has also been implicated in the response to a wide range of other stresses, elicitors and also to abscisic acid (Christie et al. 1991, Constabel et al. 1990, Jarillo et al. 1993, Matton et al. 1990, de Bruxelles et al. 1996, Peters and Frenkel 2004).

Although ADH has for a while been considered as a stress-response marker only, there is increasing evidence today that it is also part of the normal

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developmental program of organs in aerobic conditions. ADH activity has been reported in germinating seeds (Conley et al. 1999), pollen (Gregerson et al. 1993, Ingersoll et al. 1994, Tadege and Kuhlemeier 1997, van Eldik et al. 1997), and stems of tree (Kimmerer and Stringer 1988). Changes in ADH activity or in *ADH* gene expression have also been reported during the normal ripening process of several fruits (Roe et al. 1984, van der Streaten et al. 1991, Sun Chen and Chase 1993, Ke et al. 1994, Longhurst et al. 1994, Bonghi et al. 1999, Speirs et al. 1998 2002, Echeverria et al. 2004, Manriquez et al. 2006). Finally, other investigations on grapevine have shown that alcohol dehydrogenase could also be a fruit-ripening related marker (Tesniere and Verries 2000).

All these data indicate a central role for ADH both in stress survival and in the development of various organs. The apparent ubiquitous response of ADH to abiotic and biotic stresses should however be related to the fact that in plants, ADH generally belongs to a small multigene family leading (in diploids) to the expression of two or three members (Dennis et al. 1984, Dennis et al. 1985, Matton et al. 1990, Gregerson et al. 1993, Longhurst et al. 1994, Morton et al. 1996). Stress response could thus result from differential expression of *ADH* isogenes. In fact, some studies have shown that *ADH* isogenes exhibited distinct spatial and/or temporal expression in response to stress or in developing organs (Bicsak et al. 1982, Gregerson et al. 1991, Gregerson et al. 1993, van Eldik et al. 1997).

Literature data on ADH in grapevine is presented here, with emphasis on molecular characterisation of *ADH* genes, changes in transcript expression pattern in different organs, in developing berries, regulation of this gene expression by abiotic factors or by transcription control analysis and finally a transgenesis approach to investigate the importance of the ADH function in leaves and in developing berries.

2. MOLECULAR CHARACTERIZATION OF *ADH* GENES AND PROTEINS FROM GRAPEVINE

The initial molecular characterisation of the *ADH* genes from grapevine was performed from the screening of a grapevine genomic DNA library and obtaining of full-length cDNA by 5′ and 3′ RACE strategies (Sarni-Manchado et al. 1997, Tesniere and Verries 2000, Verries et al. 2000). This approach resulted in the identification of three genes: *VvADH1*, *VvADH2* and *VvADH3* whose description has been previously detailed (Tesniere and Verries 2001). Very recently, the knowledge of the complete sequence of the grapevine genome (Jaillon et al. 2007) allowed us to reconsider this gene family in order to analyze whether any other *VvADH* was present on the grapevine genome.

To search databases for *VvADH* sequences, a blast with the set of the already known *VvADH* genes on NCBI resources was performed. Then, we checked whether characteristic motifs involved in NAD coenzyme, ethanol substrate, catalytic site and conserved amino acids among ADHs were present in all these sequences, retaining only sequences belonging to the medium-chain dehydrogenase protein superfamily. The sequences recovered were then blasted against the Genoscope blast server for *Vitis vinifera* (http://www.cns.fr/cgi-bin/blast_server/projet_ ML/blast.pl), resulting in several trace names corresponding to the best blast hits on the query sequence. We identified 3 additional genes, bringing up to 6 the total number of *VvADH* belonging to the *Vitis vinifera* genome. This number is high compared to the 2 or 3 *ADH* genes generally described in other plant species. Additional sequences very closely related to these 6 genes were also found, but variations could eventually be linked to differences between cultivars or to the process used to obtain these sequences (using polymerase compared to genomic or cDNA clones directly sequenced). Table 1 summarises these different data.The genetic distribution of the six members of the alcohol dehydrogenase family on the grapevine chromosomes was performed with cns software (http://www.genoscope.cns.fr/blat-server/cgi-bin/vitis/webBlat). *VvADH1* and *VvADH3* were both found on chromosome 18, whereas the other genes *VvADH2*, *VvADH4*, *VvADH5* and *VvADH6* were anchored on chromosome 4 (Fig. 1). Interestingly, these genes were very closely located on this chromosome, as well as closely related (protein identity being higher than 96%). Moreover, the organization of the phylogenetic tree (Fig. 2) suggests that several duplication events occurred in the grapevine genome, especially between VvADH1 and VvADH3 on one hand, and between *VvADH4* and *VvADH5* on the other hand. Similar duplication events in *ADH* genes have also been detected in other plant groups (Sang et al. 1997, Small and Wendel 2000, Fukuda et al. 2005).

Table 1. Characteristics of the *VvADH* genes and proteins from *Vitis vinifera*.

	GB acc. num.	Cultivar	%	pI	MW
VvADH1	CAO60891	Pinot noir clone PN40024	99.7	5.85	41 279
	CAN98099	Pinot noir clone ENTAV 115	99.4		
	AF195867	Perlette	99.2		
	AF194173	Danuta	99.1		
VvADH2	CAN68504	Pinot noir clone ENTAV 115	100	5.97	41 122
	AF19474	Danuta	99.5		
	AF195866	Perlette	99.2		
VvADH3	CAO60889	Pinot noir clone PN40024	100	6.76	41 241
	AF194175	Danuta	100		
VvADH4	CAO46435	Pinot noir clone PN40024	100	5.97	41 280
VvADH5	CAO46433	Pinot noir clone PN40024	100	5.85	41 241
VvADH6	CAO46436	Pinot noir clone PN40024	100	5.97	41 257
	CAN68505	Pinot noir clone ENTAV 115	99.2		

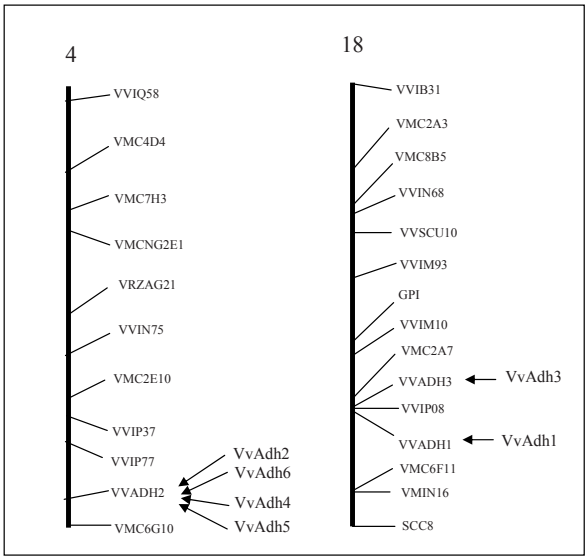

Fig. 1. Genetic distribution of the *Vitis vinifera ADH* gene family. Chromosomes are reprint from genetic map available at http://urgi.versailles.inra.fr/cmap/cgi-bin/cmap/map_set_info?_map_type_acc=genetic. The approximate location of the gene is indicated by an arrow followed by the name of the gene.

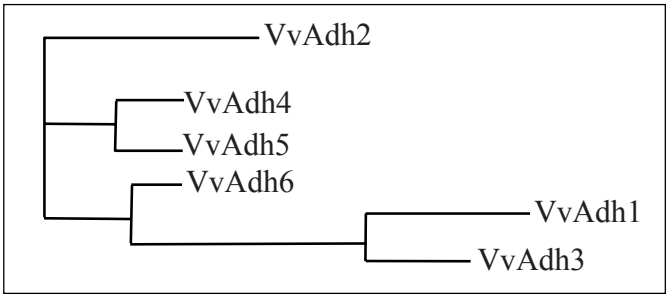

Fig. 2. Boostrap consensus tree from the parsimony analysis showing the phylogenetic relationship among the six *Vitis vinifera ADH* sequences (*VvADH*).

3. EXPRESSION OF *ADH*

Expression of *VvADH1* and *VvADH2* was examined by real-time quantitative RT-PCR in grapevine organs and during the development of grape berries (Tesniere et al. 2006a).

3.1. Expression of ADH in grapevine organs

RNA samples were extracted from young roots, buds (samplings 4-12, green buds taken from flowering axis; sampling 5-8, latent buds taken during winter 1 month before burst), tendrils (at 4 developmental stages from opening buds up to mature tendrils), inflorescences (stage 7, immature inflorescence emerging from buds; stage 17, mature inflorescence with all flowers formed and separated), flowers (at flowering time) and callus (from *in vitro* grown Portan cultivar embryogenic tissues corresponding to undifferentiated embryogenic masses, stage L, and globular to advanced torpedo embryo-like structures, stage H). Significant level of *VvADH1* transcripts were observed in tendrils as well as in inflorescence at all stages of development, whereas expression was rather limited in the other organs investigated (Fig. 3A). For *VvADH2*, transcript expression level was very low, but no organ specificity could be observed (Fig. 3B).

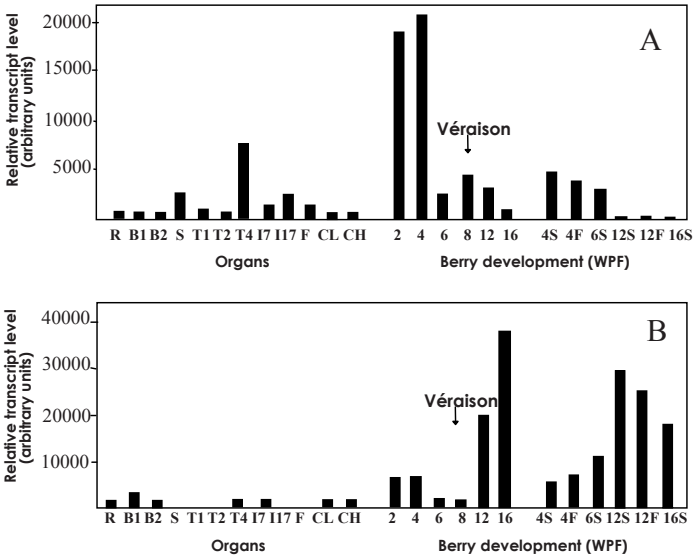

Fig. 3. Transcript profiling of *VvADH*s in various Cabernet Sauvignon grapevine organs and during berry development. Total RNA was isolated from roots (R), buds (B1: buds 4-12; B2: buds 5-8), shoots (S), tendrils at different stages of development (T1, T2 and T4), immature and mature inflorescences (I7 and I17), flowers (F), callus from undifferentiated to globular stages (CL and CH) and two to 16 weeks post-flowering (WPF) berries. *VvADH* transcript levels were determined by quantitative real-time PCR analysis. Results were normalized to EF1-α transcript level used as endogenous reference and expressed as arbitrary units; A: *VvADH1*, B: *VvADH2*. Véraison is indicated by an arrow.

3.2. Expression of ADH in developing grape berries

Expression of *VvADH1* and *VvADH2* was also examined in developing seedless berries from 2 to 16 weeks post-flowering (WPF), and in skin and flesh of berries at various developmental stages (Fig. 3). Transcripts of *VvADH1* accumulate predominantly during stage I of berry development in Cabernet Sauvignon, with a maximum observed 4 WPF, and with a significant lower level thereafter. On the contrary, *VvADH2* transcript levels were relatively high in ripening berries even if transcripts were also observed in young berries. For both isogenes, the levels of transcript accumulation were higher in the berry than in other organs, and transcript levels in berry skin and flesh were similar. Although both tissues result from very different developmental processes and have strong metabolic and functional differences, it is interesting to notice that no effect at the transcript expression level could be observed.

From these data, it is clear that except for tendrils at stage 4 for *VvADH1*, both *VvADH*s are only weakly expressed in the organs investigated, while displaying a very high expression level in berries and showing complementary expression patterns.

4. REGULATION OF *ADH* EXPRESSION

Several studies have been performed to get more insight into the factors controlling the expression of the *VvADH* in grapevine. Search for external factors like ethylene signalling, and for intrinsic factors such as regulation from promoters and 3′-ending regions were performed. Below is a review of these data.

4.1. Involvement of ethylene signalling

In grape berries, the signal that triggers fruit ripening and their transduction pathway still remain largely unknown. Fruits which display a respiration peak and an ethylene increase at the beginning of ripening are classified as climacteric but grape, which does not accumulate ethylene and lacks a respiration peak, classifies as non-climacteric fruit (Coombe and Hale 1973). However, fruit ripening and especially berry development is considered to involve both ethylene-dependent and ethylene-independent processes (Lelievre et al. 1997, Chervin et al. 2004).

Experiments have been performed to evaluate whether the ethylene signal transduction pathway could be involved in the control of *ADH* expression in grapevine (Tesniere et al. 2004). The effect of 1-methycyclopropene (1-MCP),

an inhibitor of the ethylene receptors, and of 2-chloroethylphosphonic acid (CEPA), a chemical that releases ethylene when applied to plants, was evaluated on the ADH enzyme activity and *VvADH* transcript levels in a series of berry development and in suspension cells of *Vitis vinifera* (Fig. 4). For berries, a single treatment with 1-MCP had only an effect on enzyme activity treated 10 WPF (data not shown), whereas repeated treatments resulted in a significant reduction of enzyme activity at 9 WPF (Fig. 4A). Concerning expression at the transcript levels, no significant changes in *VvADH1* and *VvADH3* levels were observed by specific Northern blotting in MCP-treated berries (data not shown). By contrast, increase in *VvADH2* expression after véraison and during ripening was significantly reduced after 1- MCP treatment (Fig. 4B). For suspension cells 24 h CEPA-treated samples exhibited a significant increase of ADH activity and *VvADH2* transcript level (data not shown). ADH activity, but not *VvADH2* expression, was also enhanced when associated with a nitrogen treatment. Conversely 1-MCP-treated cells had significantly reduced ADH activity and *VvADH2* transcript expression level. All these results indicated that *VvADH2* transcription is, at least partially, responsive to ethylene. In berries, ethylene effect was development- and dose-dependent, suggesting some changes in the regulation of ethylene signalling during fruit development. In cells, ethylene signalling was more efficient by increasing *VvADH2* transcription, when associated with low oxygen treatment. In fact ethylene appears to be implicated in the triggering of a number of responses to oxygen deficiency (Morgan and Drew 1997), and is required, although not solely responsible, for the induction of ADH during hypoxia (Peng et al. 2001). In conclusion, *ADH* could be regulated by ethylene in non-climacteric fruit, as this has also been shown in climacteric fruits, such as melon (Manriquez et al. 2006).

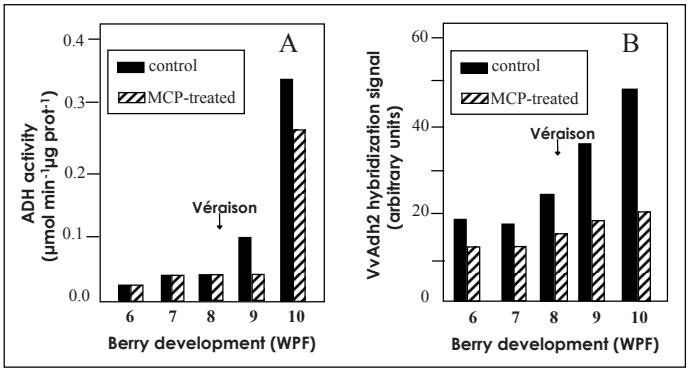

Fig. 4. (A) Specific ADH activity and (B) transcript profiling of *VvADH2* in Cabernet Sauvignon berries treated with 1-MCP at different stages of fruit development. Northern blots were probed with specific *VvADH2* 3′UTR probes. The signals were quantified and normalized in each lane to the corresponding ribosomal 18S signal. Véraison is indicated by an arrow.

As detailed below, presence of putative ethylene responsive elements (ERE) motifs on *VvADH2* promoter (Fig. 5) is another element suggesting that expression of this gene could indeed be sustained or amplified by ethylene.

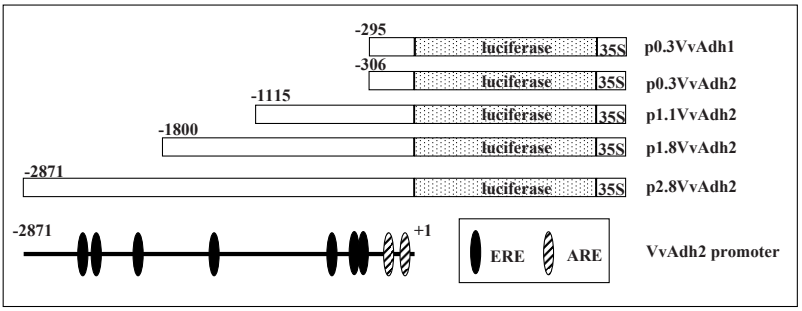

Fig. 5. Transcriptional-fusion constructs, including successive *VvADH2* 5'-deletions, used for transient analysis of the *VvADH1* and *VvADH2* promoter expression levels in suspension cells of Cabernet Sauvignon. Promoter sequences were fused to the luciferase coding region and the 35S polyA region. Location of ethylene responsive elements (ERE) and anaerobic responsive elements (ARE) are indicated on the diagram representing the *VvADH2* promoter.

4.2. Functioning of ADH promoters and 3´ ending regions

To further investigate which factors regulate the expression of *VvADH* genes, different experiments, including *VvADH* promoter and 3´ ending regions analyses, have been performed on suspension cells transiently expressing reporter genes under the control of different promoters, with or without different terminator sequences.

4.2.1. *VvADH* promoters

Sequence analyses showed distinct promoter organizations between *VvADH1* and *VvADH2* that could potentially relate to the differences found in isogene expression patterns during fruit development (Tesniere and Verries 2001). To evaluate the importance of some promoter regions in the ADH functioning, biolistic experiments were performed on cell suspensions in order to induce transient gene expression (Fig. 5). In air and under anaerobiosis, the leader version of the *ADH* promoter did not modify ADH activity in the grapevine cells (Fig. 6). Data were different concerning the luciferase reporter gene activity. In the air, proximal regions of VvADH1 exhibited a very low constitutive activity, whereas VvADH2 activity was constitutively higher (Torregrosa et al. 2002, Verries et al. 2004).

The promoter of *VvADH2*, but not of *VvADH1*, responded to anaerobiosis, suggesting a different mode of regulation. In addition, for *VvADH2* pro-

moter, differences in expression patterns were observed between 0.3 and 1.1 kb versions, indicating that the remaining 0.7 kb region was important, both in air and under nitrogen, for enhancing gene transcription (Fig. 6). Sequence organization variability between *VvADH1* and *VvADH2* could be involved in the differences between responses to anaerobiosis of the two *VvADH* promoters. In addition, data showed that the presence of anaerobic responsive elements (ARE) on the *VvADH1* and *VvADH2* promoters were necessary, but not sufficient, to confer anaerobiosis response ability. To evaluate the involvement of the two ARE elements (present in the proximal region of the *VvADH2* promoter) in the nitrogen responsiveness, mutations of these motifs were performed on the 0.3 and 1.1 kb construct versions of the *VvADH2* promoter. Results suggested that the two motifs are indeed involved in the nitrogen responsiveness, although a complete suppression of the promoter activity was never observed (data not shown). This suggests that other *cis*-elements might also be implicated in the anaerobic response.

Complementary experiments were performed to evaluate whether the *VvADH2* promoter was responsive to ethylene. Effect of a CEPA treatment was evaluated on the 2.8 kb version of the *VvADH2* promoter. Compared to controls, promoter expression response to air and anaerobiosis was significantly higher in CEPA-treated cells, at both ADH and luciferase activity levels (Fig. 6). Thus, ethylene has a significant effect on the functioning of the *VvADH2* promoter, corroborating the results obtained at the *VvADH2* transcript expression level presented above.

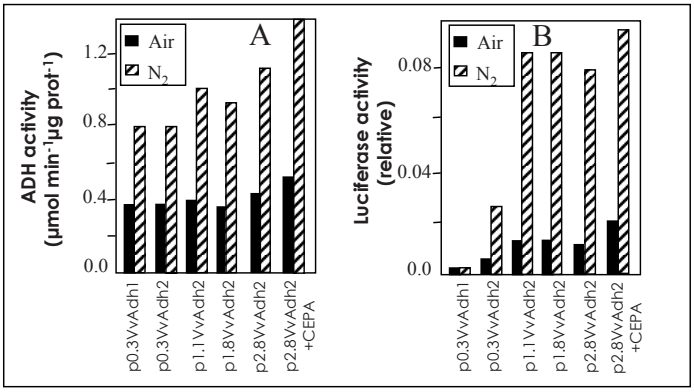

Fig. 6. Effects of the promoter types before and after CEPA treatment on (A) ADH activity and (B) on transient expression levels of chimeric genes evaluated by luciferase activity measured in Cabernet Sauvignon suspension cells kept 24h in air or under nitrogen. Each luciferase value was referred to its GUS value to obtain luciferase relative activity. Data are mean value of six bombardments within three independent experiments.

4.2.2. 3´- Ending regions

3´-ending regions also participate in gene regulation, in particular through 3´-end synthesis and stabilisation. Graber et al. (1999) suggested that variation in the 3´-end organization could lead to a modulation of gene expression. Analysis of the signal organisation of the *VvADH* 3´ending-regions (Fig. 7) allowed the identification of highly conserved motifs: the AAUAAA polyadenylation signals (Sheets et al. 1990) in *VvADH1*, *VvADH2* and *VvADH6*, and a downstream, less conserved U-rich element (Hart et al. 1985, Sanfaçon et al. 1991, Rothnie et al. 1994) for *VvADH1* and *VvADH2*. To evaluate the importance of these regions in *ADH* gene expression, different expression cassettes containing luciferase reporter gene under the *VvADH2* or CaMV35S promoters were designed and fused to different 3´UTRs (*VvADH1*, *VvADH2*, *VvADH6* and CaMV 35S), or without any terminator (Tesniere et al. 2005). First of all, a strong down-regulating effect on the *VvADH2* promoter activity, but not on the CaMV 35S, was observed in the absence of a terminator sequence (Fig. 8A). In presence of 3´-ending regions, the nature of the sequence was shown to influence the expression cassette activity. *VvADH1* and *VvADH2* terminators have similar effects in air, as well as under anaerobiosis, where luciferase expression was strongly increased. However, the highest expression level was obtained with the *VvADH6* 3´ending region (Fig. 8B). Thus these results indicated that the presence of 3´-ends was necessary for a *VvADH2* promoter activity, while the origin of the terminator sequence only modulated the expression level. The fact that the type of 3´-ending region influences reporter gene activity could be the reflect of some interactions between the promoter and 3´end regions.

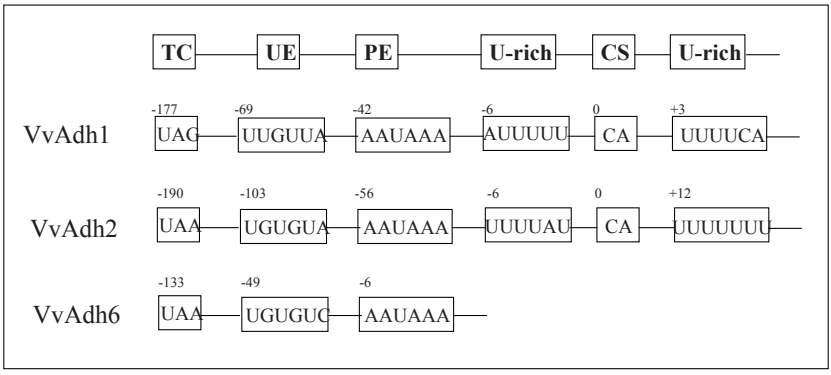

Fig. 7. Organization of the *VvADH* 3´end regions as compared to the Graber *et al*. (1999) model of 3´end processing signal. TC: terminal codon, UE: upstream element, PE: polyadenylation element, U-rich: uridine-rich region, CS: cleavage site. Reprinted from Tesniere et al. (2005) Vitis 44:1-4, with permission from Vitis.

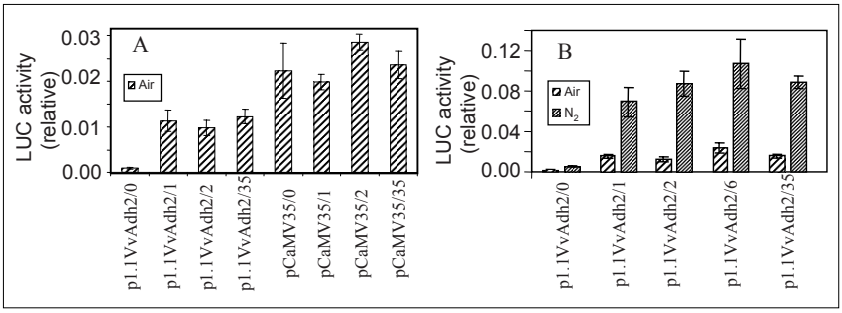

Fig. 8. Effect of different 3′-ends under *VvADH2* or CaMV35S promoters evaluated as relative luciferase activity after transient transformation. (A) Activities were measured in Cabernet Sauvignon suspension cells kept for 24 h in air or (B) in air and under nitrogen. Data are mean values of 6 bombardments within two independent experiments ± SE. Reprinted from Tesniere et al. (2005) Vitis 44:1-4, with permission from Vitis.

5. IMPORTANCE OF THE ADH FUNCTION IN GRAPEVINE

To evaluate the function of ADH in grapevine, genetic transformation was used as a tool to get more insight into the role of the *VvADH2* gene. Grapevine somatic embryos were transformed with gene constructs containing a sense or antisense orientated *VvADH2* cDNA, under the constitutive cauliflower mosaic virus 35S promoter. In the transformed plants, alterations in ADH levels did not result to any apparent effect, and no significant changes in phenological characteristics or plant phenotype were observed. In fact, the transgenic plants exhibited growth profiles comparable to control untransformed plants and field-grown plants (Torregrosa et al. 2008). The expression of the *VvADH* isogenes, the content in sugars, phenolic and volatile compounds, and the proteome were investigated in the *ADH* transformants (Tesniere et al. 2006b, Sauvage et al. 2007, Torregrosa et al. 2008).

5.1. In leaves

Plants transformed with either antisense orientation or the *ADH*-less construct displayed low but detectable constitutive ADH activity in leaves (Fig. 9A). Plants transformed with the sense-expressed transgene showed a significantly higher (100-fold) ADH activity when compared to the control. Transcription of the *VvADH2* was coherent with these results, with higher abundance of transcript in plants over-expressing *ADH*, and reduced in antisense transfor-

mants (Fig. 9B). Glucose and fructose contents were similar in control (C), sense (S) and reverse (R) lines, but sucrose content was significantly lower (by almost 90%) in S lines (data not shown). This last data suggests some link between the increase in ADH activity and the activities of enzymes related to sucrose catabolism.

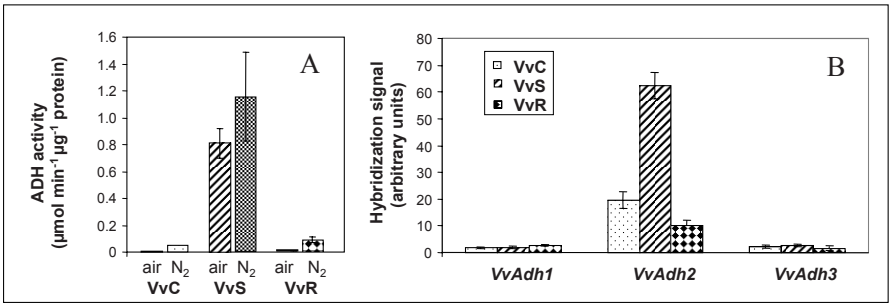

Fig. 9. Effect of genetic manipulation of ADH levels in leaves from transformed grapevine. VvC: control; VvS: sense *ADH* line, VvR: antisense *ADH* line (bars = SD. (A) ADH specific activities in air and under anaerobiosis. Analyses were performed in triplicate. (B) *VvADH* isogene expression. Hybridizations were performed with *VvADH1*, *VvADH2* and *VvADH3* specific probes. Signals were normalized relative to the 18S ribosomal hybridization. Reprinted from Tesniere et al. (2006) J. Exp. Bot. 57:91-99, with permission of the Oxford University Press.

Among the phenolic compounds, significant changes were observed for *trans*-caftaric acid, quercetin 3-glucoside, quercetol-3-glucuronide and proanthocyanidin mDP (data not shown). For this last compound, a higher level was observed in the S line compared with the C line, suggesting that a polycondensation reaction between proanthocyanidin and acetaldehyde could have somehow occurred (Fulcrand et al. 1996) at a higher rate in transformants overexpressing *ADH*. This could have resulted in the decrease in free units and in the number of terminal units for this line; the opposite was observed in the R line.

Concerning other metabolites, the free fraction of volatile compounds did not vary between the lines (data not shown). In contrast, the glycosidically bound fraction was generally found to be more important in the overexpressing S line, compared to the C and R lines (Table 2). Among the most important changes observed, benzyl alcohol accumulated to high levels in the S line. This could be related to the specificity of the ADH enzyme for this substrate (Molina et al. 1986). Other important changes concerned the C_{13}-norisoprenoid class, with increase in compound contents of all classes generally 5 to 10-fold higher in the S line than in the C line. However, the largest variation (in opposite direc-

tions), in both S and R lines with the C line (Table 2) concerned the vomifoliol and 3,6-dihydroxy-megastigm-7-ene-9-one contents which exhibited respectively 141-fold increase in the S line, and 70-fold increase in the R line.

Table 2. Effect of genetic manipulation of ADH levels in transformed grapevine leaves on the composition and content of volatile compounds (relative to internal standard 4-nonanol) from the bound fraction. Mean of three repetitions and standard deviation (SD). Values with different characters are significantly different according to Newman-Keuls test ($P<0.01$). C: control; S: sense *ADH* line; R: antisense *ADH* line; ND: not detected. Reprinted from Tesniere et al. (2006b) J. Exp. Bot. 57:91-99, with permission from the Oxford University Press.

Components µg kg^{-1} FW	C		S		R	
	mean	SD	mean	SD	mean	SD
C6 compounds						
2-Hexenal	29.01^{b}	2.97	126.65^{a}	56.29	10.54^{b}	2.81
Hexenol	52.21^{b}	3.57	370.45^{a}	164.64	32.64^{b}	5.44
Miscellaneous						
Isopentanol	5.81^{b}	0.96	331.12^{a}	147.16	7.02^{b}	0.37
Terpenes						
Trans-furan linalool oxide	5.13^{b}	0.26	40.22^{a}	17.87	4.10^{b}	0.37
cis-Furan linalool oxide	6.43^{b}	0.40	35.06^{a}	15.58	5.46^{b}	0.81
Geraniol hydrate	81.58^{b}	6.12	481.12^{a}	213.83	76.96^{b}	13.23
Sum of terpenes	93.14	-	556.4	-	86.52	-
Shikimic derivatives						
Benzaldehyde	nd	-	206.20^{a}	113.50	nd	-
Methyl salicylate	65.09^{b}	4.00	902.94^{a}	401.31	56.95^{b}	7.95
Methyl benzyl methanol (isomer 1)	16.76^{b}	1.55	156.62^{a}	69.61	20.11^{b}	1.72
Benzyl alcohol	309.52^{b}	75.76	16600.25^{a}	7377.89	407.14^{b}	17.30
4-Methyl-1-phenylethanol	18.37^{b}	1.55	133.24^{a}	59.22	25.11^{b}	4.12
2-Phenylethanol	156.39^{b}	11.55	1315.41^{a}	584.63	172.04^{b}	13.25
Methyl benzyl methanol (isomer 2)	8.10^{b}	0.78	43.54^{a}	19.35	8.40^{b}	1.40
Methyl benzyl methanol (isomer 3)	12.82^{b}	0.73	81.97^{a}	36.43	15.31^{b}	3.19
Dimethyl- benzyl alcohol (isomer 1)	15.21^{b}	1.81	84.46^{a}	37.54	7.77^{b}	1.25
Dimethyl- benzyl alcohol (isomer 2)	5.13^{b}	0.70	31.57^{a}	14.03	2.00^{b}	0.22
Zingerone	63.98^{b}	4.26	571.04^{a}	253.80	76.05^{b}	8.15
Sum of shikimic derivatives	691.91	-	20248.57	-	821.85	-
Norisoprenoids						
3,4-Dihydro-3-oxoactinidol	50.52^{b}	3.71	337.82^{a}	150.14	59.55^{b}	8.02
3-Hydroxy-β-ionone	40.14^{b}	1.82	231.28^{a}	102.79	34.79^{b}	5.36
Megastigmane-3,9-diol	640.71^{b}	56.31	3914.77^{a}	1739.90	730.87^{b}	82.05
3-Oxo- 7,8-dihydro-β-ionol	216.39^{b}	13.86	1107.64^{a}	492.28	214.52^{b}	27.31
3-Oxo-5,6-epoxy-β-ionone	502.25^{b}	32.23	3652.70^{a}	1623.42	574.86^{b}	56.76
4,5-Dihydrovomifoliol (isomer 1)	320.35^{b}	12.00	2297.87^{a}	1021.28	320.46^{b}	6.70
4,5-Dihydrovomifoliol (isomer 2)	182.14^{b}	34.93	2318.57^{a}	1030.48	376.08^{b}	25.24
3,6-Dihydroxy-megastig-7-ene-9-one	218.15^{a}	45.76	40.06^{b}	21.50	2.95^{b}	0.21
Domifoliol	42.26^{b}	19.78	6068.26^{a}	2697.00	298.54^{b}	30.52
7,8-Dihydrovomifoliol	21.34^{b}	0.89	180.38^{a}	80.17	15.83^{b}	2.53
Sum of norisoprenoids	2234.25	-	20149.32	-	2628.45	-

These two compounds show structural similarities with ABA. Indeed, vomifoliol, which can be generated from an oxidative enzymatic cleavage of ABA (Winterhalter and Schreier 1995), is considered as an analogue of ABA and used in experiments for its similar effects on stomatal aperture (Stuart and Coke 1975, Artsaenko et al. 1995, Fuchs et al. 1999).

A proteomic approach was also performed to evaluate changes in leaf protein content from plants transformed for alcohol dehydrogenase activity (Sauvage et al. 2007). Quantitative analysis of proteins separated by two-dimensional electrophoresis was performed and spots displaying significant quantitative changes between C, S and R lines were selected using Student's test (Fig. 10). Selected spots were isolated from the gels, trypsin digested and further identified by MS or LC-MS/MS analyses. In Fig. 11 the identified proteins are presented, which are mainly of chloroplastic origin: four RUBISCO large subunits, one RUBISCO binding protein, two glutamine synthetase, one elongation factor Tu, one ATP synthase beta subunit, and one plastidic aldolase. Also, an UDP-glucose pyrophosphorylase, a mitochondrial aminomethyltransferase, a linalool synthase which comigrated with the protein identified as elongation factor Tu, an enolase comigrating with a glyceraldehyde 3-phosphate dehydrogenase, and a mixture of eight proteins among which a dehydroascorbate reductase, a chalcone isomerase, and a RUBISCO activase were also identified.

The results suggested that modification of ADH activity in the leaves of S lines resulted to an alteration of photosynthetic metabolism. Moreover, the increase of UDP-glucose pyrophosphorylase in S line can be related to drastic decrease in sucrose content observed in the same transgenics, as described earlier (Tesniere et al. 2006b). UDP-glucose pyrophosphorylase is positioned at the

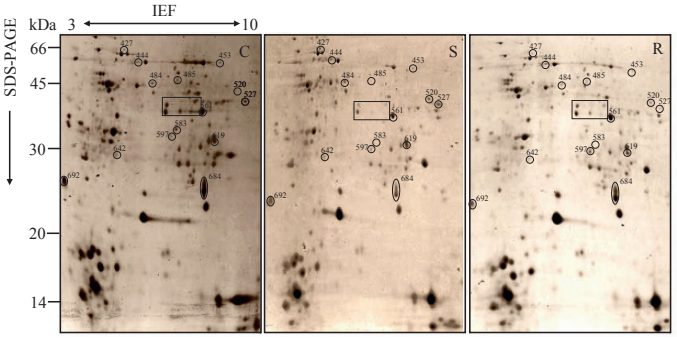

Fig. 10. Effect of genetic manipulation of ADH levels on proteins from transformed grapevine. Two-dimensional electrophoresis of proteins isolated from the control (C), sense-transformed (S), and antisense-transformed (R) plants. Gels were stained with Coomassie Brillant Blue G-250. Positions of proteins with significant changes between transformants are indicated by circles. The putative position of ADH is indicated by a rectangle. Reprinted from Sauvage et al. (2007) J. Agric. Food Chem. 55:2597-2603, with permission of the American Chemical Society.

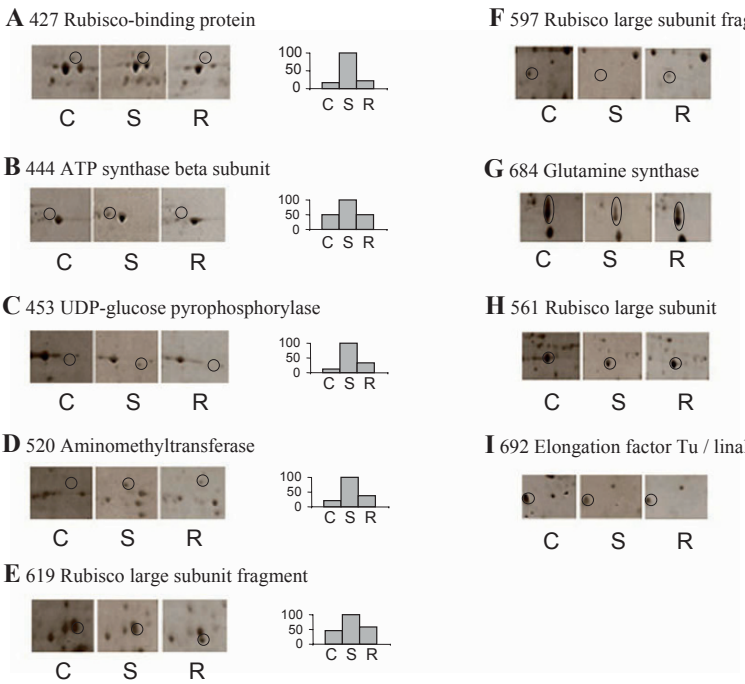

Fig. 11. Grapevine proteins whose levels were changed by the transformation and identified by MALDI-TOF: A to G, proteins whose levels were increased in leaves from S transformants (A, RUBISCO-binding protein; B, ATP synthase beta subunit; C, UDP-glucose pyrophosphorylase; D, glutamine synthetase; E, aminomethyltransferase; F and G, RUBISCO large subunits); H to K, proteins whose level decreased in leaves from S transformants (H, enolase and glyceraldehyde-3-phosphate dehydrogenase; I, plastidic aldolase; J, RUBISCO large subunit; K, glutamine synthetase); L and M, proteins whose level decreased in leaves from R transformants (L, RUBISCO large subunit, M, elongation factor Tu and linalool synthase). C: Control, S: sense-transformed, R: antisense-transformed. The level of change found within each spot is shown in bars on the right. The highest level found among transformants is shown as 100. Reprinted from Sauvage et al. (2007) J. Agric. Food Chem. 55, 2597-2603, with permission from the American Chemical Society.

crossroad of sucrose synthesis and breakdown and its enzymatic activity could be correlated to sucrose breakdown (Magel et al. 2001). The substrate/product of this enzyme is thus an important metabolite for carbohydrate metabolism in photosynthetic tissues. In addition, UDP-glucose is involved in synthesis of the carbohydrate moiety of glycoproteins and for other glycosylation reactions (Kleczkowshi et al. 2004). The higher content of UDP-glucose in S line is accompanied by the higher content of glycosylated volatile compounds observed in the S transgenics (Tesniere et al. 2006b). However, no protein directly related to the glycosidically bound fraction were identified in this study. This could be due to either the paucity of sequence data available for grapevine or to low pro-

tein content under the conditions studied.

5.2. In berries

To understand the function of *VvADH2* in fruit development, and evaluate whether ADH activity has some controlling effect on fruit ripening, *ADH* transformed plants were used to investigate the consequences of over- or under-expressed *ADH* on grape berry ripening. Biochemical, phenolic and volatile content analyses were performed in developing berries of these transformants (Torregrosa et al. 2008). Compared to control berries, *ADH* transcription and ADH enzyme activity levels were respectively enhanced or down-regulated in lines expressing sense or antisense *VvADH2* cDNA (Fig. 12). These results in fruits were coherent with those previously obtained in leaves, indicating the ectopic effect of the transformations. Concerning other analyses, only small differences between berries from the three lines were observed, in contrast to the previously obtained results in leaves from the same transformants (Tesniere et al. 2006b). For instance, no variations in total acidity, refractive index, phenolic and volatile compounds were observed between the three lines (data not shown), except some changes in mDP, which was significantly lower for the S lines. Surprisingly, no significant changes in volatile compounds (free and bound fractions) were evident. These results could suggest that different mechanisms may be involved in berries and leaves, especially concerning the variations in primary and secondary metabolism found in the leaves of the same transformants. The possibility exists of a tight control of metabolic changes in berries, even when repression of ADH induces variations in these organs.

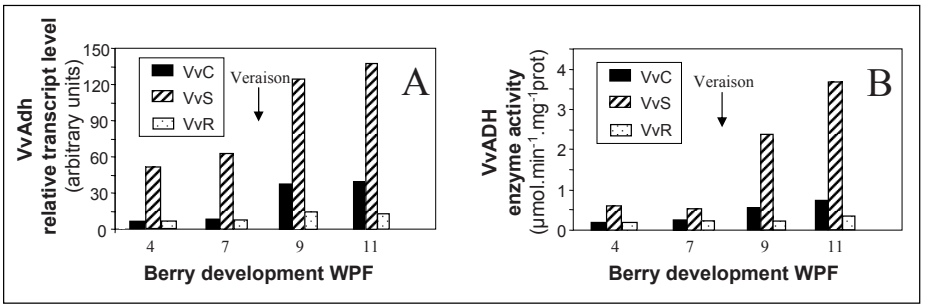

Fig. 12. *VvADH* transcript level (A) and VvADH specific activity (B) in developing berries from *ADH* transformed grapevine plants. VvC: Control; VvS: sense *ADH* line; VvR antisense *ADH* line.

6. CONCLUSIONS

This review provides a complete and up to date picture of the different data obtained so far on the genes encoding for ADH in grapevine. It highlights new aspects of the *VvADH2* gene expression controlled by the ethylene signalling pathway in a non-climacteric fruit, and by analyses of (i) some *cis*-elements present on the *VvADH* promoters and (ii) on the *VvADH* terminators. Finally, it illustrates that grapevine transgenics can provide useful tools for getting more insight on the function of a gene. Over-expression or down-regulation of *VvADH* resulted to unexpected results at the primary and secondary metabolism levels in leaves and berries. These results show the originality of the grapevine model, even when focusing on an old well-known marker in other plants, such as alcohol dehydrogenase.

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7

ADVANCEMENTS IN NITROGEN METABOLISM IN GRAPEVINE

K.A. Loulakakis[2], J.F. Morot-Gaudry[3], C.N. Velanis[1], D.S. Skopelitis [1], P.N. Moschou [1], B. Hirel [3] & K.A. Roubelakis-Angelakis[1]

[1]Department of Biology, University of Crete, P.O. Box 2208, 71 409 Heraklion, Crete, GREECE

[2]Department of Horticulture, Technological Educational Institution of Crete, 71 500 Heraklion, Crete, GREECE

[3] Unité de Nutrition Azotée des Plantes, Institut Jean-Pierre Bourgin, UR 511, INRA Centre de Versailles-Grignon, R.D. 10, F-78026 Versailles Cedx, FRANCE

1. INTRODUCTION

Nitrogen (N) plays the most important role of all soil mineral nutrients in plant growth and development. In grapevine, nitrogen is most likely to be deficient although it is the main fertilizer commonly applied to vineyards to increase productivity (Keller et al. 1998) and to influence grape juice composition (Ough and Bell 1980). Considerable new information has been obtained over the past twenty years on the uptake, translocation, distribution, partitioning, and storage of nitrogenous compounds in grapevines as well as new insights toward a better understanding on the regulation of the synthesis and degradation of amino acids and other nitrogenous compounds, and of the enzymes associated with these reactions. However, in spite of the widespread usage of nitrogen fertilization in vineyards, the physiological and biochemical effects of nitrogen on shoot and fruit growth, fruit bud initiation, flowering, fruit set, and crop yield are still poorly understood. Grapevines differ from herbaceous plants and many other woody plants in that they do not form terminal buds at the end of shoots, but they can continue to grow late into the season. The individual flower parts are formed after bud break, unlike most deciduous fruit trees.

Nitrate and ammonium ions are the most common forms of nitrogen available to plants in soils. The relative importance and concentration of each of these two ions depends on the genetic, developmental, and physiological status

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of each plant, as well as on soil properties, such as texture, structure, water content, and pH. Also, the metabolism and distribution of both nitrate and ammonium ions and the partition of their products in plants are multifactor-dependent processes. Some of these factors, such as light, temperature, nutrient species and concentration, may affect or even regulate the reactions of certain enzyme systems involved both in carbon and nitrogen primary assimilation.

There have been several reviews on nitrogen metabolism of woody plants, including that of Titus and Kang (1982) on apple, Kato (1986) on citrus, Korcak (1989) on blueberries and other calcifuges, on grapevine, Roubelakis-Angelakis and Kliewer (1992) and conifers, Canovas et al. (2007). Other general reviews on nitrogen metabolism in plants include Oaks and Long (1992), Oaks (1994), Lea and Ireland (1999). Also, information on the uptake, distribution, partitioning, redistribution and seasonal dynamics of nitrogen and on the amino acids and protein metabolism in grapevines has been reviewed by Conradie (1991), Wermelinger (1991), Williams (1991), Roubelakis-Angelakis (1991), Loulakakis and Roubelakis-Angelakis (2001).

During the past ten years, major advances in our understanding of the expression and regulation of the genes encoding for the enzymes involved in ammonium assimilation in plants in general, and grapevine in particular, have been achieved, and are presented in this Chapter, which is an updated version of a previous one (Loulakakis et al. 2001).

2. NITROGEN USE

Most plants, including grapevine, can effectively utilize either ammonium or nitrate ions. Nitrate assimilation is regulated by carbohydrate oxidation and associated with organic acid production. Consequently, nitrate can accumulate to relatively high concentrations without being detrimental to the plant (Forde and Clarkson 1999). On the other hand, the productivity of a species, when grown on ammonium salts, is related to its ability to detoxify ammonia (Britto and Kronzucker 2002), and depends directly on the availability of keto acids and indirectly on the supply of photosynthesis products. In contrast, nitrate is relatively innocuous, and plants can store high levels of the anion or they can translocate it from tissue to tissue without deleterious effect.

Nitrogen assimilation requires a complex series of biochemical reactions that are among the most energy-requiring reactions in living organisms. In nitrate assimilation, the nitrogen in NO_3^- is converted to a higher energy form in nitrite (NO_2^-), and then to a yet higher energy form ammonium (NH_4^+). It has been estimated that this process consumes nearly 15% of the plant's total energy production. Ammonium assimilation, on the other hand, consumes from 2 to

5% of total energy production (Oaks and Hirel 1985).

2.1. Reduction of nitrate

Nitrate is first taken up from the soil by means of specific transporters localized in the root cell membrane (Miller et al. 2007). Once inside the root, nitrate can be stored in the root vacuoles, reduced directly in the root cells, or translocated through xylem to the shoots and leaves for further use (Fig. 1). Several studies have shown that the proportion of nitrate reduced in the root or shoot depends, to a large extent, on the external nitrate concentration. At low concentrations, most of the nitrate can be reduced within the root tissues and translocated to the shoot as amino acids or amides. At higher concentrations of nitrate, assimilation in the roots becomes limiting and a higher proportion of nitrate is translocated and assimilated in the shoot (Sechley et al. 1992).

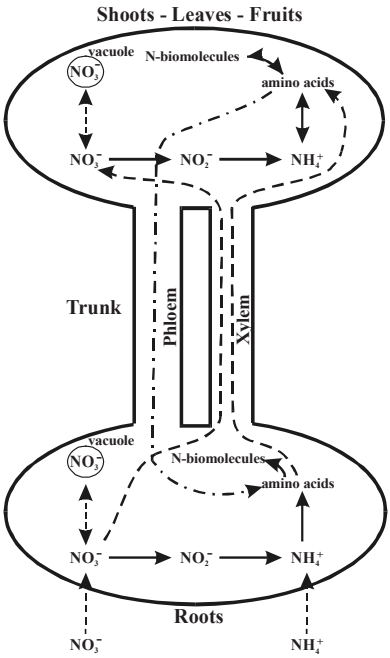

Fig. 1. Schematic presentation of the paths taken by nitrogenous species following their uptake in the plant root.

In plant cells, nitrate is reduced to ammonium by a two-step process. The first is the reduction of nitrate to nitrite catalyzed by the enzyme nitrate reductase [NR, EC 1.6.6.1. (NADH specific); EC 1.6.6.2. (either NADH or NADPH

specific) more prevalent in green algae; and EC 1.6.6.3. (NADH specific) present in molds]. The second being the reduction of nitrite to ammonium by the enzyme nitrite reductase [NiR, EC 1.7.7.1. (ferredoxin-dependent); EC 1.6.6.4. (either NADH- or NADPH-dependent)]. The reduction of nitrate to ammonium requires eight electrons (Haynes 1986). In non-chlorophyllous tissues, these eight electrons are derived from carbohydrate oxidation. In chlorophyllous tissues, the two electrons required for nitrate reduction are derived from carbohydrate oxidation while the six electrons for nitrite reduction come from the trapping of light energy, *via* ferredoxin. The available information on NR in grapevine has been presented in the previous review article (Loulakakis et al. 2001).

2.2. Ammonium assimilation

Ammonium is the primary inorganic nitrogen form involved in synthesis and catabolism of organic nitrogen. Root-specific transporters can allow a direct absorption of ammonium when available in the soil or under particular environments (Glass et al. 2002). Also, it is the product of nitrate reduction and nitrogen fixation. In addition ammonium can be liberated from several metabolic pathways, such as photorespiration, deamination of amino acids and other nitrogenous compounds and phenyl propanoid metabolism (Miflin and Lea 1980, Lea et al. 1990, Lea and Ireland 1999). Ammonium content in plant cells varies as it can be derived from several routes, which depend on the overall metabolic potential of the cell as well as its physiological status.

Although ammonium is readily available to plant cells and critical for plant growth and development, it is also considered to be toxic above a certain concentration (Gerendás et al. 1997). Ammonium dissipates trans-membrane proton gradients that are required for both, photosynthetic and respiratory electron transport and for sequestering metabolites in the vacuole. Even at low concentrations, ammonium will dissociate ATP formation from electron transport in both, chloroplasts and mitochondria. Also, in nitrogen-fixing systems, ammonium inhibits the activity of the enzyme dinitrogenase. Plant cells avoid ammonium toxicity by rapidly incorporating ammonium into amino acids, near the site of absorption or generation. Only traces of ammonium were detected in xylem exudates (Givan 1979, Roubelakis-Angelakis and Kliewer 1979), although Dintscheff et al. (1964) found ammonium ions to be one of the major N constituents reaching the shoots, leaves, and clusters. Under ammonium nutrition large concentrations of ammonium are also transported through the phloem stream (Tercé-Laforgue et al. 2004b).

The primary pathway of ammonium entry into amino acids is the Glutamine synthetase/Glutamate synthase cycle (GS, EC 6.3.1.2/GOGAT, EC 1.4.7.1

and 1.4.1.14), which involves the sequential action of GS and GOGAT (Fig. 2). The GS/GOGAT cycle is considered to play a predominant role in the assimilation of ammonium in higher plants (Miflin and Lea 1974, Stewart et al. 1980, Lea et al. 1990, Roubelakis-Angelakis and Kliewer 1992, Lea et al. 1992, Lam et al. 1996). The first enzyme, GS catalyses the ATP-dependent synthesis of glutamine from glutamate and ammonia. Subsequently, GOGAT catalyzes the reductive transfer of the amido-group of glutamine to 2-oxoglutarate resulting in the formation of two molecules of glutamate (Miflin and Lea 1976, 1980).

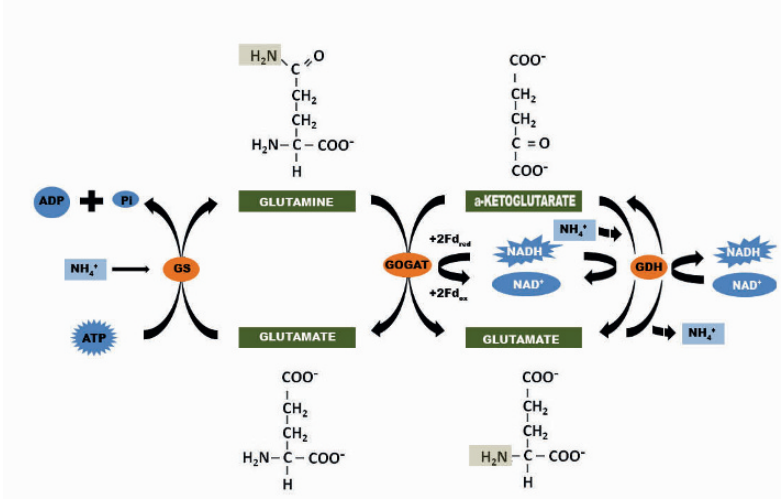

Fig. 2. Biochemical reactions catalyzed by ammonia assimilating enzymes in plants. GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase.

An alternative pathway for nitrogen assimilation could involve the direct reductive amination of α-ketoglutarate by the enzyme Glutamate dehydrogenase (GDH, EC 1.4.1.2, Fig. 2). GDH catalyzes *in vitro* a reversible reaction that synthesizes (aminating-, NADH-GDH) or deaminates glutamate (deaminating-, NAD-GDH). GDH is the primary route of nitrogen assimilation in microorganisms and was long considered as a significant enzyme in plant nitrogen metabolism. However, since the GS/GOGAT cycle was discovered (Lea and Miflin 1974), the role of this enzyme, thought before to be responsible for primary assimilation of ammonium, has been extensively debated. Biochemical, genetic and molecular evidence have shown that the major pathway for ammonia assimilation into organic molecules is the GS/GOGAT cycle (Lea and Miflin 1974, 1980, Lea et al. 1990). There is also evidence that alternative routes for assimilating ammonium may be triggered when GS activity is impaired (Hirel and Lemaire 2005, Skopelitis et al. 2006, Martin et al. 2007). Recent data from tobacco transgenic plants with altered the GDH genes have revealed that all

GDH-isoenzymes strongly deaminate glutamate and only the anionic GDH-isoforms exhibit very low aminating activity under normal trophic conditions (Purnell and Botella 2007, Skopelitis et al 2007, for more information see below). Whereas, under stress the aminating activity of GDH is induced (Skopelitis et al 2006).

Numerous studies based on the physical, biochemical and immunological characterization of the ammonia assimilatory enzymes have been performed in a variety of plants, including model and crop species. These studies, revealed important information on the regulation of nitrogen assimilation into amino acids taking into account that each reaction catalyzed by multiple isoenzymes is located in distinct organs, tissues or subcellular compartments. Originally, most of the molecular studies on ammonia assimilation in plants had focused on GS, and consequently there was greater understanding of the molecular expression and regulation of GS than with GOGAT, GDH, Asparagine synthetase (AS) and Aspartate aminotransferase (AspAT; Schultz et al. 1998, Hirel and Lea 2001, Lea et al. 2007).

The products of ammonium assimilation, glutamine and glutamate, serve as nitrogen-transport compounds and nitrogen donors in many cellular reactions, including the biosynthesis of aspartate and asparagine by AspAT and AS, respectively (Lea 1999). It has also recently been demonstrated that glutamine and glutamate play an important role as signalling molecules in a variety of metabolic and developmental processes (Coruzzi and Zhou 2001, Forde and Lea 2007). The four amino acids glutamine, glutamate, aspartate and asparagine, generated by this pathway, can account for more than 60% of the total free amino acid pool in most legumes and crop plants and are transported through the vascular tissues (Lea 1999, Ochs et al. 1999). Aspartate is an amino acid that participates in the malate-aspartate shuttle to transfer reducing equivalents from the mitochondrion and chloroplast into the cytosol and in the transport of carbon from mesophyll to bundle sheath for C4 carbon fixation. Asparagine is thought to be an important compound for transport and storage of nitrogen resources because of its relative stability and high nitrogen to carbon ratio (Lam et al. 1995, 1996, Lea et al. 2007).

The genes involved in ammonium assimilation are regulated by several factors such as nitrogen source, metabolites, light, and cell type (Lam et al. 1996). However, factors such as exogenously supplied nitrogen have been reported to have a variable influence in the synthesis of a number of isoenzymes involved in nitrogen metabolism, depending upon plant material and experimental conditions. Now we have a wider view on the regulation of ammonia assimilating enzymes including GDH, GOGAT, AS, AspAT at least in the model species *Arabidopsis* and tobacco. Extensive biochemical, genetic and molecular analyses of mutants or transgenic plants have allowed to define the *in vivo* roles

of individual isoenzymes and uncovered the regulatory mechanisms involved (Hirel and Lemaire 2005, Purnell and Botella 2007, Skopelitis et al. 2006) as well as the interactions with carbon metabolism (Foyer et al. 2001).

2.2.1. Glutamine synthetase

Plant GS is an octameric enzyme with a native molecular mass of 350-400 kD. In size and quaternary structure it strongly resembles the enzyme isolated from mammals, but it is distinct from the bacterial enzyme, which consists of 12 subunits. The subunits are assembled as two tetramers stacked one upon another, with the active site in the interface of subunits (Unno et al. 2006). The reaction catalysed by GS is believed to proceed *via* a two-step process, the first involving the formation of GS-bound glutamyl phosphate from ATP and glutamate, followed by the addition of ammonia to form a tetrahedral adduct with the subsequent liberation of glutamine, ADP, and Pi (Lea and Ridley 1989, Ishimaya et al. 2006). GS exists as multiple isoenzymes that are of two types; one type is exclusively localized to the cytosol (GS1) and the other is restricted to the plastids (GS2). Subunits of the various isoenzymes exhibit both, size (37-44 kD) and charge heterogeneity (Bennett and Cullimore 1989, Temple et al. 1995). In general, all plant species examined contain one GS2 isoform encoded by a single nuclear gene (Peterman and Goodman 1991, Stanford et al. 1993) and a number of cytosolic GS1 isoforms encoded by small gene families (up to six genes) (Peterman and Goodman 1991, Sakakibara et al. 1992, Stanford et al. 1993, Temple et al. 1995).

In addition to the dual cellular compartmentalization, the relative proportion of the individual isoforms of GS may vary according to the plant species, the physiological status of the plant, the developmental stage and the organ examined (Cren and Hirel 1999). In photosynthetic organs, light, possibly *via* phytochrome and a blue light receptor, and also ammonium induce the expression of the plastid forms of the enzyme (Edwards and Coruzzi 1989, Migge et al. 1996). This form is also preferentially degraded during plant senescence in several plant species (Masclaux et al. 2001).

In shoots and leaves, chloroplastic GS is thought to respond for the assimilation of primary ammonium reduced from nitrate and also for the reassimilation of ammonium released during photorespiration. The GS in root plastids generates amide nitrogen for local consumption (Peat and Tobin 1996). The GS1 isoforms are expressed in germinating seeds or in the vascular bundles of roots and shoots and seem to generate glutamine required as a substrate for several biosynthetic pathways and for transport of organic nitrogen throughout the plant (Miflin and Lea 1980, Lea et al. 1990, Lam et al. 1996, Oliveira and Coruzzi 1999). GS gene families have been relatively well characterized in leguminous plant species and in some non-legume annual plants, and now in per-

ennial plant species, especially at the molecular level (Cánovas et al. 2007).

In grapevine GS activity has been detected in several tissues such as leaves, shoots, roots, berries, calluses and cell suspensions (Roubelakis-Angelakis and Kliewer 1983, Ghisi et al. 1984, Loulakakis and Roubelakis-Angelakis 1996b, 2000). Grapevine leaf GS was analyzed by ion exchange chromatography into two distinct classes of isoenzymes, the cytosolic and the chloroplastic. By analogy with the corresponding chromatographic forms of other higher plants, they were designated as GS1 and GS2, respectively. The GS activity of root extracts was eluted as a single peak at position identical with the lower ionic strength-eluted GS1 peak of the leaf enzyme (Loulakakis and Roubelakis-Angelakis 1996b).

Western blot analysis revealed that grapevine GS consists of three types of polypeptides of distinct size (44, 43 and 39 kD, respectively) and differential tissue specificity. The 39 kD class of polypeptides was present in all tissues examined, the 43 kD band showed leaf-specificity, whereas the 44 kD band was detected in leaf and in lower amounts in shoot and cell suspension but not in root tissue (Loulakakis and Roubelakis-Angelakis 1996b). These bands were further analyzed into several polypeptides using 2-D electrophoresis (Fig. 3). Thus, grapevine GS seems to have similar isoenzyme and polypeptide patterns with the enzyme from other higher plants (Bennett and Cullimore 1989, Peterman and Goodman 1991, Sakakibara et al. 1992). The 39 kD class of polypeptides is cytosolic and the 43 and 44 kD class of grapevine GS polypeptides are chloroplastic that assemble into active GS1 and GS2 isoforms, respectively. The size divergence of the GS2 subunits could be attributed to post-translational modification of the product of a single nuclear gene such as glycosylation (Nato et al. 1984) or phosphorylation (Lima et al. 2006). On the other hand, cytosolic GS consists of at least 3 or 4 polypeptides of similar size but with different charge.

The existence of small gene families encoding for different cytosolic isoenzymes has been described for several plant species. For example, up to five putative cytosolic cDNA clones have been characterized in *Arabidopsis thaliana* (Peterson and Goodman 1991), and maize (Sakakibara et al. 1992, Li et al. 1993). In grapevine, three homologous but structurally distinct cDNA clones, pGS1;1, pGS1;2 and pGS1;3, encoding GS were isolated and characterized (Loulakakis and Roubelakis-Angelakis 1996b, 2000 and our unpublished results). Their structural characteristics and the expression patterns of the respective genes supported that the three clones are derived from mRNAs encoding cytosolic polypeptides.

Each clone contains an open reading frame of 1068 nucleotides that encodes a protein of 356 amino acids with a predicted molecular weight of 39 kD. The coding regions of the three grapevine GS clones show 84 to 93% nucleotide

and amino acid identity, whereas, their 5′- and 3′-untranslated sequences are quite divergent showing 40% nucleotide identity. Grapevine GS clones, as other cytosolic GS forms, lack the characteristic N-terminal and C-terminal amino acid extensions of the chloroplastic polypeptides. At the amino acid level, pGS1;1, pGS1;2 and pGS1;3 were 82-92% identical to the cytosolic GS sequences, whereas only 76-80% identity to the chloroplastic GS sequences of other higher plants was observed. Although sequence conservation among GS genes with common functions has been reported between species (Temple et al. 1995), comparison of the coding sequences and the 3′ untranslated sequences of grapevine GS cDNA clones to each member of GS1 gene families from other plants failed to reveal any difference in homologies (Loulakakis and Roubelakis-Angelakis 1996b).

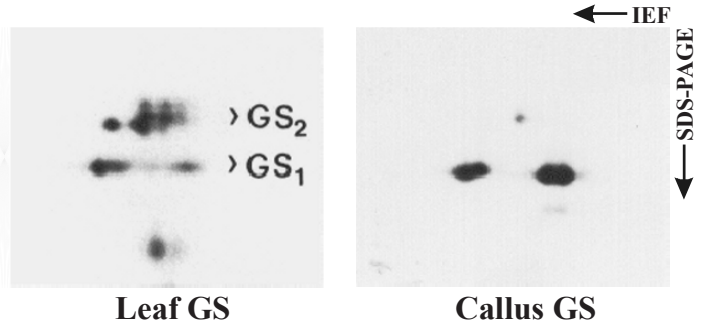

Fig. 3. Two-dimensional electrophoretic analysis (urea-isoelectric focusing/SDS-PAGE) of glutamine synthetase from grapevine leaf and callus tissues. Following electrophoresis proteins were transferred on nitrocellulose membranes and probed with antibody raised against a recombinant grapevine cytosolic GS molecule (Loulakakis and Roubelakis-Angelakis, unpublished).

Southern blot analysis results suggested that each cDNA clone represents a unique subset of the gene family, and also imply that there are more than three GS genes in the grapevine genome. This may be now verified using the full genome sequence data (Jaillon et al. 2006). Thus, grapevine GS gene family seems to be similar in complexity to the well characterized gene families of other higher plants. The 39 kD polypeptide observed in two dimensional electrophoresis are obviously encoded from at least three GS genes and further studies are required in order to correlate these polypeptides with the three cDNA clones.

Northern analysis revealed mRNAs of approximately 1.4 kb that are differentially expressed in the tissues examined (roots, shoots, leaves, callus, cell suspension and berries). *pGS1;1* related gene was expressed at relatively high levels in roots but was also highly expressed in leaf, callus and berry tissues. *pGS1;2* showed significantly higher levels of expression in root and

lower in leaf and berry tissues, whereas *pGS1;3* related gene was highly expressed in root and callus tissues (Loulakakis and Roubelakis-Angelakis 1996b and our unpublished results). These results are in accordance with previous reports on the differential tissue specificity of the individual members of GS gene families in other plants. In *Arabidopsis thaliana*, four GS1 genes are expressed differentially in leaves, roots, and germinating seeds (Peterson and Goodman 1991), while five genes of *Zea mays* GS1 show distinct tissue-specific expression (Sakakibara et al. 1992, Li et al. 1993, Martin et al. 2006). The same situation exists in rice (Tabuchi et al. 2007) and wheat (Bernard et al. 2008), for which the expression pattern of three genes encoding GS1 and one gene encoding GS2 have been thoroughly characterized.

Furthermore, GS, at the activity and protein levels, were monitored at progressing developmental stages of leaf from field-grown grapevine plants, from the stage of the newly expanding to the senescing old leaf (Loulakakis et al. 2002). During anthesis, the GS specific activity was low in the newly expanding leaves and progressively increased with leaf age exhibiting approximately 2.3-fold higher specific activity in the fully developed leaf, in which the highest protein content was observed (Fig. 4). The immunoreactive protein of cytosolic GS (GS1) remained fairly constant whereas that of the chloroplastic enzyme (GS2) was substantially lower in the young leaves (Fig. 4). On the other hand, during natural senescence of the grapevine leaves, both specific activity and immunoreactive protein of GS were progressively decreased and these changes were paralleled by chlorophyll content, Rubisco protein and photosynthetic efficiency of leaves (Loulakakis et al. 2002).

2.2.2. Glutamate synthase

Two forms of GOGAT with different reductant specificity have been identified in higher plants; one utilizes reduced ferredoxin (Fd-GOGAT, EC 1.4.7.1) and the other utilizes NADH (NADH-GOGAT, EC 1.4.1.14) as electron donor. The two forms of the enzyme are structurally distinct proteins differing in their molecular weights, kinetic characteristics and immunoreactivity (Suzuki et al. 1982, Lea et al. 1990, Hayakawa et al. 1992, Gregerson et al. 1993, Suzuki and Knaff 2005). Fd-dependent GOGAT is localized in the meshophyll chloroplasts and is major enzymatic form in green tissues, representing up to 1% of the protein content of leaves (Matoh and Takahashi 1982, Suzuki et al. 1982).

Recently, Fd-GOGAT was found to be also expressed in vascular cells suggesting that the enzyme provides amino acids for nitrogen translocation (Feraud et al. 2005). In *Arabidopsis thaliana*, for example, Fd-GOGAT accounts for up to 96% of the total GOGAT activity in leaves (Suzuki and Rothstein 1997, Somerville and Ogren 1980). The enzyme is an iron-sulphur flavo-

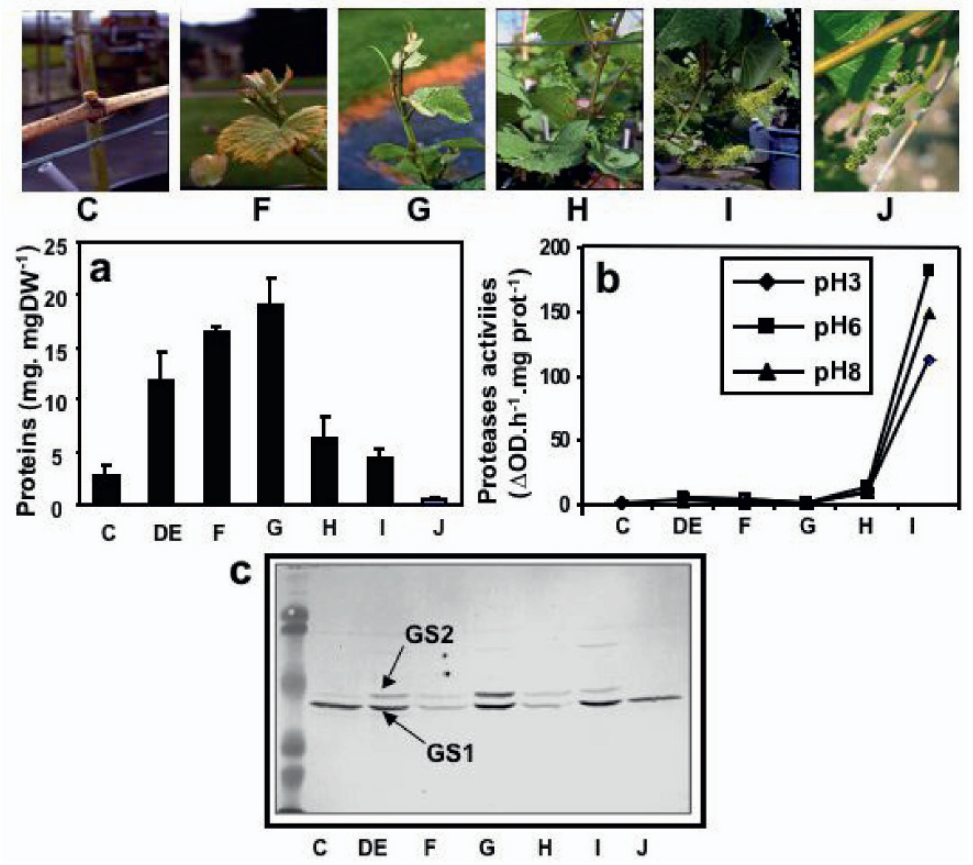

Fig. 4. GS immunoreactive protein at different developmental stages of grapevine. (a) Protein content of leaves at different developmental stages. (b) Relative protease activities (at pH 3, 6, and 8). (c) Immunoreactive GS1 and GS2 proteins (Hirel et al., unpublished).

protein with a single polypeptide of molecular mass 140-165 kD, including a transit peptide for transport into the chloroplasts. In photosynthetic tissues, Fd-GOGAT is essential for both, reassimilation of ammonium released during photorespiration and primary ammonium assimilation (Somerville and Ogren 1980, Lam et al. 1995, 1996, Coschigano et al. 1998). Fd-GOGAT transcripts and protein were also found in non-photosynthetic organs, including roots and nodules (Suzuki et al. 1982, Coschigano et al. 1998).

Fd-GOGAT genes have been cloned from several plant species, including maize (Sakakibara et al. 1991), tobacco (Zehnacker et al. 1992), barley (Avila et al. 1993), spinach (Nalbantoglu et al. 1994), grape (Loulakakis and Roubelakis-Angelakis 1997) and *Arabidopsis thaliana* (Coshigano et al. 1998). Higher than

80% homology was detected in the amino acid sequences of the enzyme among plant species. Plant genomes seem to contain a single copy of Fd-GOGAT gene, while in the amphidiploid genome of tobacco the presence of two genes was noticed (Zehnacker et al. 1992). In *Arabidopsis thaliana*, two distinct Fd-GOGAT cDNAs were recently cloned indicating the existence of two functional Fd-GOGAT genes (Lam et al. 1996, Coshigano et al. 1998).

The NADH-dependent GOGAT is a monomeric iron sulfur flavoprotein with a molecular mass of 200-240 kD (Anderson et al. 1989). The enzyme is localized in plastids of non-photosynthetic tissues, such as roots (Suzuki et al. 1982), nodules (Gregerson et al. 1993) and cell cultures (Hayakawa et al. 1990, 1992) or vascular bundles of developing leaves. In root nodules of legumes, NADH-GOGAT is involved in the assimilation of nitrogen fixed by *Rhizobium*. In non-legumes, NADH-GOGAT may participate in the primary assimilation or reassimilation of ammonium released during amino acid catabolism (Lam et al. 1996). One full-length NADH-GOGAT clone has been isolated from alfalfa (Gregerson et al. 1993) and from rice (Goto et al. 1998). The deduced amino acid sequence revealed that the alfalfa NADH-GOGAT was a fusion of two peptides having homology to the large and small NADPH-GOGAT subunits from *E. coli*, which was distinct from the known plant Fd-GOGATs.

The two types of GOGAT show different developmental patterns during the early development of plant seedlings (Matoh and Takahashi 1982) and their expression is regulated by environmental factors, such as light and exogenous nitrogen sources (Hayakawa et al. 1990, Sakakibara et al. 1991, Trepp et al. 1999, Turano et al. 1999). However, it is not yet clear if NADH-GOGAT has an overlapping or non-overlapping role with Fd-GOGAT. Changes in the content of the GS and GOGAT isoforms have led to the proposition that NADH-GOGAT and cytosolic GS are involved in the synthesis of glutamate from the glutamine, which is transported from senescing source tissues through the phloem to the non-green sink tissues in leaves (Hayakawa et al. 1993, Tabuchi et al. 2007).

In grapevine, activity of both forms of GOGAT was present in all tissues tested (Creasy and Breen 1997, Loulakakis and Roubelakis-Angelakis 1997). Green leaf Fd-GOGAT exhibited significantly higher specific activity compared to root enzyme. On the other hand, the NADH-GOGAT was detectable in all tissues examined exhibiting 7 and 75% of Fd-GOGAT activity, in leaf and root tissues, respectively. Detectable levels of both GOGAT forms were also found in cell suspension cultures. Grapevine Fd-GOGAT consists of one type of polypeptide, with a molecular mass of 160 kD revealed by protein gel blot analysis using antibodies raised against a recombinant Fd-GOGAT (Fig. 5).

The first cDNA clones of Fd-GOGAT from a perennial dicotyledonous plant species were isolated from grapevine (Loulakakis and Roubelakis-

Angelakis 1997) and pine (Cánovas et al. 2007). The two grapevine cDNAs isolated encode for the carboxy- and near to amino-terminal sequences of the Fd-GOGAT, respectively. The deduced amino acid sequence of the grapevine enzyme was significantly identical to the respective sequences of other known plant Fd-GOGATs and showed homology to the alfalfa nodule NADH-GOGAT and to the large subunit of the *E. coli* NADPH-GOGAT, indicating that Fd-GOGAT is highly conserved in higher plants (Loulakakis and Roubelakis-Angelakis 1997).

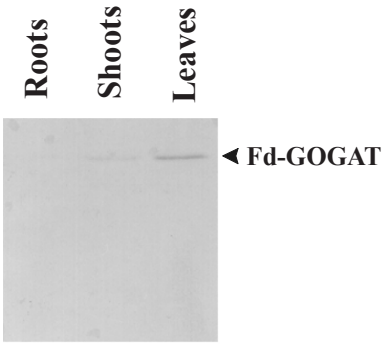

Fig. 5. Immunoblotting analysis of Fd-GOGAT in extracts prepared from grapevine roots, shoots and leaves. Protein extracts were analyzed by SDS-PAGE and immunodetected with antibody raised against a recombinant grapevine Fd-GOGAT molecule (Loulakakis and Roubelakis-Angelakis, unpublished).

DNA blot analysis revealed that Fd-GOGAT is probably encoded by two genes in the genome of grapevine similarly to *Arabidopsis thaliana* (Loulakakis and Roubelakis-Angelakis 1997, Coshigano et al. 1998). Probes from the two clones hybridized to a single transcript of about 5.7 kb showing similar tissue specificity. The abundance of Fd-GOGAT mRNA was high in leaves, low in roots and very low in shoots, cell suspensions and berries (Loulakakis and Roubelakis-Angelakis 1997). It is worth noticing that the Fd-GOGAT mRNA was accumulated at appreciable level in a non-photosynthetic tissue, such as the root. This pattern of expression could be characteristic of a particular stage of differentiation or development of *in vitro* grown grapevine plants. In maize (Sakakibara et al. 1992) and tobacco (Zehnacker et al. 1992) higher levels of Fd-GOGAT transcript were detected in photosynthetic than in non-photosynthetic organs, while in *Arabidopsis thaliana* differential expression of two Fd-GOGAT genes in shoots and roots was found (Lam et al. 1996).

Specific polyclonal antibodies for Fd-GOGAT were generated against the partial Fd-GOGAT peptide encoded by pGOGAT1 cDNA clone that was over-expressed in *Escherichia coli* cells (Loulakakis et al. 2002). This was the first

example of antibody raised against Fd-GOGAT from a perennial plant species. The antibody recognized a protein band of approximately 160 kD on nitrocellulose blots of total proteins from various grapevine tissues and precipitated most of the enzyme activity present in grapevine protein extracts. This antibody also recognized the Fd-GOGAT protein of other monocotyledonous and dicotyledonous plant species, further supporting that Fd-GOGAT is highly conserved in higher plants. Both, the grapevine Fd-GOGAT enzymatic activity and the immunodetected polypeptide co-localized to chloroplasts (Loulakakis et al. 2002).

The quantity of the Fd-GOGAT protein was significantly higher in leaves than in other grapevine tissues tested, coincident with a similar distribution of the enzyme specific activity (Loulakakis et al. 2002). In general, the level of Fd-GOGAT specific activity in shoots, internodes, roots and calluses of grapevine was correlated with the abundance of the 160-kD polypeptide and of the 5.7 kb Fd-GOGAT transcript. Also, results using grapevine tissues grown under varying environmental and trophic conditions support this observation (our unpublished results). Exceptionally, the relatively high Fd-GOGAT mRNA level observed in roots (Loulakakis and Roubelakis-Angelakis 1997) did not correspond to the enzyme activity and protein level. The results could support that Fd-GOGAT activity is controlled in the various grapevine tissues by the abundance of the transcript, due to increased gene expression and/or RNA stability, as reported for other plant species (Zehnacker et al. 1992, Turano and Muhitch 1999). However, the translational or post-translational modifications as mode(s) of regulation of the enzyme activity could not be ruled out.

Immunological comparisons of Fd-GOGAT from different grapevine tissues did not reveal any difference in their properties in agreement with results from barley, which indicated that leaf and root Fd-GOGATs are immunologically very similar (Pajuelo et al. 1997). In contrast, Suzuki et al. (1982) reported that rice root Fd-GOGAT was immunologically distinct from the leaf enzyme. Immunological results for grapevine Fd-GOGAT could not definitely support if the leaf, internode and root enzymes are products of one or two Fd-GOGAT gene(s). If a second gene exists in grapevine genome, as DNA blot analysis supports, it would have to be silent or expressed at very low levels in tissues tested or be specific to a particular stage of differentiation or development. On the other hand, there is the possibility that the two genes encode similar Fd-GOGAT polypeptides, and are expressed in these tissues (Loulakakis et al. 2002).

Grapevine Fd-GOGAT is a light regulated enzyme as its transcript level in leaves of dark-grown *in vitro* plants was increased upon illumination. In addition, enzyme activity and protein were significantly higher in light-grown than in dark-grown leaves (Loulakakis and Roubelakis-Angelakis 1997). The

presence of Fd-GOGAT in dark-grown tissue, however, supports the existence of a basic, non light-dependent level of Fd-GOGAT gene expression.

Furthermore, the specific activity and protein levels of Fd-GOGAT were followed at progressing developmental stages of leaf from field-grown grapevine plants, from the stage of the newly expanding to the senescing old leaf (Loulakakis et al. 2002). Early in the season, during anthesis, when the developing flowers and young berries are strong sinks for photosynthates and amino acids, the fully developed and active physiologically leaves, in the basal part of the shoot, showed significantly higher Fd-GOGAT protein accumulation and enzyme activity than the youngest leaves. Late in the season, following harvest, when senescence was initiated in all leaves along the shoot axis, as the chlorophyll content, Rubisco protein and the photosynthetic efficiency of leaves indicated, the levels of Fd-GOGAT protein and activity gradually decreased from the apical to the basal leaves (Loulakakis et al. 2002).

2.2.3. Glutamate dehydrogenase

An alternative pathway for nitrogen assimilation could involve the direct reductive amination of α-ketoglutarate by GDH. This enzyme catalyzes *in vitro* a reversible reaction that synthesizes (aminating-, NADH-GDH) or deaminates glutamate (deaminating-, NAD-GDH; Fig. 3). GDH is ubiquitous in plants and often it is present at high levels in plant tissues, for example in senescing and root tissues (Loyola-Vargas and de Jimenez 1984, Loulakakis et al. 1994, Dubois et al. 2003). The enzyme could operate in two ways: (1) In the assimilation or reassimilation of ammonium, thereby complementing the enzymes of the GS/GOGAT cycle in the synthesis of glutamate, especially under conditions of stress such as elevated temperature, water stress, salinity, pollution and pathogenic infection (Yamaya et al. 1984, Srivastava and Singh 1987, Oaks 1994, Skopelitis et al. 2006). Mitochondrial GDH could play this assimilatory role, in view of the high ammonium concentrations in mitochondria released from photorespiration (Yamaya et al. 1984) and of the multienzymic complexes that are present there (Pahlich 1996). (2) It could act in the deamination of glutamate, under carbon limiting conditions, connecting the GDH with the carbon metabolism rather than nitrogen metabolism (Robinson et al. 1991, Aubert et al. 2001; Purnell and Botella 2007).

Following the discovery of the GS/GOGAT cycle (Lea and Miflin 1974), the role of GDH, thought before to be responsible for primary assimilation of ammonium, has been extensively debated. Biochemical, genetic and molecular evidence has shown that the major pathway for ammonia assimilation into organic molecules in plants is the GS/GOGAT cycle (Miflin and Lea 1976, 1980, Lea et al. 1990). There is also evidence that alternative routes for assimilating ammonium may be triggered when GS activity is impaired (Hirel and Lemaire

2005, Martin et al. 2007).

2.2.3.1. *GDH* genes and GDH protein structure

The isolation of a full-length grapevine cDNA encoding for NAD(H)-GDH provided the first example of a plant GDH sequence (Syntichaki et al. 1996). Southern blot analysis revealed that more than one *GDH* genes are present in grapevine genome. The first, encoding for the α-subunit, was initially designated as *VvGDH1* (Syntichaki et al. 1996) and as *VvGDH-NAD;A1* in a refined nomenclature proposed later (Purnell et al. 2005). The second grapevine clone, encoding for the β-subunit, referred to as *VvGDH-NAD;B1* (Accession no DQ497631), was also isolated using a PCR-based strategy (Skopelitis and Roubelakis-Angelakis 2005). The *GDH* gene(s) have also been cloned from maize (Sakakibara et al. 1995), *Arabidopsis thaliana* (Melo-Oliveira et al. 1996, Turano et al. 1996), tomato (Purnell et al. 1997), *Asparagus officinalis* (accession no. AJ011096), *Brassica napus* (accession no. AY693422 and AB066298), and *Nicotiana plumbaginifolia* (Ficarelli et al. 1999). The *VvGDH-NAD;A1* and *VvGDH-NAD;B1* genes exhibit high sequence homology (Fig. 6).

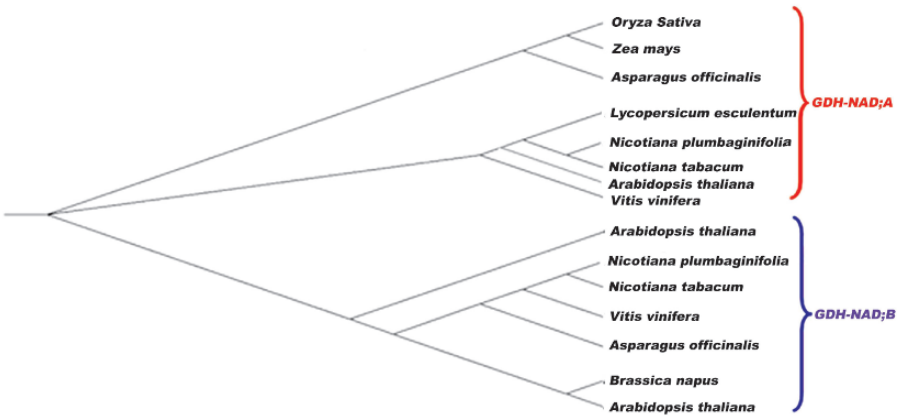

Fig. 6. Phylogenetic dendrogram of the *GDH* genes encoding for the α- and β-subunits.

The grapevine *VvGDH-NAD;A1* clone contains an open reading frame encoding for a protein of 411 amino acids with a predicted molecular weight of 44.5 kD and a relatively long 5′ untranslated region (Syntichaki et al. 1996). Since the protein is known to localize to mitochondria, this sequence corresponds to the precursor molecule and thus the difference in the molecular mass of the predicted protein (44.5 kD) from the experimentally determined value

(42.5-43.0 kD; Loulakakis and Roubelakis-Angelakis 1991) is due to the cleavage of the mitochondrial signal peptide. The presence of putative mitochondrial target sequences has also been reported in other plant *NAD(H)-GDH* gene products (Turano et al. 1997, Ficarelli et al. 1999). Both *GDH*-clones contain sequences encoding for the N-terminal mitochondrial signal peptide and sequences corresponding to the NAD(H)-binding motif of the protein and to the glutamate-binding motif. Additionally, a sequence encoding for an EF-hand motif, responsible for the ability of the protein to bind Ca^{2+} and an 2-oxoglutarate binding sequence was found only in the clone encoding for the α-subunit (Skopelitis and Roubelakis-Angelakis, unpublished).

In addition to the cDNA clones, the genomic *VvGDH-NAD;A1* and *VvGDH-NAD;B1* clones were isolated from grapevine. The full length of *VvGDH-NAD;A1* genomic clone has been submitted to the EMBL Nucleotide Sequence Database under the accession no AJ303070 (Skopelitis and Roubelakis-Angelakis 2001) and it is 3146 bp long, containing 8 introns and 9 exons. Similarly the genomic sequence of *VvGDH-NAD;B1* has been submitted in GenBank under the accession number DQ497632 (Skopelitis and Roubelakis-Angelakis 2006). It is slightly longer than its α subunit-encoding counterpart, being 4203 bp in length, but it has the same number of introns and exons.

The promoter region of *VvGDH-NAD;A1* was *in silico* analyzed and several *cis*-regulatory elements were identified. The analysis particularly identified light-responsive regulatory elements as well as sequences related to the regulation of expression by the cellular sugar content within the promoter region**.** Other regulatory elements, involved sequences related to the responses to hormones such as ABA, GA and SA, as well as sequences responsible for tissue specific expression (Skopelitis and Roubelakis-Angelakis, unpublished). The minimal promoter region of the *VvGDH-NAD;A1* gene, that can sufficiently initiate transcription was determined (Fig. 7). The full promoter (979 bp) and the promoter deletions were fused upstream of the luciferase (*LUC*) gene and were expressed in tobacco protoplasts (Skopelitis and Roubelakis-Angelakis, unpublished). The full promoter region possess the highest relative luciferase activity (29 RLU), while the deletions exhibited lowest relative activity (360 bp deletion, 22 RLU; 630 bp deletion, 5 RLU; 750 and 890 bp deletion 2 RLU).

Presence of a small NAD(H)-GDH family appears to be common among plants (Inokuchi et al. 2002, Purnell et al. 2005). Genetic and molecular analysis on maize suggested that the enzyme is encoded by two genes whereas rice data suggested that there are at least three genes (Abiko et al. 2005). In addition, two cDNAs encoding NAD(H)-GDH were identified and characterized in both

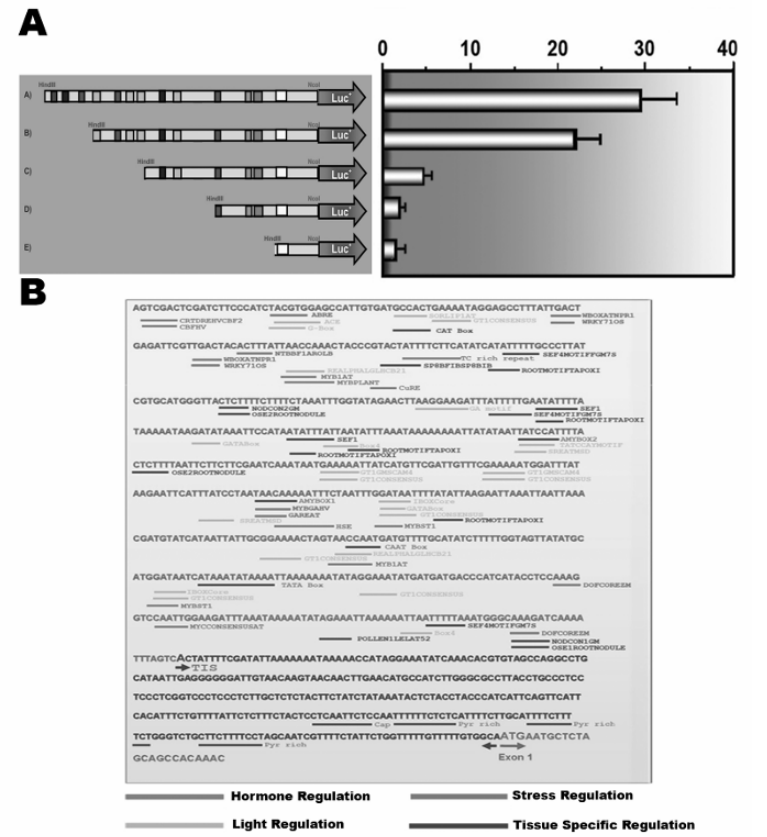

Fig. 7. *VvGDH*-NAD;A1 promoter deletion analysis and *in silico* analysis of *cis*-regulatory elements. (A) Deletions of the *VvGDH-NAD;A1* promoter fused to the luciferase gene and the corresponding Relative luciferase Activity Units (RLU) of each deletion. (B) *Cis*-regulatory elements in the promoter region of *VvGDH*-NAD;A1 gene (Skopelitis and Roubelakis-Angelakis, unpublished).

Arabidopsis thaliana and tobacco (Melo-Oliveira et al. 1996, Turano et al. 1997, Ficarelli et al. 1999). Two more genes were subsequently identified and annotated as putative *GDH* genes in *Arabidopsis thaliana* (Fontaine et al. 2006, Miyashita and Good 2008).

In *Nicotiana plumbaginifolia* the two unique genes cloned were shown to be expressed differentially under various environmental stresses, such as salinity, temperature shifts and heavy metals (Restivo 2004). However, Southern analysis in tomato indicated the existence of one *NAD(H)-GDH* gene. Based on these results, authors proposed that differential splicing of the same gene and/or post-translational modifications account for the existence of two subunits in tomato (Purnell et al. 1997). In higher plants, two major forms of GDH have been

identified: a NAD(H)-depended enzyme localized in the mitochondria and a NADP(H)-depended form associated with the chloroplasts. Following computational analysis of the *Arabidopsis thaliana* genome sequence, two other GDH genes have been identified, one using NAD(H) as a cofactor while the other uses NADP(H) (Jaspard 2006).

The phylogenetic dendrogram reinforced the notion of two evolutionary related families (Benachenhou-Lafha et al. 1993). Family I consists of *GDH* genes of eubacteria and lower eukaryotes, whereas Family II consists mainly of archaebacteria and higher eukaryotes including plants (Syntichaki et al. 1996). By constrast to GDH, GS from archaebacteria is more related to eubacteria than of higher eukaryotes (Pesole et al. 1995). Thus, apart from the yet unresolved evolutionary considerations, the observed relationship of plant GDH with archaeal extremophiles could have a functional importance. Some of the distinct conserved substitutions could be critical for the stability of the enzyme under extreme conditions. The grapevine clone expressed in the mesophyllic *E. coli* cells exhibited high levels of GDH aminating activity, which was significantly more thermostable (60^{o}C for 1h) compared to the heat labile endogenous bacterial enzyme. This observation was important since it ascertained that thermal stability is determined by the enzyme's amino acid sequence rather than by additional post-translational modifications. Moreover, in a more recent study, lateral gene transfer is proposed as an evolutionary mechanism, which has greatly affected the distribution of *GDH* genes among living organisms (Anderson and Roger 2003).

NAD(H)-GDH is hexameric or tetrameric with molecular mass of 252-310 kD and exists in multiple isoenzymes (Cammaerts and Jacobs 1983, 1985, Srivastava and Singh 1987) that have been well characterized in several plant species (Cammerts and Jacobs 1983, Srivastava and Singh 1987, Loulakakis and Roubelakis-Angelakis 1990a, 1990b, 1991, 1992, 1996a, Turano et al. 1997, 1998, Primikirios et al. 2000). The enzyme most often occurs as seven isoforms with distinct molecular mass, which would be explained by a different subunit composition of the hexameric holoenzyme.

The primary structure of GDH protein in known from a number of organisms ranging from archaebacteria to human (Benachenhou-Lahfa et al. 1993, Andersson and Roger 2003). Sequence comparisons of grapevine GDH to known GDHs established two main observations (Syntichaki et al. 1996). Firstly, the plant GDH sequence shares, with the other hexameric GDHs, conserved residues at positions of functional importance (Baker et al. 1992). A total of 45 residues are absolutely conserved among all sequences. The conserved clusters surrounding K66, K90, K102, and A140 stabilize glutamate binding, whereas the nucleotide binding motif GxGxxG (G213xG215xxG218) and the carboxyl group of D327 conform to the proposed signature for NAD^{+} binding

(Scrutton et al. 1990). Two other highly conserved regions (residues 169-181 and residues 337-347) are responsible for inter-domain linking of the monomer. Secondly, the plant GDH in a multiple alignment was grouped with all known archaebacterial enzymes. By examining the multiple alignments, we were able to define in this group a number of amino acid positions with a distinct character.

In order to examine whether any heterogenioity in the size of the enzyme subunits could be detected, GDH from various grapevine tissues was analyzed by SDS-PAGE using different concentrations of acrylamide. Analysis in 7.5% acrylamide gels, followed by western blot, revealed that GDH is composed of two polypeptides with small but distinct differences in molecular weight. By two-dimensional gel electrophoresis (urea-isoelectric focusing/SDS-PAGE) it was proven that the polypeptides have also different isoelectric points and, thus, different net charge.

Based on their mobilities in the SDS gel, the 43.0 and 42.5 were designated as α- and β- respectively. The two polypeptides were present in different quantities in the various grapevine tissues. In extracts from leaves, shoots and roots, the β-polypeptide was the predominant GDH antigen, from nitrate grown shoot calluses the β-polypeptide was slightly greater than the α, while in extracts from ammonium grown calluses the α-polypeptide dominated. Thus, grapevine GDH is hexameric consisting of two subunits, α and β, with similar antigenic properties but with different molecular weight and charge as was demonstrated by one- and 2-D gel electrophoresis (urea-isoelectric focusing/SDS-PAGE). The two subunits have molecular weights 43.0 and 42.5 kD, respectively (Loulakakis and Roubelakis-Angelakis 1991). They are associated in an ordered ratio so that the isoenzymes 1 and 7 are homohexamers of polypeptides β and α, respectively, and the isoenzymes 2 through 6 (from the less to the more electrophoretically mobile) are hybrids consisting of α- and β-subunits at ratios of 1:5, 2:4, 3:3, 4:2, and 5:1, respectively. This structure appears to be well conserved in the plant kingdom, since similar results have been demonstrated in avocado (Loulakakis et al. 1994), *Arabidopsis thaliana* (Turano et al. 1997) and *Nicotiana* (Fontaine et al. 2006). The biochemical properties of grapevine GDH protein were presented earlier (Loulakakis et al. 2001).

To determine the participation in grapevine of the α- and β-subunits in the composition of each of the seven GDH isoenzymes, the isoenzymes were resolved by two-dimensional native/SDS-PAGE and detected by western blots. The more anodal isoenzyme contained only α-polypeptide, the more cathodal one contained only β-polypeptide, and the remaining 2 to 6 consisted of different ratios of both polypeptides (Loulakakis and Roubelakis-Angelakis, 1991). Grapevine NAD(H)-GDH is a mitochondrial enzyme showing a seven-band anodal migrating isoenzymic pattern (Loulakakis and Roubelakis-Angelakis 1990a, 1991).

The enzyme displayed both NAD(H)- and NADP(H)-dependent activities with the NAD(H) being the preferred substrate. Immunological studies have revealed that the enzyme from various tissues of *in vitro* grown grapevine plants had identical immunological determinants and similar molecular mass (Loulakakis and Roubelakis-Angelakis 1990b). As for all the other higher plants examined so far, grapevine GDH is also localized mostly if not exclusively in the phloem companion cells (Paczeck et al. 2002), suggesting that the enzyme plays an active role during carbon and nitrogen metabolites translocation.

The isoenzyme with the lowest electrophoretic mobility (designated as isoenzyme 1), which accounted for 35-40% of total *in vitro* activity, was purified to homogenicity from grapevine leaves. The purification method included ammonium sulfate fractionation, DEAE-cellulose chromatography, Sephadex G-200 gel filtration and NAD-sepharose affinity chromatography (Loulakakis and Roubelakis-Angelakis 1990a). The apparent molecular weight of the native isoenzyme 1 was estimated to be 252 kD consisting of 42.5 kD subunits, as revealed by SDS-PAGE. The pH optimum for the *in vitro* aminating reaction of GDH isoenzyme 1 was 8.0 and for the *in vitro* deaminating reaction 9.3. At optimum pH conditions, the enzyme exhibited Michaelis-Menten kinetics.

Antiserum raised against purified GDH isoenzyme 1 recognized all seven isoenzymes and immunoprecipitation assay nullified the activity of grapevine protein extracts. The immunological studies revealed that GDHs from various plant organs were closely related proteins with identical immunological determinants and similar molecular weight (Loulakakis and Roubelakis-Angelakis 1990b). The *in vitro* aminating activity of grapevine leaf GDH was fully activated by 100 μM Ca^{2+}. By studying the effect of Ca^{2+} ions on individual isoenzymes, it was shown that the isoenzyme 1 was activated by Ca^{2+} whereas the isoenzyme 7 was not activated. The isoenzymes 2-6 showed different magnitude of their activating response to calcium ions (Loulakakis and Roubelakis-Angelakis 1990a). These differences in the degree of activation of the seven isoenzymes could be explained from the molecular analysis results of other plant GDHs. In *Arabidopsis thaliana* (Turano et al. 1997) and *Nicotiana plumbaginifolia* (Ficarelli et al. 1999) a putative EF-hand loop was found in one of the two GDH genes, suggesting that NAD(H)-GDH isoenzymes may differentially bind Ca^{2+} and therefore could be differentially stimulated by the ligand.

2.2.3.2. Role of GDH-isoenzymes

High levels of expression of *Vvgdh-NAD;A1* were observed in roots, shoots and calluses, intermediate in leaves and low in berry tissue (Loulakakis and Roubelakis-Angelakis 1991). GDH *in vitro* activity was detected in all grape tissues examined, both NAD(H)- and NADP(H)-dependent activities,

with the NAD(H) being the preferred coenzyme. In native electrophoresis, GDH exhibited a seven-band anodal migrating isoenzymic pattern. The isoenzyme with the less negative charge was the more abundant (Loulakakis and Roubelakis-Angelakis 1990a, 1990b, 1991). In grapevine, GDH localizes to mitochondria, as revealed by mitochondrial isolation from protoplasts (Loulakakis and Roubelakis-Angelakis, 1990a) and also by electron microscopy immunolocalization (Paczeck et al. 2002).

In germinating lupine seeds the cathodal forms of GDH originate from induction of synthesis of the β-subunits, which are encoded by the *GDH1* gene. The complete sequence of *GDH1* in yellow lupine has been determined (Dabert and Lehmann 2004), but two other, slightly different, sequences of *GDH1* have also been found (Dabert and Lehmann 2005). Interestingly, only one sequence of the *GDH2* gene, which encodes for the GDH α-subunit, has been identified (Dabert and Lehmann 2005). The presence of several isogenes of *GDH* in yellow lupine may explain the high number (over 20) of its molecular forms in germinating lupine. These forms may come from random association of at least three distinct GDH subunits into a hexamer complex.

In most studies, assessment of GDH has usually been based on the *in vitro* enzymatic activities. Based on GDH activities determined in crude plant extracts several authors have reported a considerable variation of the NADH-GDH/NAD-GDH activity ratio, depending on the developmental stages or the environmental conditions (Loyola-Vargas and de Jimenez 1984, Cammaerts and Jacobs 1985, Leon et al. 1990, Watanabe et al. 1992) and have proposed a variable anabolic and catabolic function of GDH and its isoenzymes in plant metabolism. Furthermore the two activities of GDH were found to respond differently to the supply of different nitrogenous salts and amino acids in maize root and shoot tissues (Singh and Srivastava 1982, 1983), and were proposed to be due to changes in the physico-chemical nature of the enzyme.

However, these results have been recently reconsidered; it was shown that malate in the crude extracts resulted in erroneous over-estimation of the NAD-GDH activity through the malate dehydrogenase reaction (Fricke and Pahlich 1992, Loulakakis and Roubelakis-Angelakis 1996a). Thus, in dialyzed protein extracts of grapevine the ratio of the two *in vitro* GDH activities (aminating-/deaminating-activity) remained relatively constant although the isoenzyme profile could vary, and was dependent on the nitrogen source (Loulakakis and Roubelakis-Angelakis 1996a).

Furthermore, the aminating and deaminating activities of each of the seven grapevine GDH isoenzymes were identified by staining the two activities, following separation of the isoenzymes on polyacrylamide gels (Loulakakis and Roubelakis-Angelakis 1996a). Transfer of calluses from a nitrate- to an ammonium-containing culture medium resulted in similar alterations of the isoenzyme

profile using both, the aminating and the deaminating staining methods. Also, leaf isoenzymes showed similar ratios of *in vitro* activities and affinity for their substrates (Loulakakis and Roubelakis-Angelakis 1996a). Therefore, it was concluded that each of the seven GDH isoenzymes have similar *in vitro* anabolic and catabolic activities.

However, recent studies using tobacco transgenic plants over-expressing the genes encoding for the two subunit polypeptides of GDH fed with ^{15}N-Glu or $^{15}NH_4$ and following their fate by GC/MS or/and NMR have revealed that the *in vitro* GDH enzymatic activities do not reflect the *in vivo* GDH reaction direction(s) (Skopelitis et al. 2007). Purnell and Botella (2007), using transgenic tobacco (*Nicotiana tabacum*) plants over-expressing the tomato (*Solanum lycopersicum*) gene encoding for the β-subunit polypeptide of GDH (Purnell et al. 2005), found that GDH-isoenzyme 1 deaminates *in vivo* glutamate. On the other hand, tobacco transgenic plants over-expressing the *VvGDH-NAD;A1* gene showed high *in vivo* deaminating but also very low aminating activities under standard growth conditions (Skopelitis et al. 2007).

More specifically, the *VvGDH-NAD;A1* transcript levels in transgenic plants corresponded with GDH-total protein levels, GDH α-subunit levels, and abundance of GDH-anionic isoenzymes. Interestingly, the *in vitro* aminating and deaminating activities observed in transgenic lines did not correspond to *in vivo* activities. The *in vitro* aminating activities were very high in shoots and roots of the over-expressing transgenic lines, whereas the measured deaminating activities were significantly lower. In *VvGDH-NAD;A1* over-expressing lines, the aminating activity was increased approximately 10-fold in shoots and 2-fold in roots compared to wild type plants. The average ratio of aminating to deaminating *in vitro* activities in the *VvGDH-NAD;A1* tobacco transgenics was 14 in the shoots and 16.5 in the roots. In plants over-expressing the *SlGDH-NAD;B2* gene (Purnell et al. 2005), the respective increases in shoots were approximately 17-fold and 4-fold in roots compared to non-transformed controls. In the *SlGDH-NAD;B2* transgenics, the average ratio of aminating to deaminating activities was 19.5 and 14.0 in shoots and roots, respectively (Purnell and Botella 2007).

To assess the *in vivo* GDH enzymatic activities, transgenic plants over-expressing the *VvGDH-NAD;A1* (Skopelitis et al. 2007) and *SlGDH-NAD;B2* genes (Purnell et al. 2005, Purnell and Botella 2007, Skopelitis et al. 2007) and the respective wild type and non-transformed controls, respectively, were supplied with ^{15}N-Glu or $^{15}NH_4$. The low GDH-deaminating activities measured *in vitro*, could imply that the over-expressing lines should exhibit low *in vivo* rates of glutamate deamination but high aminating activities (Fig. 8). However, both shoots and roots of all *VvGDH-NAD;A1* or *SlGDH-NAD;B2* over-expressing lines supplied with ^{15}N-Glu for 4 h contained significantly lower levels of ^{15}N-

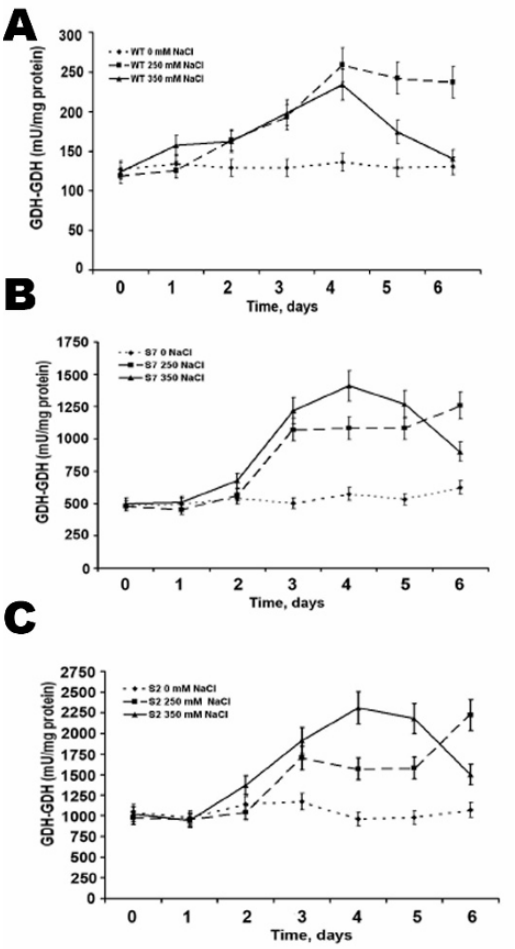

Fig. 8. Aminating activities of GDH in (A) wild-type, (B) S7 and (C) S2 overexpressing lines *VvGDH-NAD;A1* under normal or stress conditions (Skopelitis et al., unpublished).

Glu compared to wild type and non-transformed controls, respectively. More specifically, the shoots of the transgenic lines contained approximately 30% of the total amount of ^{15}N-Glu present in the shoots of wild type plants. Very similar trends were found in the shoots of the *SlGDH-NAD;B2* lines. To further confirm these findings, the accumulation of ^{15}N-NH_4 in roots and shoots of wild type and *VvGDH-NAD;A1* transgenic lines was assessed 4 h post-treatment with ^{15}N-Glu by NMR analysis. The transgenic lines accumulated more ^{15}N-NH_4 in both root and shoot compared to that of wild type, fully confirming the findings

of GC/MS analysis that the principal role of GDH is the deamination of glutamate.

In light of the high GDH-aminating activities measured *in vitro*, all overexpressing transgenic lines would be expected to exhibit high *in vivo* rates of ammonium assimilation in the presence of MSX, a potent inhibitor of GS. To test this hypothesis, plants from all transgenic lines and wild type and non-transformed plants were supplied $^{15}NH_4$ in the presence of MSX. Presence of the inhibitor resulted in approximately 90% inhibition of *in vitro* GS activity. In the presence of MSX, the levels of ^{15}N-Glu in the shoots of the wild type and non-transformed controls was approximately 25% of the amount without MSX. This labelled glutamate could be the product of the residual GS activity, together with any GDH aminating activity. If the ^{15}N-Glu originated exclusively from the residual GS activity, then the amount of ^{15}N-Glu in the presence of MSX in both groups of transgenic lines would not differ significantly from the respective controls.

This was the case for shoots and roots of the *SIGDHh-NAD-B2* overexpressing lines, in which ^{15}N-Glu levels in the presence of MSX were not statistically different from the non-transformed control. Interestingly, in shoots and roots of the *VvGDH-NAD;A1* overexpressing lines, in the presence of MSX, there was a 30% more ^{15}N-Glu than in the wild type control. These results support that GDH-isoenzyme 7 strongly deaminates glutamate but also exerts low aminating activity under normal growth conditions. In addition, it is shown that the *in vitro* aminating/deaminating GDH enzymatic activities do not reflect the *in vivo* activities and explain the existing paradox and conflict in the literature.

In conclusion, using ^{15}N-NMR and ^{15}N-GC/MS analyses, it was shown that all GDH- isoenzymes strongly deaminate glutamate whereas only the isoenzymes consisting of α-GDH subunit are able to assimilate ammonium at very low rate (Purnell and Botella 2007, Skopelitis et al. 2007). In line with these studies, both the use of mutants and transgenic plants modified for the activity of the two GDH isoenzymes should be able to give more information on the exact physiological role of the enzyme both in the context of its cellular localization restricted to the phloem and its role under carbon limiting conditions and under stress (Tercé-Laforgue et al. 2004b, Fontaine et al. 2005, Skopelitis et al. 2006, Miyashita and Good 2008). The role of GDH during the process of leaf senescence also requires further investigation considering that it is unlikely that the enzyme plays a direct role in the reassimilation of the nitrogen released during protein hydrolysis (Tercé-Laforgue et al. 2004a, Maclaux-Daubresse et al. 2006).

2.2.3.3. GDH during development

In germinating lupine seeds the cathodal isoenzymes of GDH originated from induction of *de novo* synthesis of the β-subunits. The presence of several

isogenes of *GDH* in yellow lupine may explain the high number (over 20) of its molecular forms in germinating lupine. These forms may come from random association of at least three distinct GDH subunits into a hexamer complex. The number and activity of cathodal forms increased when embryo axes devoid of cotyledons were cultured under sugar starvation (Morkunas et al. 2000, Lehmann et al. 2003), while the opposite was true after the addition of sucrose to the medium. These results indicate that the induced GDH has primarily a catabolic function and serves to facilitate the pathway (Lehmann and Rajczak 2008).

Masclaux et al. (2000) attempted to identify the turning point in the developmental cycle of tobacco leaves, where primary assimilation of nitrogen in sink leaves switches to remobilization of nitrogen in source leaves. They reinforced previous results (Loulakakis and Roubelakis-Angelakis 1991, 1992) according to which a high ammonium content was correlated with increased expression of the *VvGDH-NAD;A1* gene, with *de novo* synthesis and accumulation of the α-subunit polypeptide of GDH and also with the assembly of the more anodal GDH isoforms. Masclaux et al. (2000) reported that GS2 and Fd-GOGAT decreased in the senescing tobacco leaves, whereas GDH immunoreactive protein increased, accompanied by increased intracellular ammonium content. GDH could act in these source leaves in the aminating direction, assimilating the remobilized ammonium and complementing the GS/GOGAT pathway.

In grapevine during anthesis, when the developing flowers and young berries are high-power sinks for photosynthates and amino acids, the fully developed and physiologically active leaves in the basal part of the shoot are the ones with high Fd-GOGAT and GS protein accumulation and enzyme activity. On the other hand, it is of interest to note that GDH specific activity and protein increase in the young highly expanding leaves. This could suggest that during this period of high demand for amino acids, the two pathways of ammonium assimilation act in concert. Thus, GDH could show under these circumstances high *in vivo* aminating activity.

Post-harvesting, when senescence is initiated in all leaves along the shoot axis, as the chlorophyll content, Rubisco protein and the photosynthetic efficiency are reduced, the levels of Fd-GOGAT and GS immunoreactive protein and activity gradually decrease from the apical to the basal leaves while GDH increases substantially. As senescence is accompanied by increased proteolysis and increase in intracellular ammonium content (Masclaux et al. 2000), the role of GDH as a stress-related enzyme (Syntichaki et al. 1996, Skopelitis et al. 2006) is supported, acting probably in the aminating direction. The ammonium-detoxification effect of GDH has been shown to be linked to the entrance of glutamate in the Krebs-Henseleit cycle, and is accompanied by an increase in the enzymic activities of ornithine transcarbamoylase (OTC) and arginine decar-

boxylase (ADC), and concomitant increase in the intracellular putrescine content (Primikirios et al. 2000).

Leaf senescence is a long developmental process leading to death. Plants are static and, most of the time, nutrients are limited. Leaf senescence was selected through evolution to allow plants to improve nutrient economy and to survive upon starvation. Indeed, nitrogen depletion is known to accelerate leaf senescence and to increase its recycling and mobilization (Schulze et al. 1994). Rubisco is the main leaf N storage protein, and the degradation of this enzyme during senescence has been widely studied even though Rubisco degradation is still poorly understood. Proteolytic cleavage of Rubisco releases amino acids available for phloem loading and other interconversions. The way by which amino acids are loaded to the phloem of senescing leaves for translocation is poorly documented (van der Graaff et al. 2006). One possibility is that amino acids would be interconverted to increase the synthesis of amino acids dedicated to transport, like glutamine and asparagine.

Through the catabolism of glutamate, the deaminating activity of GDH provides ammonium as a substrate for neosynthesis of glutamine by cytosolic GS1 (Masclaux-Daubresse et al. 2006). Although GS1 and GDH are mostly root-localized, during senescence GS1 protein is detected in both leaf mesophyll and companion cells (Brugiere et al. 2000). In addition, in early senescing *Arabidopsis thaliana* lines glutamate and asparagine decrease more rapidly in contrast to late senescing lines (Diaz et al. 2008). The measurement of NR and GDH activities suggested that N assimilation decreased and glutamate catabolism increased with aging, since NR decreases and GDH increases. Interestingly, *GDH* transcript accumulated more rapidly in the early senescing *Arabidopsis thaliana* lines. The good correlation between GS1 accumulation and leaf senescence severity suggested that the N remobilization was more efficient and occurred earlier in the early-senescing lines. In addition, the increase of GDH activity with aging in *Arabidopsis thaliana* suggests that GDH participate in amino acid degradation and recycling (Masclaux-Daubresse et al. 2006).

2.2.3.4. Nitrogen metabolim during flower development

It was known long ago, especially in the Champagne area, that shortages in assimilate supply could cause flower abortion called ‘coulure’ in French, ‘shatter’ in English, thus leading to a significant decrease in crop production. This phenomenon generally occurs at the end of spring, when shoots are stronger sinks than reproductive organs, leading to an imbalance in the supply of carbon and nitrogen assimilates to the flowers (Broquedis et al. 1996). Moreover, this imbalance can be greatly affected by environmental conditions such as drought, high temperature or the amount of fertilizers applied to the soil (Ewart and Kliewer 1977). It is therefore likely that the regulation of nitrogen metabolism in relation to carbon assimilate supply is of major importance during

flower development. Therefore, studies were conducted to characterize the main reactions of nitrogen assimilation during the main steps of grapevine flower development (Fig. 4), by monitoring the changes in metabolites and enzymes classically used to depict the source to sink transition in a number of model and crop species (Masclaux et al. 2000, Hirel et al. 2005, Kichey et al. 2006).

During the early stages of flower development soluble protein content increased steadily from stage C (flower bud formation) to stage G (florets formation). This increase was followed by a sharp decrease occurring at stage H **(**separated flowers**)** occurring until fruit set (stage J). From stage G to H, proteolytic activity increased drastically concomitantly to a release of ammonia usually observed during protein hydrolysis. As shown in Fig. 4, this late phase of lower development was also characterized by the predominance of GS in the cytosol (GS1) and a strong induction of GDH localized in the phloem companion cells (Paczek et al. 2002). These two marker enzymes are representative of the transition sink to source in a variety of organs, GS1 being probably involved in the reassimilation of ammonium released as the result of protein breakdown and GDH being induced concomitantly to a built up of ammonium (Tercé-laforgue et al. 2004a, Skopelitis et al. 2006). Although the exact physiological function of these two enzymes, particularly GDH, remains to be fully elucidated it is likely that they play a major role in the supply and recycling of nitrogen assimilates from senescing leaves to set berries (Espinoza et al. 2007). Interestingly, the control of nitrogen assimilate supply for grain production in cereals has been highlighted in several studies, thus confirming that the period bracketing flowering is critical for the elaboration of yield and its components whatever the species considered (Hirel et al. 2007).

2.2.3.5. GDH during stress

A nodal point and common response in most biotic and abiotic stresses is the generation of reactive oxygen species (ROS), particularly $O_2\cdot^-$ and H_2O_2, both extra- and intracellularly, that originate in chloroplasts, mitochondria and by activation of the plasma membrane-NADPH oxidases, the cell wall-bound NAD(P)H oxidase-peroxidase, and amine oxidases (Apel and Hirt 2004, Mittler et al. 2004, Papadakis and Roubelakis-Angelakis 2005, Paschalidis and Roubelakis-Angelakis 2005b, Skopelitis et al. 2006, Moschou et al. 2008a). ROS have been viewed as toxic by-products of cellular metabolism; however, a growing body of evidence suggests that they function as signalling molecules in eukaryotes, leading to specific downstream responses (Mittler et al. 2004 and references therein), involving among others the activation of two of the *Arabidopsis thaliana* mitogen-activated protein kinases, which are essential for signal transduction in response to H_2O_2 (Kovtun et al. 2000).

Recently, Moschou et al. (2008b) showed that H_2O_2 derived from apoplastic oxidation of polyamines by polyamine oxidases participates in signal trans-

duction pathways. Efficient scavenging of ROS plays a significant role in the tolerance of plants, although the exact contribution in the response to abiotic stresses remains unknown (Hasegawa et al. 2000).

Under stress conditions, such as salinity, increased proteolytic activity results in increase of the intracellular titers of ammonia, and eventually hyperammonia and toxicity, if not efficiently removed (Lutts et al. 1999, Skopelitis et al. 2006). In grapevine suspension cells, calli and leaf discs NaCl resulted in increased intracellular ammonium ions, due to increase in protease activity, and induced the expression of the *VvGDH-NAD;A1* gene, which encodes for the α-subunit (Fig. 9; Skopelitis et al. 2006). Thus in grapevine, salinity resulted to increased GDH immunoreactive protein and α-subunit, while in native electrophoresis the anionic iso-GDHs predominated resulting to increased GDH *in vitro* aminating activity. The previous were also observed by the addition of menadione to grapevine cell suspension cultures, a prooxidant, suggesting that ROS are responsible for the induction of the *VvGDH-NAD;A1* gene, which results to similar induction of the corresponding immunoreactive protein and activity (Skopelitis et al. 2006).

To confirm the validity of *in vitro* measurements of aminating activity of GDH, tobacco plants were supplied with $^{15}NH_4^+$, and GC-MS analyses of ^{15}N-Glu, ^{15}N-Gln, and ^{15}N-Pro were performed at different time points (Fig. 10). Be-

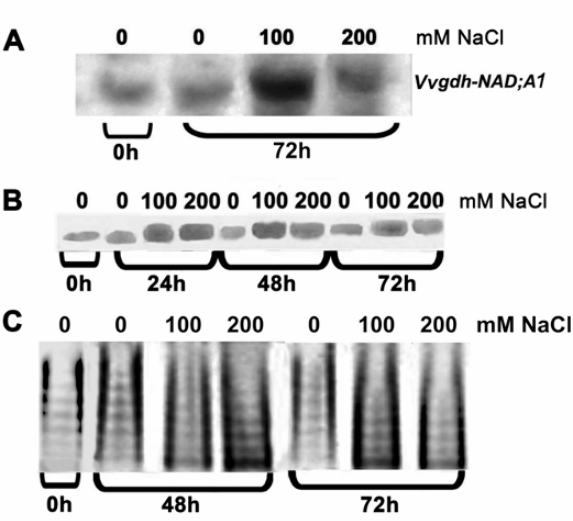

Fig. 9. RNA-gel blot analysis of the *Vvgdh-NAD;A1*, Western blot analysis for the GDH protein, and in-gel GDH aminating activity assay under normal or stress conditions. (A) RNA-gel blot analysis for the *VvGDH-NAD;A1* mRNA 0 and 72 h post-treatment at 0, 100 and 200 mM NaCl. (B) Western blot analysis for the GDH protein 0, 24, 48, 72 h post-treatment at 0, 100 and 200 mM NaCl. **(C)** Isoenzymic profile of GDH; assembly of the anionic iso-GDHs 0, 48, 72 h post-treatment at 0, 100 and 200 mM NaCl (modified from Skopelitis et al. 2006).

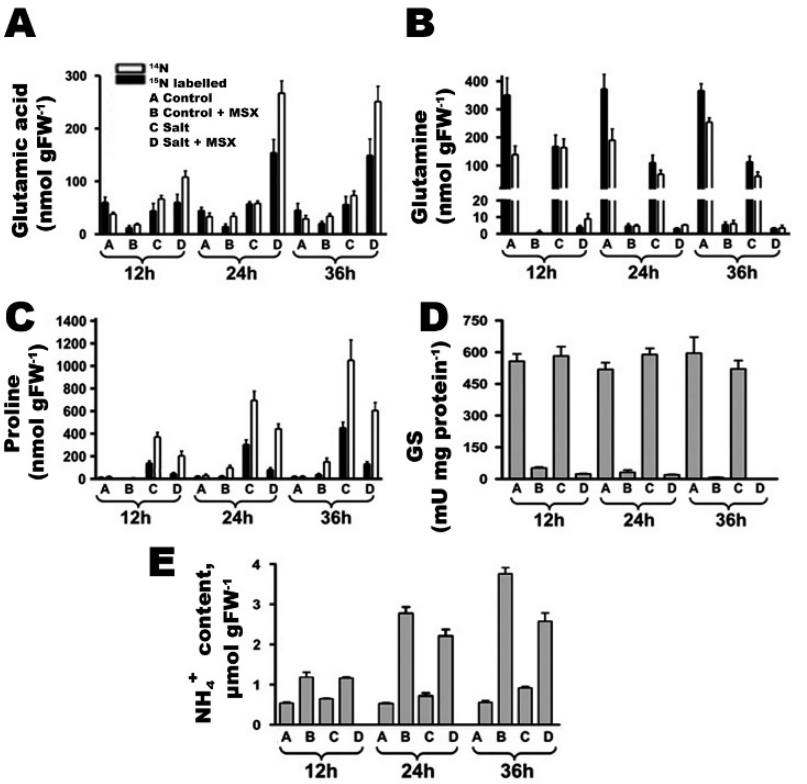

Fig. 10. GC-MS analysis of the fate of $^{15}NH_4$ in shoots of *N. tabacum* cv Xanthi plants grown in the presence of NaCl. Labelled and non-labelled (A) Glu, (B) Gln, (C) Pro, (D) GS activity and (E) ammonium ion accumulation in plants grown in the presence of 250 mM NaCl at 12, 24, and 36 h after $^{15}NH_4$ application. Data are the means of four replicates, and bars represent ±SE. FW, fresh weight (modified from Skopelitis et al. 2006).

cause expression of *GDH-NAD;A1* was evident 24 h following salt treatment, plants were treated with 250 mM NaCl for 24 h prior to the addition of $^{15}NH_4^+$. To exclude the possibility of assimilation by the GS, addition of $^{15}NH_4^+$ was performed in the presence of MSX in the shoots of control. A 3-fold higher glutamate was observed in the salt-treated plants in comparison with the corresponding controls. Labelled and non-labelled glutamine were negligible in MSX-treated plants, in agreement with the drastic reduction of GS activity (data not shown). ^{15}N-Pro showed a timely-dependent accumulation post-treatment with salt, showing a 4-fold increase 36 h post-treatment, compared to control plants. In the presence of MSX, ^{15}N-Pro accumulation occurred at a lower rate, explaining also the higher level of ^{15}N-Glu. Similar trends were observed for the non-labeled Pro (Skopelitis et al. 2006).

Ammonium is not the principal signal for *GDH-NAD;A1* gene induction, since *GDH-NAD;A1* followed the increase of ROS rather than that of ammonium increments in salt-treated grapevine cells/calluses/leaf discs/plants. For ammonium to induce *GDH-NAD;A1*, it has to reach an intracellular threshold before being able to generate sufficient levels of ROS to act as a signal. These results allow to propose that ROS participate in the signalling pathway, and either alternatively or additively, they result in proteolysis contributing to intracellular hyperammonia.

Although GS/GOGAT are the principal enzymes for the assimilation of ammonium in plant cells (Lea and Miflin, 1974), their activities in salt-treated tobacco remained largely unchanged in contrast with the *in vivo* aminating activity of GDH, which increased. When photorespiratory ammonium accumulates, as a result of impaired GOGAT activity (Ferrario-Mery et al. 2002) or when GS becomes limiting (Harrison et al. 2003), alternative metabolic pathways may be activated to avoid ammonium accumulation. One pathway could be the GDH activity.

The fact that the ^{15}N-Glu produced in salt-treated plants was shifted toward ^{15}N-Pro synthesis further supports the idea that these molecular responses are induced as a reaction to stress. Proline accumulation in stressed plants has been associated with enhanced tolerance to conditions of abiotic stress (Nanjo et al. 1999a, 1999b). In addition to proline synthesis, the ammonia detoxification effect of GDH links glutamate with the Krebs-Henseleit (urea) cycle, thus providing the substrates for polyamine biosynthesis. Under hyperammonia, glutamate is shifted to synthesize ornithine, which is recycled *via* arginase as part of the urea cycle (Paschalidis and Roubelakis-Angelakis 2005a) and may be the substrate for proline synthesis. Both ornithine and arginine are used for the biosynthesis of polyamines (Paschalidis and Roubelakis-Angelakis 2005a, 2005b), which may also play a role in the protection of plants against abiotic stress (Capell et al. 2004, Moschou et al. 2008b).

As the induction of *GDH-NAD;A1* by salt is accompanied by increased *in vivo* aminating GDH activity, it is expected that the genes encoding for the enzymes that mediate α-ketoglutarate synthesis should be upregulated as well. Two types of I(C)DH have been identified in plants (Hodges 2002). The first is IDH, a tricarboxylic acid cycle enzyme localized in the mitochondria that uses NAD^+. The second is ICDH, consisting of several isoenzymes, localized to the cytosol (Gálvez et al. 1996), chloroplasts (Gálvez et al., 1994), mitochondria (Gálvez et al. 1998), and peroxisomes (Corpas et al. 1999). Both enzymes are potential candidates for supplying α-ketoglutarate for the amination reactions of GOGAT and GDH. I(C)DHs supply the cell with α-ketoglutarate to form glutamate (Lancien et al. 1999). Increase in the transcript of *NtGDH-NAD;A1* and immunoreactive GDH protein as a result of NaCl treatment was accompanied

by similar trends in the transcript(s) and protein of I(C)DH. Thus, the abundance of *NtIDH;A* and *NtcytIDHh* transcripts (Gálvez et al. 1994) and the levels of immunoreactive mtICDH and cytICDH proteins were examined in the shoots of tobacco seedlings 36 and 48 h after salt treatment (250 mM NaCl) with and without MSX. RNA gel blot analysis revealed that the increase in the *NtGDH-NAD;A1* transcript in the shoots of salt-treated plants (with and without MSX) at 36 h was accompanied by a concomitant increase in the transcript of *NtIDH;A* but not in plants treated only with MSX (Skopelitis et al. 2006).

With regard to *NtcytICDH*, a 3-fold increase was observed only in the shoots of salt-treated plants. Westnern blot analysis showed an increase in the levels of mtICDH 48 h post-treatment in the presence and absence of MSX. Similarly, a 5.2- and 2-fold increase in the levels of cytICDH immunoreactive protein was found in salt-treated plants with and without MSX, respectively. The increase in the *NtGDH-NAD;A1* transcript in the shoots of salt-treated plants was accompanied by a concomitant increase in the transcripts of *NtIDH;A* and *NtcytICDH* and in the levels of mtICDH and cytICDH immunoreactive proteins. These results suggest that, in the presence of salt, both enzymes could supply α-ketoglutarate for glutamate synthesis by GDH (Skopelitis et al. 2006).

Quantitative genetic approaches strongly suggest that the reaction catalyzed by NAD(H)-GDH is of major importance in the control of plant growth and productivity (Dubois et al. 2003). Compared with wild type plants, transgenic tobacco over-expressing bacterial *GDH* (α-subunit of GDH) showed increased tolerance to herbicides and to toxic levels of ammonia as well as higher biomass (Ameziane et al. 2000). Plants transformed with the α- and β-subunits of GDH from *Chlorella sorokiniana* showed increased growth rates and improved stress tolerance (Schmidt and Miller 1999), whereas a maize (*Zea mays*) *gdh* null mutant was cold temperature sensitive (Pryor 1990). Fruits from transgenic tomato plants carrying a gene for NADP-GDH from *Aspergillus nidulans* contained 2- to 3-fold more free amino acids and 2-fold more Glu (Kisaka and Kida 2003), which has been proposed as a signalling molecule in plant growth and environmental response (Glevarec et al. 2004). Moreover, grapevine cultivars tolerant to drought stress showed increased GDH activity during stress (Toumi and Roubelakis-Angelakis, unpublished).

In line with these studies, both the use of mutants and transgenic plants modified for the activity of the two GDH isoenzymes should be able to give more information on the exact physiological role of the enzyme both in the context of its cellular localization restricted to the phloem and its role under carbon limiting conditions (Tercé-Laforgue et al. 2004b, Fontaine et al. 2005, Miyashita and Good 2008). The role of GDH during the process of leaf senescence also requires further investigation considering that it is unlikely that the enzyme

plays a direct role in the reassimilation of the nitrogen released during protein hydrolysis (Tercé-Laforgue et al. 2004a, Maclaux-Daubresse et al. 2006, Skopelitis et al. 2006).

3. REGULATION OF AMMONIA ASSIMILATING ENZYMES BY NITROGEN SOURCE IN GRAPEVINE

The effect of exogenously supplied nitrogen on expression of genes encoding for GS, GOGAT and GDH in plants remains uncertain and varies with species, experimental conditions and the specific member of each gene family examined. The expression of the three enzymes in grapevine, especially GDH, under different nitrogen sources has received considerable research attention over the past decade. The results from grapevine cell suspensions and calli clearly show that the nitrogen source in the culture medium strongly affects expression of GS, Fd-GOGAT and GDH at activity, protein and mRNA levels (Loulakakis and Roubelakis-Angelakis 1996b, 1997, Skopelitis et al. 2006).

Presence of ammonium or glutamine in the culture medium of grapevine cells caused a decrease in both, enzyme activity and protein of chloroplastic and cytosolic isoforms of GS, while nitrate had a positive regulatory effect. Furthermore, change in the nitrogen source resulted in differential pattern of expression of the *pGS1;1 pGS1;2*, *pGS1;3* genes (Loulakakis and Roubelakis-Angelakis 1996b). Depending on the plant species, the type of tissue and the experimental conditions, either positive or negative regulatory effects of the nitrogen source on *GS* gene expression have been reported (Hayakawa et al. 1990, Stanford et al. 1993, Sukanya et al. 1994). These results indicate that differential regulation of gene expression significantly affected the evolution of GS_1 gene families (Li et al. 1993). In fact, the multiple GS genes in plants could provide a mechanism for the differential regulation of GS synthesis in various tissues in response to different requirements for ammonium assimilation (Cren and Hirel 2002).

In the case of Fd-GOGAT, the results confirmed a clear inductive effect of nitrates on *Fd-GOGAT* transcript level in grapevine cell cultures. In contrast, supply of ammonium, as the sole source of nitrogen, resulted in decreased *Fd-GOGAT* transcript levels (Loulakakis and Roubelakis-Angelakis 1997). In other plant species, a positive response to nitrate for *Fd-GOGAT* transcript, polypeptide content and activity has been noticed (Hayakawa et al. 1990, Redinbaugh and Campbell, 1993), while ammonium either did not affect or suppressed the expression of *Fd-GOGAT* (Hecht et al. 1988, Hayakawa et al. 1990, Oaks 1994). The inhibitory effect of ammonium on Fd-GOGAT could be related to a more general ammonium toxicity phenomenon, as previously reported (Hecht et al. 1988). Further study on the expression of GDH, GS and GOGAT in both cell

cultures and whole grapevine plants, under varying trophic and environmental conditions, would further elucidate their contribution in ammonia assimilation.

The total quantity of α- and β-subunits and the isoenzymic pattern of GDH were altered by the exogenous nitrogen source. Protein extracts derived from calli grown on medium containing nitrates or glutamic acid contained a slightly greater amount of β-subunit and cathodal isoenzymes, whereas α-subunit and the more anodal isoenzymes predominated in callus grown in the presence of either ammonium or glutamine (Loulakakis and Roubelakis-Angelakis 1991, 1992, Skopelitis et al. 2006). This positive effect of ammonium on the GDH enzyme system was concentration-dependent. Transfer of grapevine calli from culture medium containing only nitrates, as a nitrogen source, to culture media containing increasing concentrations of ammonium resulted to a significant increase in NADH-GDH activity and total GDH protein. These changes were accompanied by a decrease in the protein of the more cathodal isoenzymes and an increase in the protein of the more anodal ones (Roubelakis-Angelakis et al. 1991).

Furthermore, *in vivo* labelling of *de novo* synthesized proteins in grapevine callus with ^{35}S-methionine revealed a considerable increase in the synthesis of α-subunits in the presence of ammonium while there was not detectable synthesis of β-subunits. Thus, the increase in the activity, α-subunit content and in the intensity of staining of the more anodic isoenzymes is caused by the *de novo* synthesis of the α-subunit of GDH (Loulakakis and Roubelakis-Angelakis 1992). Two-dimensional analysis of the *de novo* synthesized isoenzymes of GDH indicated a low rate of synthesis of all isoenzymes in the presence of nitrates and confirmed the synthesis of the more anodal isoenzymes in the presence of ammonium. These results support that in nitrate-grown calli the combination of the two *de novo* synthesized subunits results in the appearance of radioactivity in all the isoenzymes.

In contrast, in ammonium-grown callus the radioactive more anodic isoenzymes are produced by the assembly of *de novo* synthesized α-subunits and pre-existing β-subunits (Loulakakis and Roubelakis-Angelakis 1992). These results could support that ammonium does not cause activation of GDH, as it was proposed (Srivastava and Singh 1987), but that it induces expression of the gene encoding for the α-subunit, and therefore the regulation of GDH is exerted by differential expression of the respective genes. In fact, grapevine cell suspensions cultured in the presence of ammonium expressed significantly higher levels of the mRNA corresponding to *pGDH5* gene. In addition, similarly to calli, cell suspensions cultured in ammonium containing media showed significantly higher specific activity of GDH accompanied by an accumulation of the α-subunit of the enzyme and predominance of the more anodal isoenzymes (Skopelitis et al. 2006). The increased accumulation of GDH in the ammonium-grown cells may suggest a role of this enzyme in the direction of nitro-

gen assimilation, at least as a detoxification reaction (Skopelitis et al. 2006).

Immunolocalization experiments were performed to study the compartmentalization of N metabolism during the development of grape berries. This study highlighted the complex compartmentation of aspartate amino transferase, alanine amino transferase, GS and GDH between and within different cell types (Famiani et al. 2000). This complex compartmentalization may be important in determining fruit quality during maturation since alanine and arginine are the most abundant amino acids detected in fruit juice (Kluba et al. 1978). Alternatively the isoenzymatic profile of some of these enzymes may be used as marker for screening grape varieties (Collet et al. 2005).

4. FUTURE PERSPECTIVES

Nitrogen metabolism is a prerequisite for normal plant growth and development, since almost all the 'vital' biomolecules belong to the class of nitrogenous substances. In particular, glutamate and glutamine, which are the central amino acid synthesized by the principal enzymatic reaction(s) of ammonium assimilation, are both the starting substrates for the synthesis of numerous nitrogenous molecules to ensure plant growth and development. Although, these two molecules are essential in the nitrogen economy of grapevine, further research will be required to identify if other associated or independent metabolic pathways are involved in the control of nitrogen use efficiency at different stages of plant development.

As already developed for perennial species (Hirel et al. 2007), an approach that integrates genetic, physiological and agronomic studies of grapevine nitrogen response will be necessary to elucidate the regulation of nitrogen use efficiency for adjusting fertilization and thus obtaining optimal yield and berry quality (Jackson and Lombard 1993). This type of approach will allow to identify which are the main drivers involved in the control of nitrogen assimilation and recycling during grapevine growth and development, particularly during the supply of assimilates to the developing flowers and berries. This will include extensive genetic and biochemical phenotyping on different genotypes grown under low and high nitrogen fertilization applications to identify which are the key genes, proteins, enzymes and metabolites involved. Although grapevine is a woody species, has a long generation time and possesses a complex system for N reserve partitioning and mobilization (Zapata et al. 2004), it is now possible to take advantage of the sequence of the grapevine genome (Jaillon et al. 2007) to further investigate the plant genomic response to nitrogen fertilization and its regulation.

Nothing is known about the genetic determinism of nitrogen use effi-

ciency in grapevine, although this trait is likely to be of major importance for both wine and table grape breeding. It will be therefore interesting to identify the QTLs (Quantitative Trait Loci) for berry yield and quality (Doligez et al. 2006), in relation to nitrogen use efficiency, using a similar approach that was already successfully conducted both in model and crop species (Hirel et al. 2007). Hopefully, the contribution of these approaches will improve our knowledge of the genetic determinism for nitrogen assimilation and recycling, as well as their potential usefulness for marker assisted breeding of new grape varieties with improved nitrogen use efficiency.

In addition, the possibility to perform genetic engineering in grapes (Paoletti and Pimentel 1996) opens new perspectives toward a better understanding of the control of N metabolism on plant growth and development in this species, as already successfully achieved in another woody species, such as poplar (Kirby et al. 2006).

Our recent findings on the role of N assimilation during stress which involves the anodal isoforms of GDH are summarized in a model (Fig. 11). Thus,

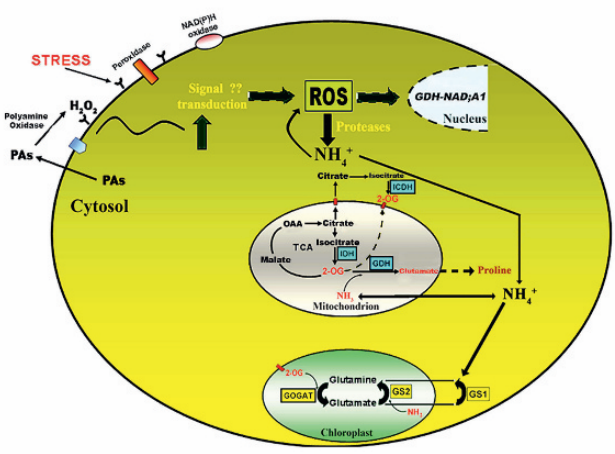

Fig. 11. Proposed model for the assimilation of stress-induced proteolysis-derived ammonium anions by the GDH pathway (Skopelitis et al. 2006, Moschou et al. 2008b).

a perceived exogenous stimulus which initiates a cascade of events that generates ROS accumulation, induces the *GDH-NAD;A1* gene encoding for the anodal GDH isoforms. High ROS accumulation induces, among other, proteases that are responsible for proteolysis-dependent hyperammonia. The increased ammonium anions are re-assimilated, at least partly, through the anodal GDH isoforms, to produce glutamate shifted to proline.

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8

POLYAMINES IN GRAPEVINE: An Update

K.A. Paschalidis[1], P.N. Moschou[1], A. Aziz[2], I. Toumi[3] & K.A. Roubelakis-Angelakis[1]

[1]Department of Biology, University of Crete, P.O. 2280, 71409 Heraklion, GREECE

[2]URVVC Stress & Environment EA 2069, Lab. PPDD, University of Reims, B.P. 1039, 51687 REIMS Cedec 02, FRANCE

[3]Laboratory of Grapevine Molecular Physiology, Route Touristique de Soliman 95, Hammam-Lif 2050, TUNISIA

1. INTRODUCTION

The polyamines (PAs) putrescine (Put), spermidine (Spd) and spermine (Spm) exist as free (soluble; S-), or as conjugated to small (SH-) or to large (PH-) molecules. Increasing evidence supports that PAs represent important intrinsic developmental signals, which may affect developmental processes that range from cell division and morphogenesis to stress responses (Perez-Amador et al. 2002, Fos et al. 2003, Arias et al. 2005, Paschalidis et al. 2001, Paschalidis and Roubelakis-Angelakis 2005a, b, Cona et al. 2006, Vuosku et al. 2006, Kusano et al. 2007, Agalou et al. 2008, Moschou et al. 2008a, b, c).

The first step in PA biosynthesis is the formation of Put from Orn and Arg *via* the rate limiting enzymes Arg decarboxylase (ADC; EC 4.1.1.19) and Orn decarboxylase (ODC; EC 4.1.1.17), respectively. Put is converted into Spd and Spm by Spd synthase (EC 2.5.1.16) and Spm synthase (EC 2.5.1.22), respectively, which add aminopropyl groups generated from *S*-adenocylmethionine (SAM) by SAM decarboxylase (EC 4.1.1.50; Tiburcio et al. 1997). PAs are also catabolized through the activity of one or more diamine oxidases (DAO; EC 1.4.3.6), which oxidize the primary amino groups of the diamine Put, and PA oxidases (PAO; EC 1.5.3.11), which oxidize the secondary amino groups of Spd and Spm (but not other PAs) yielding Δ^1-pyrroline and 1,5-diazabicyclononane, respectively, along with 1,3-diaminopropane (Dap) and H_2O_2 (Fig. 1, Cohen 1998). A follow-up of our previous review on PAs in grapevine (Paschalidis et al. 2001) is attempted in this edition.

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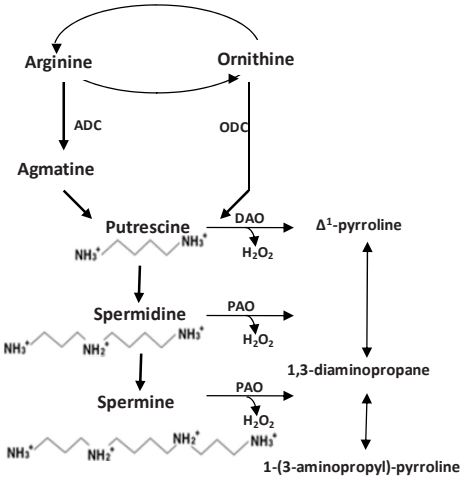

Fig. 1. Biosynthetic and catabolic pathways of polyamines in higher plants. Arginine decaroxylase (ADC), ornithine decarboxylase (ODC), diamine oxidase (DAO), polyamine oxidase (PAO).

2. POLYAMINE TITERS DURING GRAPEVINE DEVELOPMENT

Recently, PA titers were analyzed in a tissue/organ specific manner along the tobacco (Paschalidis and Roubelakis-Angelakis 2005a,b) and grapevine (unpublished) plant axis and were correlated with detailed expression patterns of the anabolic and catabolic genes. In tobacco plants PAs homeostasis in meristems, leaves, petioles, internodes and roots was documented as a part of the control of cell cycle progression, cell division/expansion and differentiation in the developing tobacco plant (Paschalidis and Roubelakis-Angelakis 2005a,b). In the woody perennial grapevine plant, Orn transport from young to old tissues with parallel enhancement of the ODC pathway led to an inverse, to the other PAs, Put gradient with high levels of Put accumulating in the basal plant part and in roots, suggesting different homeostatic mechanisms between the two species. Furthermore, PA catabolism by enhanced expression of amine oxidases, which is induced by abscisic acid (ABA), generated H_2O_2 which correlated with the levels of peroxidases (POXs) and phenolics during vascular differentiation.

More specifically, in grapevine plants total (S+SH+PH)-Put increased with explant age, whereas total Spd and Spm decreased (Table 1); the total PA content in leaves (lamina and petiole) of grapevine decreased from 878±74 to 613±56 nmol g^{-1} FW in the youngest (1^{st}) to the oldest (25^{th}) leaf, respectively (Table 1). The individual tot-Put, tot-Spd and tot-Spm contents ranged from

190±13 to 464±14, from 538±10 to 195±8 and from 150±6 to 37±3 nmol g^{-1} FW, respectively, from the youngest to the oldest leaf. All fractions (S, SH and PH) of Spd and Spm decreased with increasing ontogenic stage and age of plant organs and reached very low levels in the senescing (25th) ones. Spd decreased more than 2-fold from younger to older lamina tissues and even more in the vascular tissues (petioles and internodes); in the youngest internodes, Spd levels were almost 6-fold higher than in the oldest ones. Furthermore, very low levels of Spm were found in roots while PH-Spm was below the detection limit in old leaves (Table 1). However, all the three fractions of Put increased with age. High Put content has been correlated with higher biomass production, meaning that pool sizes of Put increase when strong growth occurs (Tkachenko et al. 2001, Ioannidis and Kotzabasis 2007); in grapevine, high Fv/Fm values were accompanied by high values of cell volume and area, and coincided with high Put titers in old leaves (Table 1).

Table 1. Polyamine titers (nmol g^{-1} FW), polyamine ratios (%) in apical (1st) and basal (25th) leaves of grapevine and Fv/Fm ratios in the 1st, 5th, 15th and 25th leaves of grapevine. Data are the means ± SE (Paschalidis et al. unpublished).

Polyamine titers		Leaf age 1st	Leaf age 25th
Put	S	87 ± 6	259 ± 4
	SH	76 ± 5	145 ± 7
	PH	27 ± 2	63 ± 3
	Total	190 ± 13	464 ± 14
Spd	S	169 ± 4	57 ± 3
	SH	359 ± 5	135 ± 5
	PH	9 ± 1	3 ± 0
	Total	538 ± 10	195 ± 8
Spm	S	74 ± 3	17 ± 1
	SH	72 ± 2	20 ± 2
	PH	4 ± 1	ND
	Total	150 ± 6	37 ± 3
Put+Spd+Spm	S	330 ± 28	333 ± 22
	SH	508 ± 41	300 ± 19
	PH	40 ± 5	67 ± 15
	Total	878 ± 74	700 ± 56
Polyamine ratios (%)		Leaf age 1st	Leaf age 25th
S/Total		37.59 ± 2.6	40.13 ± 2.3
SH/Total		57.86 ± 4.7	48.94 ± 4.1
PH/Total		4.56 ± 3.2	10.93 ± 5.7
Fv/Fm Ratios			
1st leaf		0.58 ± 0.06	
5th leaf		0.72 ± 0.05	
15th leaf		0.74 ± 0.03	
25th leaf		0.81 ± 0.05	

3. PUTRESCINE COINCIDES WITH HIGH PHOTOSYNTHETIC EFFICIENCY

In most annual species, plant leaf area depends on individual leaf area/expansion because the number of leaves is genetically determined; in contrast, in most woody species, such as the grapevine, plant leaf area depends more on leaf number than on individual leaf area (Lebon et al. 2004). In order to determine whether PA abundance in the grapevine leaf precisely correlates with expansion, it was necessary to map spatial and temporal changes during development of grapevine plants. As grapevine leaves got older, Spd and Spm levels and their synthesis decreased, whereas Put increased (Table 1 and Fig. 2). On the contrary, both mesophyll cell volume (and area) and epidermal cell area consistently increased (data not shown); maximum quantum yield of photosystem II (Fv/Fm) also increased early from the youngest (1^{st}; 0.58±0.06) to young (5^{th}; 0.72±0.05) grapevine leaves and remained at consistently high values thereafter until the 25^{th} leaf (0.81±0.05). Therefore, in grapevine, Spd and Spm abundance and distribution negatively correlated with the extent and distribution of cell expansion and cell size; Put, in contrast coincided with these parameters and also with maximum photosynthetic efficiency, as reflected by Fv/Fm (Table 1).

4. POLYAMINE ANABOLISM IN GRAPEVINE

In grapevine leaves, all the biosynthetic enzyme activities (ADC, ODC SAMDC, SPDS and SPMS) decreased with leaf age (Fig. 2A). The transcripts of *ADC, ODC, SAMDC,* and *SPDS* also decreased with increasing tissue age (Fig. 2B). Both ADC and ODC activities decreased basipetally from marginal to central leaf area and to petiolar and internodal regions (Fig. 2A and data not shown). The *ODC* transcripts (Fig. 2B) were in accordance with ODC activities and counteracted both spatially and temporally the endogenous Put levels. However, the expression of the *ADC* gene (Fig. 2B) along with the immunoreactive ADC protein (Fig. 2C) were higher in the vascular than in the leaf laminae regions, coinciding with the Put spatial gradient. The expression of SAMDC, in terms of both SAMDC specific activities and the abundance of *SAMDC* mRNA followed a similar to ADC expression pattern, whereas SPDS and SPMS activities, along with *SPDS* transcripts, generally spatially increased from leaf margins to internodal regions (Fig. 2).

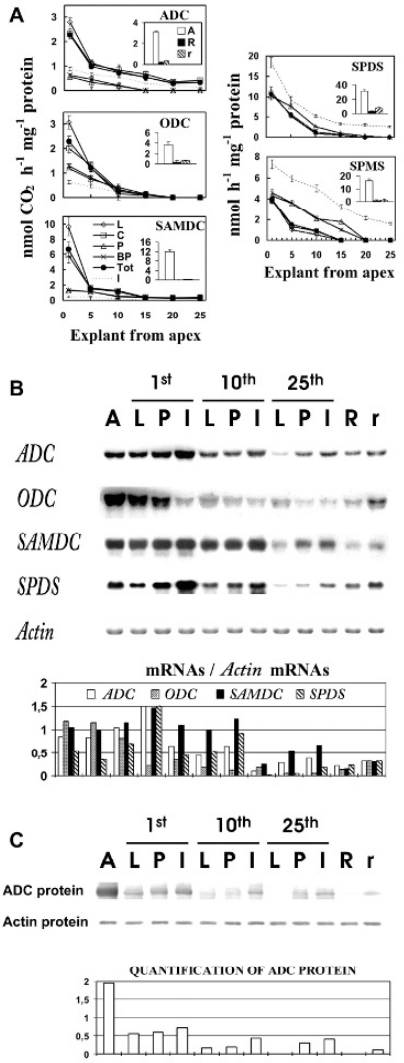

Fig. 2. Expression of PA anabolic enzymes in the grapevine plant. A, Specific activities of ADC, ODC, SAMDC, SPDS and SPMS in the soluble fractions from grapevine explants at different developmental stages. L, apical and marginal leaf lamina; P, acropetal petiole; Tot, total leaf with petiole; I, internode. Numbering started from the apical organ being designated as 1st. Inserted graphs depict specific activities in: A, shoot apex; R, primary root; r, secondary root. Error bars represent ± SE. B, RNA gel blot analysis of the expression patterns of *ADC, ODC, SAMDC* and *SPDS* genes. Quantitation of mRNA levels based on actin mRNA levels (mRNAs/actin mRNAs) is shown on graph. C, Western gel blot analysis of the ADC protein expression patterns (top). Hybridization with anti-actin polyclonal antibody (bottom). Graph shows the quantitation pattern after densitometric analysis of the western blots (Paschalidis et al., unpublished).

5. POLYAMINE CATABOLISM IN GRAPEVINE

The specific activities of POXs and amine oxidases increased with age in hypergeous grapevine tissues; they were also higher in internodes, lower in petioles, and even lower in leaves (Fig. 3). POX activity and expression of amine oxidases (Fig. 3) are therefore associated mostly with tissues undergoing lignification and less with photosynthetic ones. We have previously shown a similar pattern of POXs and amine oxidases in the tobacco plant (Paschalidis and Roubelakis-Angelakis 2005b) and an association of the three main PAs (Put, Spd, and Spm) with thylakoid membranes and with various photosynthetic subcomplexes (Kotzabasis et al. 1993)

Reactive oxygen species (ROS) are engaged in the biogenesis and modification of cell wall structure (Papadakis and Roubelakis-Angelakis 1999, Papadakis et al. 2001, Papadakis et al. 2005, Papadakis and Roubelakis-Angelakis 2005) and in ammonia detoxification (Skopelitis et al. 2006). These data are reinforced by this work and allow envisioning a new scenario of the molecular events regulating cell wall differentiation and vascular tissue formation during development. In older vascular tissues, the synergistic action of amine oxidases and POXs plays a role in differentiation. Progress in plant development results in the induction of DAO, PAO and POX (H_2O_2-dissipitating function) expression in vascular tissues throughout the grapevine plant axis, along with an increase in H_2O_2 accumulation. This event is probably linked to the physiological requirement for higher production of H_2O_2 *via* amine oxidases in the apoplast to drive POX-catalyzed cross-linking and lignification to complete cell wall stiffening and differentiation.

6. POLYAMINES AND OSMOTIC STRESS

6.1. Free PAs and osmotic stress

Here, we report the response of grapevine leaf discs to osmotic stress induced by polyethylene glycol (PEG-6000) and mannitol. We propose a model consistent with a mechanism linking PA metabolism to osmotic tolerance and possibly tolerance to biotic stress.

PEG-treated grapevine leaf discs showed a 2-fold increase in free agmatine (Agm), Put and Spd contents compared with control, while the levels of Spm and Dap, a product of polyamine oxidation, were not altered (Fig. 4). Similarly, leaf discs treated with mannitol, a non metabolic sugar osmotic agent, also exhibited a high accumulation of free Put and Spd. The results suggest that grapevine has a large capacity to enhance PA amounts in leaves in response to water or osmotic stress.

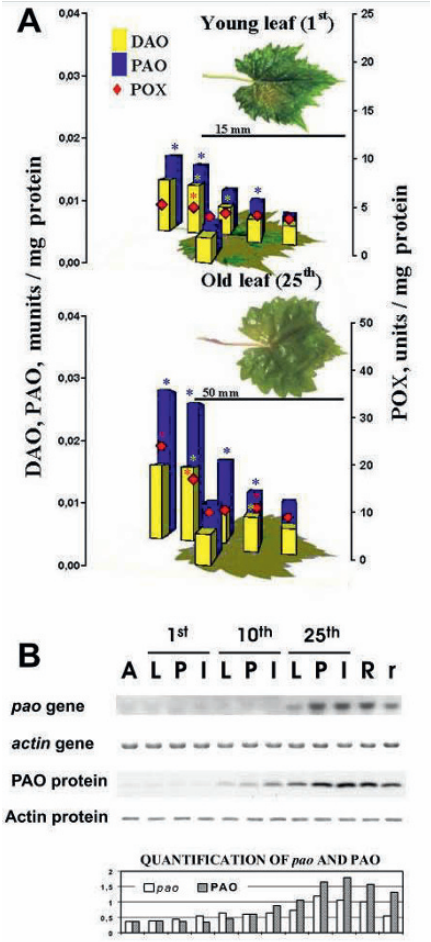

Fig. 3. Expression of PA catabolic enzymes in the grapevine plant. A, Specific activities of DAO, PAO and POX in soluble fractions of the youngest (1st) and the oldest leaf (25th). Asterisks represent statistically significant differences from the preceding acropetal tissue-point. B, RNA and western gel blot analysis of the expression patterns of the *PAO* gene and PAO protein. Quantitation of mRNA (mRNAs/actin mRNAs) and protein levels (PAO protein/actin protein) are shown (Paschalidis et al., unpublished).

The role of PAs in plant defense to water stress could be dependent on PA forms and stress degrees. In other experiments performed with different cultivars of grapevine, we also showed a positive correlation between high PA titers and osmotic stress tolerance under *in vitro* conditions. Pinot noir, which is characterized by high accumulation of free PAs, exhibited an enhanced tolerance to osmotic stress than Chardonnay and Merlot (Aziz et al. unpublished). Under these conditions, the levels of conjugated Put and Spd in both the soluble

and insoluble fractions were also significantly increased with respect to controls (data not shown). The levels of PA conjugates remained however much lower than the levels of free PAs, whereas no significant differences were found among the cultivars in the conjugated forms of Put, Spd, or Spm.

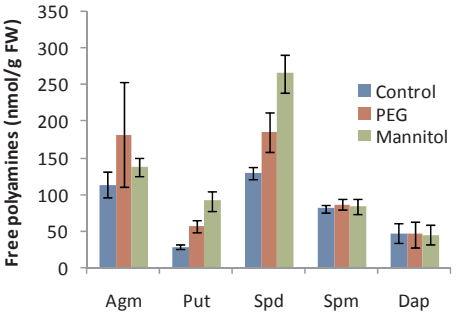

Fig. 4. Free polyamine levels in grapevine leaf discs in response to osmotic stress. Discs excised from mature leaves were incubated in a reference medium (control) in the presence of PEG 6000 or mannitol (-1.5 MPa). Data are means ± SE, each from 24 leaf discs after 50 h of incubation (Aziz et al., unpublished).

6.2. PAOs and osmotic stress

Treatment of grapevine leaf discs with aminoguanidine (AG), a DAO inhibitor, resulted in a strong accumulation of free Agm, Put and Spd (Fig. 5). This accumulation was amplified in the presence of osmotic stress, except for Spd. The Spm and Dap content did not show significant changes.

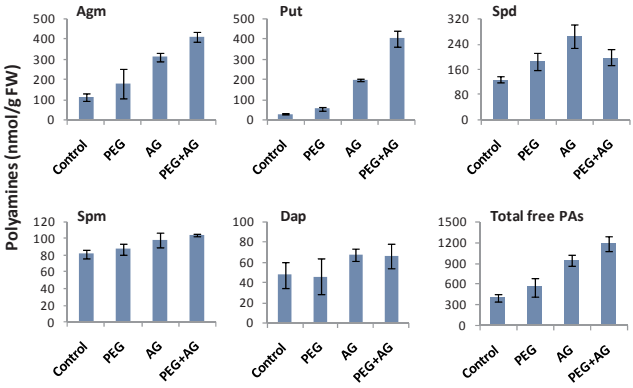

Fig. 5. Effect of osmotic stress and the DAO inhibitor, aminoguanidine (AG), on free polyamine levels in grapevine leaf discs. Discs excised from mature leaves were incubated in a reference medium (control) or in a PEG 6000 solution (-1.5 MPa) alone, or in the presence of 1 mM AG. Data are means ± SE, each from 24 leaf discs after 50 h of incubation (Aziz et al., unpublished).

The total free PA content was higher in leaf discs treated with AG alone or after PEG-mediated stress induction. These results suggest that the stress responses monitored at the PA level involve not only an increase in PA biosynthesis, but also a stimulation of diamine oxidation. Use of AG indicated that Put oxidation was responsive to osmotic stress conditions. The results supported that the biosynthesis of Spd from Put and SAM in grapevine leaf discs was not blocked. AG promoted also an increase in Agm level in both, the control and the stressed leaf explants and slightly enhanced Dap accumulation. This could rely on stress-enhanced ADC activity, and Put and Spd catabolism in leaf tissues.

7. POLYAMINE METABOLISM AND RESISTANCE RESPONSES

7.1. PA biosynthesis and phytoalexin production

To determine whether osmotic stress and PA biosynthesis pathways can modulate basal resistance or susceptibility of grapevine to pathogen infection, we examined the effect of PEG treatment with or without α-difluoromethylarginine (DFMA) and α-difluoromethylornithine (DFMO), specific inhibitors of ADC and ODC, respectively, on the induction of phytoalexins and photosynthetic efficiency in grapevine leaf discs. Phytoalexin biosynthesis in grapevine plants is an important element of defense responses to (a)biotic stress. Resveratrol and its dimer, ε-viniferin, are the major phytoalexins produced by grape (*Vitis* spp.) in response to microbial attacks and abiotic elicitors (Coutos-Thévenot et al. 2001, Aziz et al. 2006, see also Chapters 12 and 16 in this Book). Here, we showed that the amounts of both phytoalexins were very low in control leaf discs treated or not with DFMA or DFMO (Fig. 6A).

However, high amounts of phytolaexins were produced by grapevine leaf discs in response to osmotic stress, with a substantial accumulation of ε-viniferin (Fig. 6A). The presence of both resveratrol and viniferin in grapevine leaf tissues could result from the induction of phenylalanine ammonia lyase (PAL) and stilbene synthase, as key enzymes involved in phenylpropanoid pathway, as previously reported in grapevine cells subjected to an oligosaccharide elicitor (Aziz et al. 2003). The presence of large amounts of ε-viniferin suggests a rapid dimerization of resveratrol.

When the osmotically shocked discs were pre-treated with DFMA or DFMO, their PA contents strongly decreased and the osmo-induced phytoalexin accumulation was reduced. This inhibition was particularly evident in the production of ε-viniferin. The level of osmo-induced resveratrol remained unchanged. This could indicate that in stressed leaf discs both ADC and ODC are

required for synthesis of Put, and thus that under high osmotic stress the synthesis of phytoalexins might involve both PA biosynthetic pathways. This is the first report suggesting that activation of PA biosynthesis is involved in the regulation of defense responses in grapevine under osmotic stress. A similar picture was found during the HR of a tobacco (*Nicotiana tabacum*) cultivar resistant to tobacco mosaic virus (TMV). In this case, ODC and ADC activities were found to increase, thus leading to elevated free and conjugated PAs, mainly in necrotic lesions (Torrigiani et al. 1997). It was also known from some studies that PAs could be associated with cell wall pectic polysaccharides (Angelini et al. 1993), and that pectin fragments (α-1,4-oligogalacturonides) were effective in inducing various defense responses in grapevine cells (Aziz et al. 2004). Also, PAs are interconnected with ethylene biosynthesis, and the balance between the two pathways could be important in the priming of plant resistance or sensitivity.

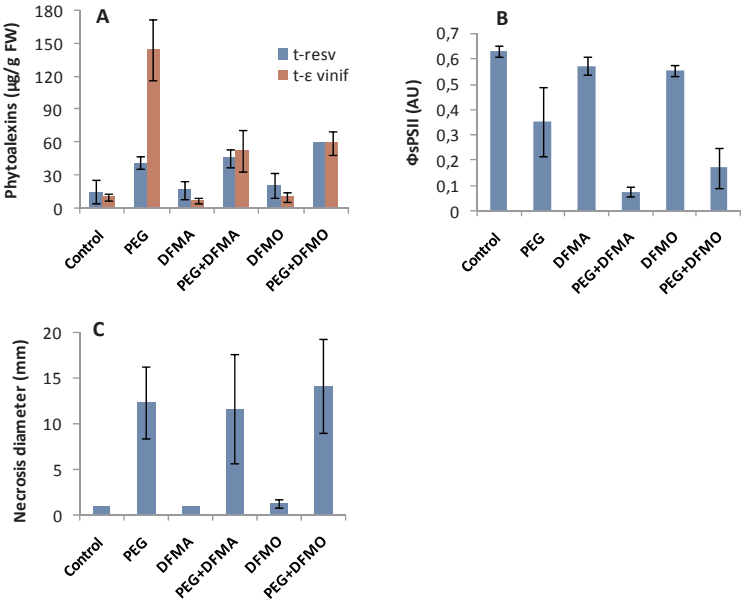

Fig. 6. Effect of osmotic stress and inhibitors of Put biosynthesis on phytoalexin accumulation, photosynthetic efficiency (ΦsPSII) and disease resistance of grapevine leaf discs. Discs excised from mature leaves were incubated for 12 h in a reference medium (control) containing 1 mM DFMA or 1 mM DFMO, specific inhibitors of ADC and ODC, before application of osmotic stress with PEG 6000 (-1.5 MPa) during 50 h. Phytoalexins (A) and ΦsPSII (B) are means ± SE, each from 30 leaf discs. After 50 h of elicitation, the leaf discs were challenged with *Botrytis cinerea* and disease was estimated by measuring necrotic lesions (C). Data are means ± SE, each from 30 leaf discs. t-resv: *trans*-resveratrol, t-ε-vinif: *trans*-ε-viniferin, Ctl: control, PEG: polyethylene glycol (Aziz et al., unpublished).

7.2. PA biosynthesis and photosynthesis efficiency

The cellular redox state plays a central role in the regulation of plant defense responses. To elucidate the role of photosynthesis in osmotic adaptation and basal disease resistance we measured different parameters of chlorophyll fluorescence in grapevine leaf discs including the quantum yield of PSII (Φ_{PSII}). The results (Fig. 6B) showed that high osmotic stress reduced photosynthetic capacity in grapevine leaf discs. This decrease of Φ_{PSII} could essentially be due to the strong increase in the reduction state of the primary quinine acceptor, suggesting that a fraction of the PSII reaction centers was closed during osmotic stress.

Treatment of leaf discs with DFMA or DFMO under non-stress conditions did not show any change in Q_{PSII} capacity. However, in stressed leaf discs both DFMA and DFMO reduced Φ_{PSII} activity (Aziz et al. unpublished). The data suggest that under osmotic stress conditions, both ADC and ODC pathways could be involved in plant cell homeostasis, probably through a regulatory effect on photosynthetic apparatus. That PAs could play a pivotal role in photosynthesis has been proposed since they are capable to reverse stress-induced damages in photosynthetic apparatus (Sfakianaki et al. 2006), possibly by affecting the regulation of the reaction centers at the PSII level and hence the rate of electron transport, which seems to be reduced in stressed leaf discs.

7.3. PA biosynthesis and protection against Botrytis cinerea

In order to understand the consequences of osmo-induced changes in PA level on the fungal disease development, grape leaf discs with altered PA levels were inoculated with *B. cinerea* and the size of necrotic lesion was subsequently measured, as an indicator of disease severity.

Inoculation of grapevine leaf discs with *B. cinerea* at a concentration of 10^5 conidia/mL, within 3 days, did not provoke symptoms of grey mold over the inoculation site in control or DFMA- or DFMO-treated leaf discs (Fig. 6C). However, osmotically stressed leaf discs exhibited enhanced susceptibility to *B. cinerea* infection, which resulted in necrosis of more than half surface of the PEG-treated leaf discs within 3 days. Similarly, stressed leaf discs pre-treated with DFMA or DFMO showed severe disease symptoms. These data indicated that although ADC and ODC seemed to be involved in the regulation of phytoalexin (viniferin) production, they are not required for basal resistance against *B. cinerea*.

7.4. Effect of DAO inhibition on phytoalexin production

Adjustment of Put, Spd and Spm levels in grapevine leaf discs under osmotic stress is attributed, at least in part, to activation of the PA catabolic pathways. To determine whether DAO could be involved in basal resistance to *B. cinerea*, we studied the effect of AG, with or without osmotic stress on the induction of phytoalexins in grapevine leaf discs. The results showed that enhancement of free PA titers in AG-treated leaf discs (Fig. 5) led to increased levels of phytoalexins, especially *trans*-resveratrol and *trans*-ε-viniferin (Fig. 7A). Under these conditions, the metabolic flux from Put seemed to be in favor of Spd synthesis through the Spd synthase pathway, which could be further oxidized through PAO. Surprisingly, pretreatment of osmotically stressed leaf discs with AG resulted in a strong reduction of ε-viniferin production, whilst the level of resveratrol remained unchanged. These data point to the osmo-induced DAO-dependent accumulation of phytoalexins as a major defense reaction in grapevine.

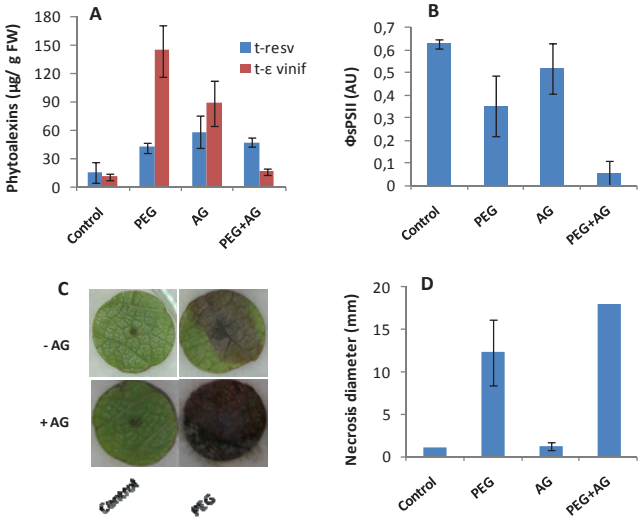

Fig. 7. Effect of osmotic stress and DAO inhibitor, aminoguanidine (AG), on phytoalexin accumulation, photosynthetic efficiency (ΦsPSII) and disease resistance of grapevine leaf discs. Discs excised from mature leaves were incubated for 12 h in a reference medium (control) containing 1 mM AG (AG) before application of osmotic stress with PEG 6000 (-1.5 MPa) during 50 h. Phytoalexins (A) and ΦsPSII (B) are means ± SE, each from 30 leaf discs. After 50 h of elicitation, the leaf discs were challenged with *Botrytis cinerea* and disease symptoms were photographed (C) at 3 days post-inoculation and estimated by measuring necrotic lesions (D). Data are means ± SE, each from 30 leaf discs. t-resv, trans-resveratrol; t-ε-vinif, trans-ε-viniferin; Ctl, control; PEG, polyethylene glycol (Aziz et al., unpublished).

Changes in free PAs and their catabolism through an increase in DAO activity and, in some cases, of PAO have been shown to occur in response to biotic and abiotic stresses (Walters 2003, Moschou et al. 2008a, b, c). The activities of these two enzymes produce H_2O_2, which may act as a signal molecule in structural defense, or as an antimicrobial compound in host resistance (Aziz et al. 2003, Cona et al. 2006). Hydrogen peroxide derived from PA oxidation has been shown to contribute to cell wall reinforcement during plant-pathogen interaction (Rea et al. 2002) and programmed cell death in response to salt stress (Moschou et al. 2008b). These results are in part consistent with other reports showing that inhibition of elicitor-induced H_2O_2 lowered the accumulation of PAL transcript in grapevine cells (Aziz et al. 2004).

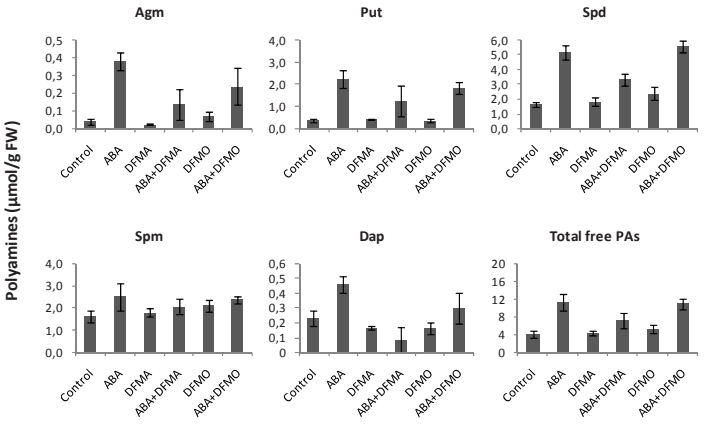

Fig. 8. Effects of ABA and inhibitors of Put biosynthesis on free PA titers in the leaves of *in vitro* grapevine plantlets. Four week-old rooted plantlets were transferred from the growing medium to new containing 100 µM ABA alone or in the presence of 0.5 mM DFMA or 0.5 mM DFMO, as specific inhibitors of ADC or ODC, respectively. Data are means ± SE, each from 24 plantlets at 15 days post-elicitation (Aziz et al., unpublished).

7.5. Effect of DAO inhibition on photosynthesis efficiency

As expected, osmotic stress triggered by PEG reduced chlorophyll fluorescence and photosynthetic efficiency (Fig. 7B). Application of AG, an inhibitor of DAO, under non-stressed conditions can disrupt photosynthesis by a slight inhibition of Φ_{PSII}. However, the combination of AG and PEG resulted to a strong inhibition (by about 90%) of Φ_{PSII} (Fig. 7B). The PSII reaction centers were apparently not damaged but they were not able to carry out electron transport. It is well known that the catabolic degradation of Put by DAO is a means to regulate the cellular content of this diamine (Kumar et al. 1997, Bagni and Tassoni 2001). The reaction product of DAO, pyrroline, can be further catabo-

lized to γ-aminobutyric acid (GABA), subsequently transaminated and finally incorporated into the Krebs cycle. In addition to H_2O_2, GABA has also been shown to play a key role in signal transduction pathways during stress response of many plants (Shelp et al. 1999). Another study suggested a complex relationship between photosynthesis and cell death (Allen et al. 1999).

7.6. Effect of DAO inhibition on protection against B. cinerea

To investigate the effect of PA oxidation on grapevine resistance to *B. cinerea* infection, we treated leaf discs with AG prior to their exposure to osmotic stress, then challenged with conidial suspension of *B. cinerea* (10^5 conidia/mL). In non-stressed leaf discs incubated with or without AG, necrotic lesions remained confined to the inoculation site within 3 days (Fig. 7C, D). However, inhibition of DAO by AG enhanced the PEG-induced leaf necrosis during fungal infection (Fig. 7C, D). It can be suggested that accumulation and further oxidation of free PAs under osmotic stress can affect metabolic defense reactions in grapevine. Thus, modulation of plant PA levels may lead directly or through their oxidation products to significant changes in basal host resistance to pathogen. Alterations in PA metabolism have also been detected in barley seedlings expressing systemically induced resistance against powdery mildew (Walters et al. 2002). This systemic protection was accompanied by increased activation of the defense-related enzymes PAL and POX, but also by significant increases in levels of Put and Spd and increased activity of the biosynthetic enzymes and of DAO.

8. ABA ALTERS POLYAMINE METABOLISM

Abscisic acid is an important phytohormone that plays critical roles in plant development and adaptation to various abiotic stresses. A large number of transcripts involved in ABA metabolism or responsive to ABA were increased with water deficit or salinity in grapevine (Cramer et al. 2007). In addition, recent studies have shown that ABA plays important roles in disease susceptibility, resistance to pathogen infection, and interaction with other signalling molecules (Allègre et al. 2007, Yasuda et al. 2008). Several genes involved in PA metabolism expressed by abiotic stresses are also inducible by ABA (Urano et al. 2003). Because photosynthetic capacity was strongly reduced following osmotic stress, we suspected that changes in ABA responses could be responsible for the stress enhanced disease. Thus, we postulated that ABA can modulate PA metabolism in grapevine.

8.1. ABA and PA biosynthesis

We have investigated the role of ABA in the regulation of polyamine biosynthesis in grapevine plantlets under *in vitro* conditions. The levels of all free PAs in both shoots (Fig. 8) and roots (data not shown) increased after 15 days of exposure to 100 μM ABA. Accumulation of Agm indicates that ADC is involved in the synthesis of PA in response to ABA as observed under osmotic stress. Without ABA, DFMA an irreversible inhibitor of ADC, but not DFMO, an irreversible inhibitor of ODC, inhibited only slightly free Agm and Dap production. However, in ABA-treated plantlets, the use of DFMA resulted in a strong inhibitory effect on free Agm, Put, Spd and Dap (by about 50 to 80%) accumulation in leaf tissues (Fig. 8). DFMO did not exhibit significant reduction of ABA-increased PA levels. The results suggest that ADC pathway could be a rate-limiting step in PA biosynthesis in response to ABA. The role of ABA in the regulation of PA biosynthesis by water stress has also been demonstrated in *A. thaliana* (Alcazar et al. 2006). These authors proposed that up-regulation of *ADC2*, *SPDS1* and *SPMS* genes by drought stress was an ABA-dependent response, since up-regulation was not observed in ABA deficient (*aba2*) and insensitive (*abi1*) mutants. This cannot exclude the possibility that ABA might be involved in PA conjugation, flux and degradation processes.

8.2. ABA and PA catabolism

In order to evaluate the participation of DAO and PAO in the adjustment of free PA levels in response to ABA, two specific inhibitors were tested: AG and β-hydroxyethylhydrazine (HEH), which inhibits PAOs. In control plantlets treated with 1 mM AG, only Agm content increased slightly. However, in the presence of ABA, AG enhanced the levels of Agm, Put and Spd in both shoots (by about 2-fold, Fig. 9) and roots (data not shown), while Spm and Dap remained unchanged in both organs. HEH had also no effect on PA levels in control plantlets. In ABA-treated plantlets, HEH caused also an increase in Spd level in both leaves (Fig. 9) and roots (data not shown). The Put, Spm and Dap levels remained, however, unchanged.

The addition of AG or HEH revealed a possible contribution of the DAO and PAO pathways in modulating PA concentrations in both leaves and roots in response to ABA. This is also in accordance with the recent findings of Moschou et al. (2008c) showing that ABA induced the expression of *AtPAO2* and *AtPAO3* genes in *A. thaliana*. Similarly, exogenous ABA stimulated apoplastic DAO activity, increased H_2O_2 production and $[Ca^{2+}]_{cyt}$ levels in *Vicia faba* guard cells, and induced stomatal closure (An et al. 2008). These processes were all impaired by DAO inhibitors.

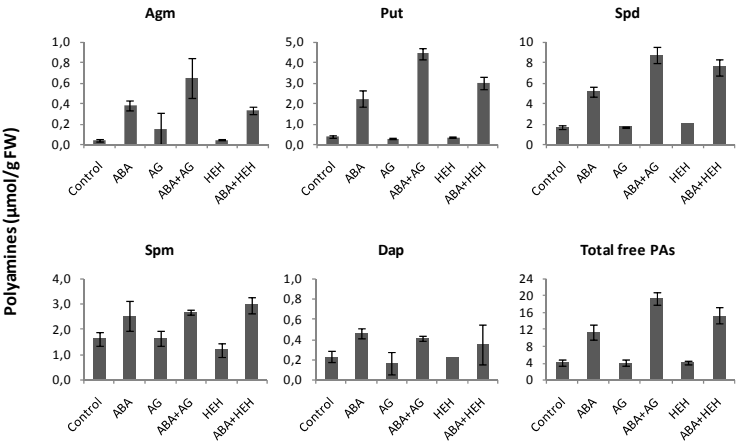

Fig. 9. Effects of ABA and inhibitors of diamine-oxidase, aminoguanidine (AG), and polyamine-oxidase, β-hydroxyethylhydrazine (HEH), on free PA titers in grapevine leaves of *in vitro* plantlets. Four week-old rooted plantlets were transferred from the growing medium to a new one which contains 100 µM ABA alone or in the presence of 1 mM AG or HEH 1 mM. Data are means ± SE, each from 24 plantlets at 15 days post-elicitation (Aziz et al., unpublished).

8.3. ABA and defense responses

The role of ABA in plant defense is poorly understood and even controversial. Whereas several reports have shown an inverse correlation between ABA level and resistance to pathogens in several plant species, others have suggested a positive role of ABA in activation of defense gene expression and pathogen resistance (Mauch-Mani and Mauch 2005). We have investigated how treatment of grapevine plantlets with ABA compromises a basal resistant interaction with *B. cinerea*. We found that ABA treatment dramatically reduced the basal level of phytoalexins and PAL activity. We also show that after 1 month of growth in the presence of ABA, the younger leaves from plantlets displayed visible symptoms of injury. In other experiment, Hamiduzzaman et al. (2005) showed that there was a small reduction in the number of sporangia of *Plasmoprara viticola* in plants treated with ABA at 50 µM. Minor phytotoxic effects were also observed in leaf veins (brownish color) upon ABA treatment. Interestingly, the level of resistance against *P. viticola* in ABA-treated plants was significantly lower than in JA-treated plants (Hamiduzzaman et al. 2005).

ABA could also interfere with PAs and defense signalling pathways at several common points. The initial responses to ABA in stomatal closure in-

clude increases in ROS (Vilela et al. 2007), which are important signalling molecules in defense. This is in accordance with osmotic stress-induced strong inhibition of photosynthetic efficiency in grapevine leaf discs. Hydrogen peroxide, one of the degradation products of PAs, has been shown to contribute to some defense responses and programmed cell death during (a)biotic interaction (Rea et al. 2002, Aziz et al. 2004, Moschou et al. 2008a, b). This cannot exclude the possibility that other derivative products of PA oxidation could be involved in disease resistance modulation under biotic stress.

8.4. ABA induces polyamine exodus to the apoplast

Polyamine exodus to the apoplast is a process that takes place during abiotic and biotic stress conditions and facilitates H_2O_2 production, which in turn acts as a second messenger directing tolerance responses in tobacco plants (Moschou et al. 2008b). ABA, on the other hand, has been correlated with early responses to abiotic stress challenge. Thus, chemical signals like ABA most probably dominate during early stages of stress before hydraulic signals are produced, and become less important under severe drought in which case leaves wilt (Schachtman and Goodger 2008). To estimate ABA contribution to the induction of PA exodus in grapevine we used cell suspension cultures of cv Kahli kerkennah (Toumi et al. unpublished). Thus, ABA efficiently induced PA exodus into the culture medium, which can be considered as the apoplast (Fig. 10). Interestingly, this exodus took place as early as 30 min post-treatment. More specifically, Put, Spd and Spm followed a progressive increase post-treatment and increased up to 4-fold 48 h post-treatment with ABA (Fig. 10). Similarly to ABA, mannitol as well induced PA exodus to the apoplast (data not shown).

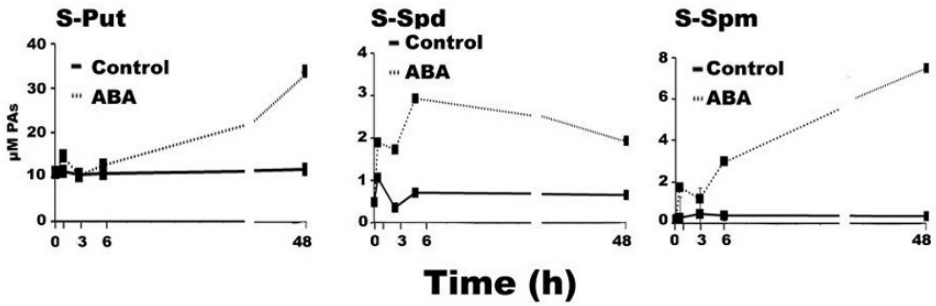

Fig. 10. Exogenous ABA application induces PA exodus into the apoplast. Application of 10 μM ABA in cell suspension cultures of cv Kahli kerkennah and Put, Spd and Spm titers in the cell suspension medium were monitored at 0, 0.5, 3, 6 and 48 h post-treatment. Data are the means of three independent experiments ± SD (Toumi et al., unpublished).

ABA is a potential inducer of PA exodus into the apoplast, while the most interesting result is its contribution to this early response. This correlation of ABA and PAs could direct responses correlated with additional stress responses, including also stomatal closure that could be induced by the H_2O_2 produced through PA oxidation by the apoplastic PAO.

8.5. Drought stress and polyamines

To evaluate the possible contribution of polyamines to the enhancement of tolerance to drought stress, we have used two Tunisian grapevine cultivars, Kahli kerkennah and Guelb Sardouk that show differential susceptibility to drought stress. The former is tolerant to drought, whereas the latter is sensitive (Toumi et al. 2008). To initiate drought stress, the method of withholding irrigation was used. Interestingly, the tolerant one, when subjected to prolonged drought stress (5 to 8 days), exhibited significantly higher PA biosynthetic activity (Fig. 11). More specifically, ADC specific activity was by 2-fold higher in the tolerant cultivar, but the most striking differences between the tolerant and the sensitive cultivars were observed for ODC and SAMDC specific activities that were 3- and 4-fold higher 8 d of withholding irrigation, respectively (Fig. 11). Thus, the tolerant cultivar exhibited significantly higher potential for PA synthesis under drought stress, in contrast to the sensitive one that failed to readjust its PA biosynthetic capacity. As a consequence, Put and Spd were

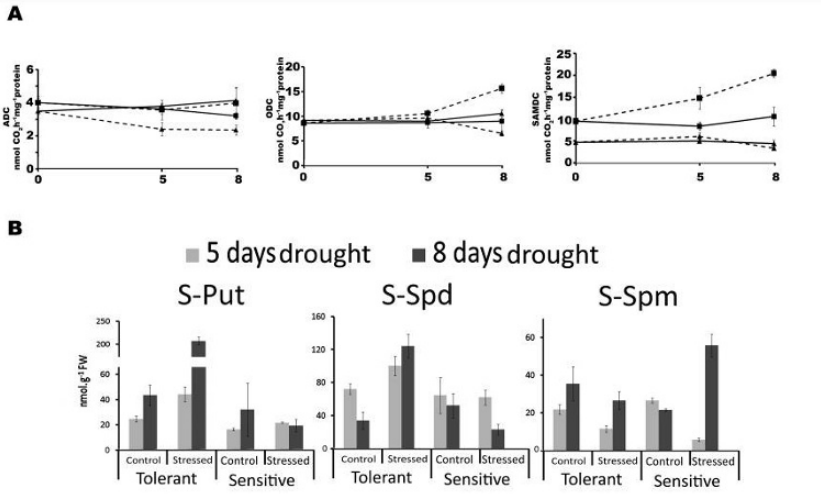

Fig. 11. Biosynthetic PA activity and PA titers in cv Kahli kerkennah (Tolerant) and Guelb Sardouk (Sensitive) subjected to drought stress. A, Specific activities of ADC, ODC and SAMDC 5 and 8 days of with-holding irrigation. B, Free PA titers 5 and 8 days of with-holding irrigation. Data are the means of three independent experiments ±SD (Toumi et al., unpublished).

significantly increased only in the tolerant cultivar, reaching a 2- and 5-fold increase, 5 and 8 d of with-holding irrigation, respectively (Fig. 11). On the other hand, in the sensitive cultivar no significant increase in Put titers were observed, while Spd showed a decrease 8 d after with-holding irrigation (Fig. 11). Interestingly, Spm increased only in the sensitive cultivar, while decreased in the tolerant one.

The previous reinforce the view that high PA biosynthetic capacity and/or PA titers are a tolerance trait for grapevine. Interestingly, the high content of Spm titers that is observed under prolonged stress in the sensitive cultivar could be due to the inability of the plant to oxidize this amine.

FUTURE PERSPECTIVES

That PAs play important roles in plant growth and development but also in the responses to biotic and abiotic stresses has been reinforced in the last years. Only recently the possible molecular mechanisms, which underline some of these responses have started to be understood. PA homeostasis but also PA catabolism seem to be key factors. The recent availability of transgenic plants with altered the genes encoding for the biosynthetic and catabolic PA genes as well as the respective mutants will help to further understand how PA act in modulating cellular behavior.

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9

HORMONAL CONTROL OF GRAPE BERRY RIPENING

C. Davies & C. Böttcher

Commonwealth Scientific and Industrial Research Organisation
Plant Industry, P.O. Box 350
Glen Osmond, South Australia 5064, AUSTRALIA

1. INTRODUCTION

Grape berry ripening, like many other developmental processes, clearly involves the coordination of a large number of events. Some metabolic activities that occur prior to véraison, such as photosynthesis and organic acid accumulation, are either turned off at véraison or are at least down regulated. Other processes, such as the accumulation of anthocyanins in berry skins, commence at véraison. In this chapter we will use the last time-point before an accumulation of sugars is recorded as the working definition of véraison. Where possible the data from papers has been reinterpreted to align with this definition. The changes that occur as the berry begins to ripen have received considerable attention at the physical and biochemical level (Ollat et al. 2002, Conde et al. 2007), but their overall control and coordination remains poorly understood. Not only are the timing and extent of changes in the berry coordinated but this control has to extend over a range of diverse primary and secondary metabolic pathways. In addition, many of the 'subprograms', that make up the greater ripening 'program', such as colour and sugar accumulation, are responsive to environmental conditions which influence their progress. The control system that initiates and maintains the ripening phase must therefore be complex, able to coordinate diverse portions of metabolism and must be to some extent flexible.

Control over metabolism and growth can occur at a number of different levels including transcription, translation, enzyme activity etc. This regulation may be exerted in different ways, for example gene transcription can be controlled at the individual gene level or through the regulation of an entire pathway. Recent developments in the analysis of gene transcript levels have revealed how extensive the changes in gene expression are that occur during fruit ripening. For example, differential screening, cDNA and oligonucleotide mi-

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croarray analysis has shown that the expression of thousands of genes, including large numbers of transcription factors, change during grape berry ripening (Davies and Robinson 2000, Terrier et al. 2005, Waters et al. 2005, Deluc et al. 2007, Pilati et al. 2007). This information has been supplemented by the use of 2-D gel analysis, which has documented the changes in levels of many proteins during ripening (Deytieux et al. 2007, Giribaldi et al. 2007).

Hormones have long been known to regulate plant development through coordinating change. Regulating metabolism requires a complex system of interactions as is evidenced by the increasing number of reports of crosstalk between regulatory networks (Gazzarrini and McCourt 2003, Vandenbussche and Van Der Straeten 2007). In this chapter we discuss the role of various plant hormones in the control of grape berry ripening. The timing and extent of ripening is of considerable scientific interest, but also has implications for the various grape industries. The timing of commercial ripeness impacts on marketing of fresh fruit, processing of dried fruit, and on winery logistics and processing. The extent and rate of ripening can also greatly modify grape 'quality' and wine style.

In general, all fruit go through similar changes as they ripen, for example, they store energy-rich metabolites, soften and accumulate colour, flavour and aroma compounds. However, despite these similarities in fruit development there seem to be significant differences in the way the ripening process is controlled across plant species. Traditionally plant physiologists have categorized different fruit species as being either climacteric or non-climacteric, based on physiological differences in their ripening (Tucker 1993). Much of the research on the development and ripening of fruit has been conducted using climacteric fruit such as tomato and banana, which undergo a peak in respiratory activity during the ripening process (Seymour 1993). In some fruit an ethylene burst is coincidental with, or lags behind, the respiratory peak.

The significance of the respiratory peak in climacteric fruit ripening has been questioned because of its apparent absence in fruit attached to the plant and it has been suggested that a change in ethylene level is the critical marker that characterises climacteric fruit (Bower et al. 2002). In contrast to climacteric fruit, non-climacteric fruit tends to show a slow decline in respiratory activity during ripening with no major peak in ethylene levels (Seymour 1993). As we will discuss in this chapter, the classification of climacteric and non-climacteric fruit on the basis of ethylene production at the time of ripening, is less distinct than at first thought. Grapes are regarded as non-climacteric (Coombe and Hale 1973) along with fruits, such as strawberries, olives and capsicum. While the role of ethylene in climacteric fruit ripening, especially in tomato, is well established, the hormonal triggers for grape berry ripening remain the subject of debate as, despite some correlative data, no direct role for ethylene in non-climacteric fruit ripening has been proven.

2. PROMOTERS OF RIPENING

Not all of the recognized plant hormones have been shown to affect grape berry ripening, and of those that do, an even smaller number have been shown to have patterns of accumulation that suggest they might be involved during 'normal' berry development. It is convenient to divide the hormones into two groups on the basis of their ability to either promote, or delay, berry ripening, or some part of it. As we shall see, even this relatively simple distinction is not clear cut.

2.1. Ethylene

2.1.1. Grape ethylene biosynthesis and accumulation

As indicated above, the role of ethylene, whilst well established in climacteric fruit ripening, is the subject of ongoing debate in non-climacteric fruit species. This is partly due to the comparatively low levels of ethylene produced by non-climacteric fruit and the inherent difficulties in quantifying low levels of a gas. Although ethylene itself is relatively easy to measure, for example by gas chromatography coupled with flame ionisation detection, there are technical challenges associated with capturing the gas. A number of different techniques have been used to collect ethylene released during berry ripening from a range of different cultivars. Not surprisingly, this has led to differing outcomes. Some procedures rely on capturing evolved ethylene; others involve vacuum treatments to extract 'internal' ethylene. Presumably, the latter method also extracts dissolved ethylene to some extent. Early studies, using *V. vinifera* L. cv Doradillo fruit removed from the vine, did not detect an increase in ethylene at the onset of ripening (Coombe and Hale 1973). The authors were attempting to measure internal ethylene as they collected the gas released following a vacuum treatment. Similarly, Inaba et al. (1976) reported no peak in ethylene production at, or before, véraison using bagged bunches of seeded and seedless Delaware grapes on vines. They did, however, observe a very small ethylene peak (on a µl/cluster basis) after véraison. Ethylene levels (µl/kg fresh weight) did not increase at véraison in Thompson Seedless and Carignane bunches removed from the vine and placed in jars (Weaver and Singh 1978).

In contrast to the above results, some reports have indicated changes in ethylene production during or before ripening. Alleweldt and Koch (1977) measured ethylene, essentially as described by Coombe and Hale (1973), in B-6-18, Bacchus and Optima berries removed from the vine. They saw a 15-fold increase in ethylene evolution at around véraison, just prior to an increase in abscisic acid (ABA) levels, but concluded that grape berries should be considered to be non-climacteric fruit as the levels were still relatively low and the ob-

served increase in respiration was small. A similar result for B-6-18 was reported by During et al. (1978).

As none of the measurements in the above papers were replicated, evaluation of the data is difficult. Chervin et al. (2004) performed a replicated study of ethylene levels in field grown Cabernet Sauvignon berries. Ethylene was extracted from detached bunches at different stages of development by vacuum treatment. A modest peak in ethylene occurred at 7 weeks post-flowering, accompanied by berry coloration and peaks in 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) transcript level and enzyme activity. These results give credence to the view that there may be some modest increase in ethylene evolution at, or just before, véraison.

The interpretation of experiments conducted to measure changes in ethylene and respiration at ripening may be confounded by the methods used. Studies in other plants have shown that the detachment of fruit from the plant can affect ripening. This, along with the methodology of ethylene measurement, may influence the experimental outcome in grapes. For example, in attached tomatoes and melons the expected climacteric rise was not detectable (Saltveit 1993, Bower et al. 2002). In melons, ethylene evolution was much lower in detached fruit with a barely significant rise at ripening compared to that seen in attached fruit (Bower et al. 2002). Conversely, detachment triggers autocatalytic ethylene production in apples (Lin and Walsh 2008). Inaba et al. (1976) measured ethylene in grapes on the vine while in other studies (Coombe and Hale 1973, Alleweldt and Koch 1977, Weaver and Singh 1978, Chervin et al. 2004, Lin and Walsh 2008) the fruit was removed for measurement. Therefore, some of the differences reported by these authors could be due to the measurement methodologies used.

Reports of modest increases in ethylene levels in non-climacteric fruit are not confined to grape. Recently, laser photo-acoustic spectroscopy has been used to measure the small changes that occur in ethylene and CO_2 evolution during strawberry ripening while attached to the plant (Iannetta et al. 2006). Although strawberries are considered to be a non-climacteric fruit, several peaks in ethylene levels at different stages of development were detected. A linear increase in ethylene levels when strawberries ripened (fruit going from pink to red) was accompanied by an increase in CO_2 production. Rises in ethylene levels and/or in the transcript levels of ethylene biosynthetic genes, have also been reported in other non-climacteric fruit e.g. pineapple and litchi (Cazzonelli et al. 1998, Wang et al. 2007).

Further support for an increase in ethylene production in grapes at around véraison comes from other studies investigating gene transcript and protein levels. Pilati et al. (2007) used microarrays to analyse changes in transcript levels in field grown Pinot noir. The profiles produced were of limited resolution as only three time-points (one pre-véraison sample at maximum acidity, one two

weeks later at about the time of véraison and one three weeks after that when ripening was well under way) were analysed. They showed that *ACO* transcript levels were elevated at around véraison and that 1-aminocyclopropane-1-carboxylic acid synthase (*ACS*) was expressed up to véraison, after which time its transcript level declined. A different pattern of *ACO* expression was observed in a more detailed analysis of transcript changes during Cabernet Sauvignon development by Deluc et al. (2007), who looked at seven different developmental stages. Like Pilati et al. (2007), they found that the transcript levels of *ACS* decreased after sugar levels began to increase but found that *ACO* transcript levels decreased fairly steadily from their first sample taken at the green 'pea' stage (just less than three weeks after flowering). Two-D gel analysis of protein levels during the development of Nebbiolo Lampia berries showed that, like the transcript levels, the levels of ACS protein declined after véraison (Giribaldi et al. 2007). On balance, it seems that both *ACS* and *ACO* transcript levels and enzyme activity decrease after véraison but there could be small peaks in levels before véraison consistent with a possible role for ethylene in the control of berry ripening.

It can be concluded from this discussion that any change in ethylene gas levels during grape berry ripening must, if it occurs at all, be small when compared to the changes that occur in ethylene levels in fruit, such as tomatoes, at ripening. However, it is possible that the response to ethylene may be modulated by changes in the sensitivity of perception during development. Whatever their role during 'normal' development, the effect of applied hormones can vary considerably depending on the developmental stage of the fruit. This suggests that the ability to perceive, transduce and act upon hormone signals must vary through development. Also, there is evidence that the transcript levels of some grape ethylene receptors do change during berry development. One receptor (*ERS1*) was expressed most highly at the pea size stage [stage 31, modified E-L system (Coombe 1995), no earlier time-points were assayed], after which the transcript levels decreased at bunch closure (stage 32) and then remained fairly stable until ripening (Deluc et al. 2007). The expression of another receptor (*EIN4/ETR5*) increased at the commencement of sugar accumulation and was most highly expressed at harvest. A more detailed study in strawberries (Trainotti et al. 2005) showed that the transcript levels of three putative ethylene receptors change during fruit development. One receptor (*FaETR2*), whose expression was up-regulated at the beginning of ripening, belongs to the more sensitive type-II group.

2.1.2. The effect of ethylene and ethylene releasing compounds on grape berry ripening and gene expression

The application of ethylene gas (and propylene, an active analogue), and the ethylene producing compound 2-chloroethylphosphonic acid (CEPA) to climacteric fruit has a profound effect on ripening and this is often used commercially to hasten the ripening of fruit, such as bananas. Even in climacteric species, fruit that are treated when too young do not respond as well as fruit that are closer to the normal time of ripening. This has been shown by experiments in tomato using propylene (McGlasson et al. 1975). This suggests that there are other changes that occur during development which affect sensitivity to the hormone (the possibility of receptor changes has been discussed above).

The effects of ethylene (or propylene) and CEPA application to grape berries appear to be less dramatic than those described for climacteric fruit. Due to the difficulty in applying ethylene to grape bunches, much of the research in this area has been conducted through the use of CEPA sprays. However, it should be noted that although CEPA releases ethylene upon decomposition above pH 4.5, it also yields chloride and phosphate and may have some non-specific effects not related to the response to ethylene.

The response to CEPA application is obviously complex and influenced by numerous factors. There is not sufficient space here to discuss the many experiments that have been done on a wide variety of cultivars. A review by Szyjewicz et al. (1984), which quotes over 100 papers on the effects of CEPA, summarises the most important issues. For each case, where an effect of application has been observed, for example an increase in colour, there are examples where no effect on this parameter was discerned. The reason for this could be due to any number of factors, such as the developmental stage of the fruit at application, varietal differences in uptake and response, the method of application (including concentration, use of surfactants etc.), and growth conditions.

Despite this, there are a number of reports where CEPA application has increased colour development in a wide range of grape cultivars (Szyjewicz et al. 1984). It appears that CEPA treatment induces an increase in the transcript levels and enzyme activities of a number of genes involved in anthocyanin synthesis. Roubelakis-Angelakis and Kliewer (1986) showed that phenylalanine ammonia-lyase (PAL) activity was increased in the skin of isolated berries treated with light and sucrose and that this effect was enhanced by the addition of CEPA. CEPA sprayed on Cabernet Sauvignon berries at a stage when 50% of the berries were coloured increased skin colour but did not affect berry weight (El-Kereamy et al. 2003). Northern blot analysis showed that chalcone synthase (*CHS*), flavanone-3-hydroxylase (*F3H*), leucoanthocyanidin dioxygenase (*LDOX*), UDP-glucose flavonoid 3-*O*-glucosyl transferase (*UFGT*), but not dihydroflavonol 4-reductase (*DFR*) transcript levels were elevated by the

treatment. UFGT protein levels were also induced by CEPA application.

Increases in total soluble solids and a hastening of the timing of the initiation of ripening through treatment with CEPA, have also been extensively documented in *Vitis* species (Szyjewicz et al. 1984). Interestingly, there are also reports of CEPA delaying ripening. Some examples of the early work conducted in this area serve to illustrate this point. Hale et al. (1970) demonstrated that grape berries exhibit a biphasic response to CEPA application. When CEPA was applied to Shiraz berries as single treatments at 4, 5, 6, or 7 weeks post–flowering (wpf), colour development (percent coloured berries) was delayed compared to untreated fruit which commenced to colour between 9 wpf and 9 weeks and 5 days post-flowering. Treatments with CEPA at 8 and 9 wpf, i.e. just before véraison, appeared to hasten colouration. Measurement of the sugar to acid ratio suggested that ripening in general was delayed by the earlier treatments and advanced by treatments nearer to the time of véraison in control fruit. Therefore, CEPA (and by inference ethylene) can be considered to be an inhibitor or promoter of ripening depending on the developmental stage of the berries at the time of application. The existence of a biphasic response to ethylene itself was supported by later experiments, where CEPA was applied to Doradillo grapes (Coombe and Hale 1973). Changes in the response to ethylene during development have also been reported in citrus, another non-climacteric species (Katz et al. 2004).

Despite the difficulties in application some experiments using ethylene have been conducted with grapes. In general, the results appear to agree with those obtained using CEPA. Hale et al. (1970) treated Doradillo grapes in polythene bags with ethylene for 10 days from midway through the lag phase (the phase of no, or slow, growth which separates the two phases of berry expansion in most cultivars) and measured berry compressibility. They suggested that this treatment advanced both the timing and rate of berry softening; the commencement of softening was accelerated by about six days. El-Kereamy et al. (2003) using Cabernet Sauvignon berries, showed that ethylene increased the level of *CHS* transcripts more rapidly than CEPA.

Experiments using ethylene gas have provided some evidence that the induction of anthocyanin pathway gene expression through exposure to ethylene operates through a different mechanism than that which operates during 'normal' berry ripening. When Tira-Umphon et al. (2007) treated field grown Cabernet Sauvignon grapes with ethylene, they observed an increase in *UFGT* transcript levels but saw no increase in the expression levels of the transcription factor, *MybA*. *MybA* is the gene which controls anthocyanin accumulation during 'normal' ripening (see Chapter by Boss and Davies this edition). This effect was confirmed in CEPA-treated Gamay suspension cells (Tira-Umphon et al. 2007). The reasons for these observations are unknown but changes in berry development induced by hormone application, although superficially similar to

changes occurring during normal ripening, may not be achieved through the same mechanism.

Information about the possible role of ethylene in the control of grape genes which are up-regulated at véraison can also be gained from the study of the promoter sequence of ripening-induced genes. The promoter region of a grape alcohol dehydrogenase (ADH) gene, *VvADH2*, the predominant grape *ADH* gene expressed during berry ripening, was found to contain sequences related to ethylene responsive elements (Verries et al. 2004). The transcript levels of *VvADH2* and to a lesser extent ADH enzyme activity in berries at véraison were reduced by 1-methylcyclopropene (1-MCP) treatment (Tesniere et al. 2004, see also Chapter by Tesniere and Abbal, this edition). 1-MCP is an inhibitor of ethylene perception that works through irreversible binding to ethylene receptors (Huber 2008).

CEPA treatment of suspension cells increased *VvADH2* transcript levels in air and nitrogen and, as might be expected, 1-MCP treatment appeared to counteract the CEPA induction of *ADH2* transcript levels. However, the changes in transcript levels were not always mirrored by similar changes in ADH activity and so some post-transcriptional control may be involved. The other two *ADH* genes in grapevine were not expressed at significant levels (Tesniere et al. 2004). The role of ethylene in the control of *VvADH2* expression during ripening is likely to be complicated and may not necessarily be direct.

2.1.3. The effect of 1-MCP on berry ripening

The effects of hormones can be modified through changing synthesis, breakdown or perception. Various inhibitors of ethylene synthesis and perception are available but much of the more recent work has been conducted using 1-MCP.

Chervin et al. (2004) treated Cabernet Sauvignon berries for 24 h periods with 1-MCP at weekly intervals (5, 6, 7, 8 and 9 wpf). Treatments at 6, 7, and 8 wpf appeared to inhibit colour development and berry size increase and resulted in less organic acid loss. Ripening in the control berries began sometime between 7 and 8 wpf. The 1-MCP treatments were most effective at the time of the observed peak in ethylene evolution (7 wpf).

Therefore, it seems that 1-MCP was able to delay ripening when applied around the time of véraison. One way that 1-MCP could affect ripening is through the control of sugar accumulation. The expression of two sucrose transporters, *VvSUC11* and *VvSUC12,* increases at véraison (Davies et al. 1999, Manning et al. 2001). Chervin et al. (2006) treated Cabernet Sauvignon bunches (at a stage with 50% coloured berries) with 1-MCP for 24 h and sampled at 5 and 19 days after treatment. Not only were sucrose levels decreased by 1-MCP but the transcript levels of *VvSUC11* and *VvSUC12* were also decreased.

Whether the effect of ethylene on these sucrose transporters is direct or indirect is unknown but sugars are known to be extensively involved in signalling in plants (Rolland et al. 2002). Again these results could be explained by 1-MCP delaying ripening.

2.1.4. Expression of putative ethylene-related genes during berry ripening

The expression of some putatively ethylene-related transcripts has been determined by microarray analysis. Deluc et al. (2007) listed a number of genes reported to be involved in ethylene synthesis and signalling in Cabernet Sauvignon. Their data regarding the expression of *ACO* and *ACS* genes has been discussed above. They grouped the genes that change with time over seven different berry developmental stages into 21 different clusters based on their expression profile. Apart from the five genes involved in ethylene synthesis, they listed 16 other genes suggested to be involved in ethylene response. These genes fall into eight different clusters which exhibit widely differing profiles. Another study, also discussed above in regard to ethylene biosynthesis gene expression, used arrays to detail changes in gene expression during Pinot noir berry development (Pilati et al. 2007). They listed 14 ethylene-associated genes falling into three (out of eight) different clusters.

Without further study it is difficult to draw firm conclusions from this data other than to say that, if these genes are indeed implicated in the response to ethylene, then the signalling process is very complex. It also means that ethylene has a number of roles throughout much of berry development, as both studies show that the expression patterns of putative ethylene-associated genes are diverse. Grimplet et al. (2007) used arrays to compare gene expression between the seeds, flesh and skin of mature Cabernet Sauvignon berries. In well-watered vines, five genes involved in ethylene synthesis (two *ACO* genes, two *S*-adenosylmethionine (SAM) synthetase genes and one encoding an ethylene overproduction protein) were all more highly expressed in skin than in seed or pulp. This presumably leads to a localized distribution of ethylene biosynthesis enzymes and hence ethylene concentrations within the berry. These studies also investigated the effect of water deficit on gene expression. The expression of two *SAM* synthetase genes and the two *ACO* genes was induced in skins by water stress and the two *ACO* genes and two ethylene response factors were also expressed more highly in the pulp of water stressed berries, suggestive of a role for ethylene during berry water stress.

On the basis of the low levels of ethylene and the low, and fairly stable, respiration rates in berries, grapes can be justifiably referred to as non-climacteric fruit using the accepted definition. Although the segregation of fruit species into climacteric and non-climacteric has proved convenient, and may still be useful, the role of ethylene in non-climacteric fruit ripening should be

reconsidered. A number of examples have been given above which illustrate this point. In summary, there is evidence that ethylene levels may increase in grape berries at around the time of the initiation of ripening, that transcript levels of biosynthesis genes increase at the same time, that ripening (or certain aspects of it) can be advanced by ethylene application and that ripening can be negatively affected by inhibitors of ethylene perception. However, it appears that the response to ethylene is dependent on the stage of development, suggesting that there is modulation of ethylene perception and/or signalling. More work needs to be done to establish the role and mechanism of action of ethylene during grape berry ripening and how the ethylene signalling pathway interacts with other hormones implicated in the control of ripening. Much of the evidence supporting an involvement of ethylene in ripening is correlative. Proof of the role it plays will require the analysis of transgenic plants with modified ethylene accumulation and/or perception.

2.2. ABA

2.2.1. Grape ABA synthesis/accumulation

While there is still some debate regarding the changes in ethylene levels that occur around véraison, the situation for ABA accumulation is much clearer. A reproducible pattern of ABA accumulation has been reported by a number of groups, who have used different systems for measurement in a wide range of cultivars and in some cases inter-specific crosses. All reports, where changes in ABA levels were measured at around the time of véraison, show that there is an increase in free ABA levels concomitant with sugar accumulation and colour development (Coombe and Hale 1973, Hale and Coombe 1974, Cawthon and Morris 1982, Kataoka et al. 1982, Okamoto et al. 2004, Baydar and Harmankaya 2005, Deytieux-Belleau et al. 2007).

More complete developmental series show that ABA levels are high early in berry development after which they decrease to be low just prior to véraison. They increase again at about the time of the initiation of sugar accumulation and reach a peak two to three weeks later, after which time they decline as the fruit approach ripeness (Inaba et al. 1976, Scienza et al. 1978, Davies et al. 1997, Kondo and Kawai 1998, Zhang et al. 2003). Due to this pattern of accumulation, ABA has been mooted to be involved in ripening and perhaps in the initiation of ripening (see below).

2.2.2. Distribution of ABA within the berry

Free ABA appears to be unequally distributed within the grape berry. The levels in skin are reported to be higher than in the flesh (Coombe and Hale 1973, Kataoka et al. 1982). Seeds also have a higher concentration of ABA than

flesh (Zhang et al. 2003). A comparison between the ABA levels in the outer wall (skin with some flesh including the peripheral conductive tissue), the inner wall (the remainder of the flesh) and the placenta of Muscat Bailey grapes was conducted by Shiozaki et al. (1999). Highest ABA levels were detected in the placenta with slightly less in outer wall and least in the inner wall. All tissues showed a sharp increase in ABA levels at véraison followed by a decrease (Coombe and Hale 1973, Kataoka et al. 1982, Shiozaki et al. 1999). Using immuno-labelling, Shiozaki et al. (1999) showed that both the peripheral and central phloem was strongly labelled in ripe berries suggesting that ABA levels were elevated in these cells. Immunogold labelling of very young berries has shown that in phenol-rich cells labelling was seen mainly in the nucleus but also in the cytoplasm and vacuole (Zhang et al. 1999). No labelling was observed in the vacuole of non phenol-rich cells.

The localization of ABA to the phloem is consistent with a role in assimilate unloading and uptake. The localization to the vacuole may be related to such things as metabolite sequestration. Interestingly, there is evidence that high affinity, highly specific ABA-binding proteins are localized to the tonoplast and may be involved in ripening as their affinity for ABA (in terms of dissociation constant) is highest at véraison (Zhang et al. 1999). Additional evidence supporting a role for ABA in sugar accumulation is detailed in the following section.

2.2.3. Expression of ABA biosynthesis genes

Not all of the genes associated with the ABA biosynthesis pathway (Nambara and Marion-Poll 2005) have been clearly defined in grapevine. Two plastidial enzymes, 9-*cis*-epoxy-carotenoid dioxygenase (NCED) and zeaxanthin epoxidase (ZEP), catalyze crucial steps in ABA synthesis. NCED, which catalyzes the first committed step in ABA biosynthesis by producing xanthoxin, is usually thought of as catalysing the key step in the pathway (Finkelstein et al. 2002). Soar et al. (2004) described the cloning of two cDNAs corresponding to *NCED* genes (*VvNCED1*, AY337613; *VvNCED2*, AY337614) from Shiraz. The function of these genes has not been directly demonstrated but phylogenetic analysis suggests that they are likely to be *NCED*s rather than the closely related carotenoid cleavage dioxygenases, as they cluster most closely with other NCEDs. A cDNA (*VvZEP*; AY337615) corresponding to a single *ZEP* gene was isolated from Cabernet Sauvignon. Southern analysis suggested that there are likely to be only two *NCED* and one *ZEP* gene in grapevine (Soar et al. 2004).

There is surprisingly little information about the expression of *NCED* and *ZEP* genes during grape berry development. However, some information comes from microarray studies. Expression profiles for two putative *NCEDs* and one

ZEP were described by Deluc et al. (2007), who used Affymetrix chips to study Cabernet Sauvignon berry development. *VvZEP* transcript levels steadily decreased from their maximal level at the first time point (stage 31) until flattening off at stage 38. The level of *VvNCED1* transcripts increased during the lag phase and reached maximal levels at stage 35 when berries began to colour. The putative NCED4 that Deluc et al. (2007) describe is in fact a carotenoid cleavage dioxygenase on the basis of sequence analysis (data not shown). The *VvNCED2* gene described by Soar et al. (2004) does not appear to be represented on the Affymetrix *V. vinifera* GeneChip. The expression of *VvNCED1* seems to be roughly in line with the profile of ABA accumulation (see above), which suggests that at least some of the ABA accumulated in the berries could be made within the fruit.

Differences in transcript profiles between the skin, flesh and seed of ripe berries have also been detected using the Affymetrix grapevine chip (Grimplet et al. 2007). *VvNCED1* was more highly expressed in pulp than in either skin or seed and its transcript levels were increased under water stress. The higher *VvNCED1* transcript levels in pulp compared to skin and seed tissue is in contrast to the distribution of ABA reported above (see section 2.2.2.). Again the available gene expression data is consistent with ABA having a role in ripening and/or a response to abiotic stress.

2.2.4. Expression of putative ABA-related genes during berry ripening

In a recent paper Yu et al. (2006) described the isolation of a Ca^{2+}-dependent protein kinase, which may be involved in the ABA-signalling pathway during berry development. A 58-kD protein (ACPK1) was isolated from the microsomal fraction from the mesocarp of Kyoho grapes. ABA stimulated the kinase activity and increased the transcript and protein levels of this Ca^{2+}-dependent protein kinase in a dose- and time-dependent manner (Fig. 1 and Yu et al. 2006). These effects were shown to be specific to the (+) ABA isomer and were indirect, as the activation required intact tissues. The *ACPK1* transcript was found at the highest levels in berry flesh but was also detected in skins. No expression was detected in root, stem or leaf tissue. Transcript, protein and activity levels were highest in berries 10 days after flowering and later during ripening but were reduced pre-véraison and during the lag phase. This pattern roughly followed that for ABA levels. ACPK1 was localised to the plasma membrane and chloroplasts and appears to activate a plasma membrane H^+-ATPase through phosphorylation (Yu et al. 2006). The authors suggested that ACPK1 may activate the plasma membrane H^+-ATPase thereby further enhancing the proton gradient which may promote ABA-stimulated sugar uptake, an obvious link with ripening.

Deluc et al. (2007) detailed the expression of a number of genes puta-

tively involved in ABA response. The expression profiles of genes that varied more than two-fold (and for which the data was deemed statistically reliable) were clustered on the basis of the transcript levels, over the seven developmental stages examined. The 18 genes defined as having a function in ABA response were found to be in 11 different clusters out of a total of 21 clusters. Similarly, Pilati et al. (2007) used the Affymetrix grapevine chip to study transcript profiles during the development of field-grown Pinot noir berries over three seasons (2003, 2005 and 2006). The resolution of this study was limited by the analysis of only three time-points during development. The transcript profiles of six genes encoding ABA responsive proteins were in a cluster, whose levels increased after véraison, and the other three were members of a cluster whose expression decreased after véraison. Genes deemed to be ABA regulated, involved in ABA signalling or ABA response factors were expressed in a range of different patterns, when their transcript levels in flesh, skin and seeds were compared (Grimplet et al. 2007).

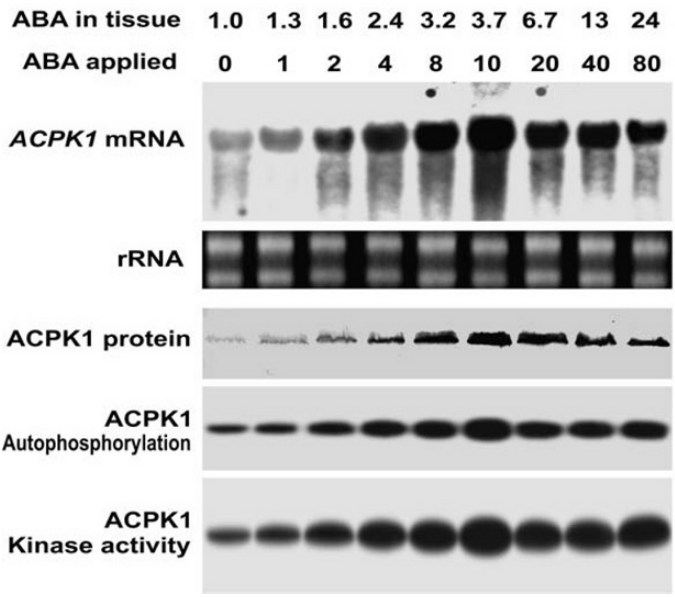

Fig. 1. ABA upregulates gene expression, autophosphorylation, and kinase activity of ACPK1. Discs of berry mesocarp were incubated in different concentrations of (+/-)-ABA, 0-80 um (ABA applied) and after incubation ABA levels in the tissue were determined by radioimmunoassay (ABA in tissue). The level of *ACPK1* RNA was detected by Northern blot analysis (*ACPK1* mRNA). Total microsomal proteins were immunoprecipitated with anti-ACPK1-N antiserum, the proteins were analysed by SDS-PAGE (ACPK1 protein) and assayed for autophosphorylation (ACPK1 Autophosphorylation) or histone-phosphorylating kinase activity (ACPK1 Kinase activity). Reprinted from Yu et al. (2006), with permission from the American Society of Plant Physiologists.

This variation in pattern may reflect a combination of the differing expression pattern of genes involved in ABA response and signalling and the fact that the gene annotation is somewhat erratic and requires further attention. The NCED4 example quoted above illustrates that reliance on the current grapevine gene annotation could lead to misunderstandings.

Clearly, there is considerable scope to further investigate the changes in gene expression of both, the biosynthesis pathway genes and those genes likely to be regulated by ABA. Putative ABA responsive genes are expressed differentially between berry tissues and with different patterns during berry development. Although there are errors in gene annotation, this diversity is not surprising due to the acknowledged complexity of hormone signalling and the number of roles that ABA could potentially play during berry development and stress response.

2.2.5. ABA application affects berry ripening and ripening-related gene expression

As discussed above for ethylene, the application of ABA can also positively influence grape berry ripening in a range of different cultivars. In contrast to ethylene there is little evidence that ABA can delay ripening. However, the timing of treatment with ABA is still crucial to its effectiveness and the types of changes induced. In general, ABA treatment one- or two-weeks before véraison can hasten the initiation of ripening, as judged by earlier increases in sugar levels (often measured as TSS) and colour accumulation (Hale and Coombe 1974, Matsushima et al. 1989). Earlier treatments do not seem to have any effect.

Many reports indicate that there was little effect of ABA treatment on sugar accumulation but that anthocyanin accumulation was increased (Pirie et al. 1976, Kataoka et al. 1982, Yakushiji et al. 2001, Delgado et al. 2004, Mori et al. 2005, Peppi et al. 2006, Cantin et al. 2007, Peppi et al. 2007, Peppi and Fidelibus 2008). Many, but not all, of the treatments in these reports were applied at, or after, véraison which may be the reason for the lack of effect on sugar accumulation. While ABA may be able to hasten the initiation of sugar accumulation it may not be able to further enhance it once ripening has commenced.

The effect of ABA on the expression of genes in the anthocyanin pathway in grapes has been studied in some detail. One report showed that ABA treatment of berries at véraison increased berry anthocyanin levels and PAL activity and overcame the inhibition of anthocyanin accumulation caused by high temperatures and darkness (Kataoka et al. 1984). Anthocyanin levels and chalcone isomerase (CHI) activity (but not PAL activity) were increased by ABA treatment of pre-véraison Olympia (*V. labruscana* L.) cultured berry halves (Hiratsuka et al. 2001). More recent studies have shown that the expression of many of the anthocyanin biosynthesis pathway genes is increased by ABA. Ban

et al. (2003) showed that ABA application to Kyoho grapes (*V. vinifera* L. x *V. labrusca* L.) at the onset of berry softening increased anthocyanin accumulation throughout development. Using Northern blot analysis they showed that the levels of *PAL*, *CHS*, *CHI*, *DFR* and *UFGT* transcripts were elevated at seven days after treatment, but at 21 days after treatment the transcript levels had returned to those found in the control. This suggests that the observed increase in anthocyanins results from a temporary stimulation of gene transcription, the amount and activity of enzymes were not determined. Similar experiments in Cabernet Sauvignon showed that ABA application at véraison also enhanced anthocyanin accumulation. In contrast to the experiment described above, Jeong et al. (2004) used real time Q-PCR to show that in addition to *CHS*, *CHI*, *DFR* and *UFGT*, the expression of *F3H*, *LDOX* and the transcription factor gene *MYBA* was also induced, at two and four weeks after treatment with ABA. They also noted that the increase in transcript accumulation was only temporary and at six weeks after treatment expression levels were the same as those in the controls. These results suggest that ABA application stimulates the activation of the entire anthocyanin biosynthesis pathway.

It appears that ABA application can also affect the synthesis of flavonoids other than anthocyanins. Quercetin levels increase after véraison. ABA sprayed onto berries at véraison increased the concentration in the skin tissue of quercetin compared to that found in control fruit in Merlot, but not Cabernet Sauvignon and also increased transcript levels of two putative flavonol synthase genes in Merlot (Fujita et al. 2006).

Apart from the accumulation of anthocyanins during ripening, berries also accumulate high sugar levels in the form of glucose and fructose. As noted above there is some evidence that ABA application can stimulate the uptake and storage of sugars by berries. Acid invertases are localized in the apoplast, cytoplasm and vacuole and are likely to play important roles in the uptake and accumulation of sugars. Invertases are therefore prime targets for control by ABA. Pan et al. (2005) demonstrated that ABA increased the activity of both soluble and cell wall acid invertases in Kyoho berry discs in a pH- (5.5 was the most efficacious), time- and concentration-dependent manner. The induction of activity was shown to be due to the *cis*-(+) form of ABA.

A further experiment using intact isolated berries confirmed these effects, except that the pH and ABA analogue dependence was not tested. In both cases, the change in activity was reflected in corresponding changes in the amounts of enzyme present. Although the transcript levels were not tested, their increase seems a likely cause of the elevated activities, as the addition of a transcriptional inhibitor nullified the ABA-induced sugar metabolism. Invertase activity was also shown to be controlled through post-translational modification. The protein kinase inhibitor quercetin, suppressed the ABA induction of both cell wall and soluble invertase activity in berry discs without substantially altering

the amount of enzyme (which had been increased by ABA addition). The effect was less evident in enzyme extracts, but it appears that phosphorylation was involved in the control of invertase activity. This conclusion is supported by the decrease in activity of both forms of invertase, when acid phosphatase enzyme was added to the enzyme extract.

There may also have been a slight stimulation of activity of both types of invertase by other protein kinase inhibitors, but these results are less clear cut. In summary, it is likely that a complex series of reversible phosphorylation events are involved in the control of invertase activity, in addition to an ABA-induced increase in enzyme level, which probably derives from increased transcription. These results are important, as they provide evidence of a link between ABA, invertase activity and sugar metabolism. ABA-induced increases in invertase activity could be an important part of the initiation of grape berry ripening.

2.2.6. Interaction between ABA and sugars

The possibility that ABA can induce sugar uptake and accumulation as part of its proposed role in the promotion of grape berry ripening has been discussed above. The relationship between sugars and ABA has been studied in some detail in other plants, where sugar and ABA signalling pathways have been shown to share genes in common (Rook et al. 2006). There is further evidence for interaction between ABA and various sugars in the control of ripening-associated processes in grapes. Pirie and Mullins (1976) showed that both sucrose (20 μmol/berry) and ABA (0.04 μmol/berry) increased anthocyanin levels in the skin of detached berries, when applied through the pedicel. They went on to suggest that there was a synergistic effect when ABA and sucrose were applied together, although the differences were small.

Somewhat contrasting results were obtained by Hiratsuka et al. (2001), who used halved Olympia (*V. labruscana*) berries and two sugar concentrations (2.5% and 10%). Sucrose at the higher concentration tended to inhibit anthocyanin accumulation. ABA applied at 1g/L and rhamnose applied at 10% significantly increased anthocyanin levels. When added together, ABA and rhamnose acted synergistically. What the results of the studies in half berries mean in terms of berry development is debatable. Rhamnose is present in berries during ripening at much lower levels than used in these experiments. Also, the concentration of ABA used is high, even though the actual uptake was not analysed. However, the interaction between sugar and hormone signalling is likely to be important, as shown by work describing the interaction between ABA and the HT1 hexose transporter.

One gene, which may provide a link between sugar metabolism and ABA in grapes is *MSA,* an *ASR*-like gene (Cakir et al. 2003). ASR (ABA-, stress-, and

ripening-induced) proteins have been isolated from a number of species (but not *Arabidopsis*). The grape *ASR*-like gene was isolated using a yeast one-hybrid system with the proximal promoter of *HT1,* a hexose transporter whose transcription is induced by sucrose and glucose (Atanassova et al. 2003), as the bait sequence. This sequence contains two putative sugar motifs. In grape berries, *MSA* and *HT1* transcript levels were highest pre-véraison but were expressed at lower levels from véraison onwards. In berry cell suspensions *MSA* expression was induced by sucrose, but not by ABA alone, however, ABA acted synergistically in the presence of sucrose (Fig. 2). The time-course of the induction of *MSA* and *HT1* transcript accumulation by sucrose in cell suspensions differed as *MSA* was induced transiently, before *HT1*. Gel-shift assays showed that the MSA protein binds to the sugar-responsive elements found in the *HT1* promoter.

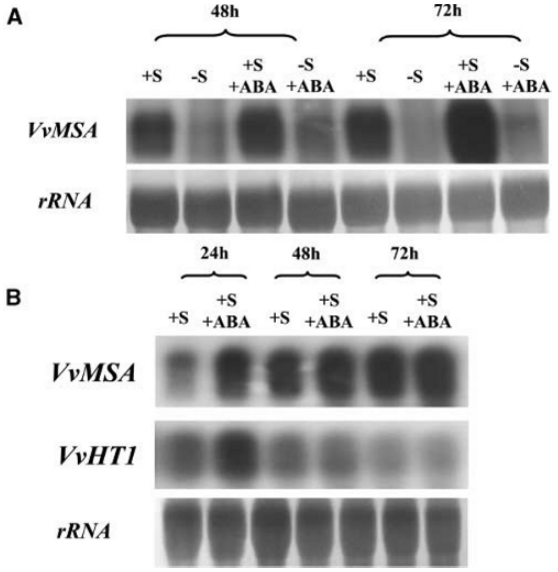

Fig. 2. ABA induction of *VvMSA* and *VvHT1* gene expression in grape berry cell suspension. A, RNA gel blot analysis of *VvMSA* messenger accumulation at 48 and 72 h after ABA treatment (10 μM) in either the presence (+S) or absence (-S) of sucrose. B, RNA gel blot analysis of *VvMSA* and *VvHT1* transcript amounts at 24, 48, and 72 h after ABA treatment in sucrose-supplemented medium. Twenty micrograms of total RNA was loaded in each well. *VvMSA*- and *VvHT1*-specific labeled probes were used for hybridization. Equal loading was checked by 25S rRNA staining with methylene blue. Reprinted from Cakir et al. (2003), with permission from the American Society of Plant Physiologists.

The interaction was also shown through co-expression studies in tobacco leaves using promoter/reporter gene fusions. The presence of a nuclear localiza-

tion signal was confirmed by the localisation of the MSA protein to the nucleus. Together these results suggest that ABA, with sucrose, can influence the cells' hexose transporter activity through changing the level of MSA protein, which in turn interacts with the *HT1* promoter to control its activity. More work needs to be conducted to define the role of MSA in sugar/ABA interactions in grape berries.

On the broader scale, we still have a poor understanding of ABA metabolism and signalling during grape berry development in general. The difficulties involved in the development of a genetic approach will have to be overcome in order to tackle these issues, as the unique aspects of grape berry development mean that knowledge gained in model species may not always be transferable and current research methods tend to yield correlative rather than definitive results.

2.3. Brassinosteroids accumulation and influence on ripening

Brassinosteroids (BRs), which have been shown to have roles in growth promotion and stress response (Haubrick and Assmann 2006), are the latest plant hormones implicated in the control of berry ripening (Symons et al. 2006). The levels of castasterone (CS) and its precursor 6-deoxoCS increased sharply at about the time of véraison in field grown Cabernet Sauvignon berries (Fig. 3a,b). The biosynthesis of castasterone in grapes is thought to occur through the late, rather than the early, C-6 oxidation pathway, due to the low levels of typhasterol. Brassinolide (BL), which is the most active BR, was not detected (Wang et al. 2001). The application of Epi-BL hastened berry ripening whereas brassinazole (an inhibitor of BR biosynthesis) delayed ripening (Symons et al. 2006).

The transcript levels of two biosynthesis genes, *VvDWF1* and *VvBR6OX1*, and a gene encoding a putative receptor, *BRI1*, were followed throughout development. *VvDWF1*, which encodes an enzyme catalysing a reaction early in the BR biosynthesis pathway, was expressed most highly in two week-old berries, after which the levels declined until around the time of véraison, when they again increased and were maintained during the later stages of ripening. A similar pattern was observed by Castellarin et al. (2007), except that in their studies *VvDWF1* transcript levels declined later in ripening. The putative receptor gene, *VvBRI1*, was expressed throughout berry development with a peak in transcript levels shortly after véraison (Symons et al. 2006). The peak in *VvBRI1* transcript levels was also observed in Merlot (Castellarin et al. 2007). Microarray analysis yielded similar results (Deluc et al. 2007, Pilati et al. 2007). The expression pattern of *VvBR6OX1,* which converts 6-DeoxoCS to CS, was most interesting. *VvBR6OX1* transcript levels were low in flowers but increased

steadily to reach a maximum at véraison (8 wpf) after which they declined sharply (Fig. 3c). Microarray data from a study of Pinot noir berry development also indicated that a decrease in *VvBR6OX1* transcript levels occurred after véraison (Pilati et al. 2007).

The negative correlation between *VvBR6OX1* transcript levels and the amount of the corresponding enzyme substrate is characteristic of negative feedback regulation of this gene in other species (Nomura et al. 2001, Bancos et al. 2002, Goda et al. 2002). The presence of high levels of *VvBR6OX1* transcripts at times (e.g. at 8 wpf) when little castasterone accumulation was occurring (Fig. 3), indicates that the control over BR synthesis is quite complex. The expression of a putative *BRH1* ortholog, which encodes a protein involved in BR response, was shown by microarray analysis to increase sharply as berries begin to colour, which agrees with the proposal that BRs have a role during the ripening phase of berry development (Deluc et al. 2007).

The mechanism by which BRs appear to affect ripening is unknown. In other plants, BRs, like ABA, have been found to participate in a range of processes including the control of growth and the response to stress (Finkelstein and Rock 2002, Haubrick and Assmann 2006). Both of these roles may be relevant as during ripening the berry is expanding as well as accumulating large amounts of osmotically active sugars which may induce a stress response.

2.4. Gibberellins

Gibberellins (GAs) are regulators of many processes during plant development involving cell division and expansion. In grapevine GAs are thought to be involved in early berry expansion and fruit set. Changes in the levels of GAs in berry flesh during development are consistent with GAs having a role in the early stages of berry development. The consensus is that GA levels in the flesh of seeded berries are high at around flowering and early in berry development and decrease steadily thereafter (Zhang et al. 2003, Baydar and Harmankaya 2005, Symons et al. 2006).

It is possible that this pattern of accumulation may indicate a role for GAs in delaying ripening, however any delaying of ripening by GA treatments may be attributable to changes in sink source relationships rather than a direct effect on the control of ripening as such. The application of GAs early in florescence and berry development is used in the table grape industry to control such parameters as bunch size, fruit size, bunch openness and berry set. In many cases these effects are not accompanied by consistent changes in berry qualities such as sugar levels. For example, GA_3 sprayed onto Kyoho grapes at 10 days after bloom resulted in increased cluster length, berry weight and size but total soluble solids and titratable acidity were unaffected (Han and Lee, 2004). Sato

et al. (2004) also found that GA applications at full bloom and 10-16 days thereafter had no effect on total soluble solids levels or titratable acidity but they did suggest an effect on flesh firmness.

In contrast Teszlak et al. (2005) suggested that GA treatment of two red-skinned *V. vinifera* cultivars at flowering generally delayed ripening. However, given the possible effects on vine and berry growth it is difficult to suggest that this represents support for a direct effect of GAs on berry ripening. If GAs are used to make the berries on a vine much bigger, it may delay ripening simply because a greater amount of sugar needs to be accumulated to reach ripeness or because of some other general effect on vine growth/performance. Any effects of GA on ripening would appear, in the main, to result from the changes in berry and bunch size and how they relate to the balance between sink and source rather than to any direct effect of GAs on ripening.

There has been some suggestion that GAs can alter the level of invertase, an enzyme thought to be involved in hexose accumulation. Treatments of Sul-

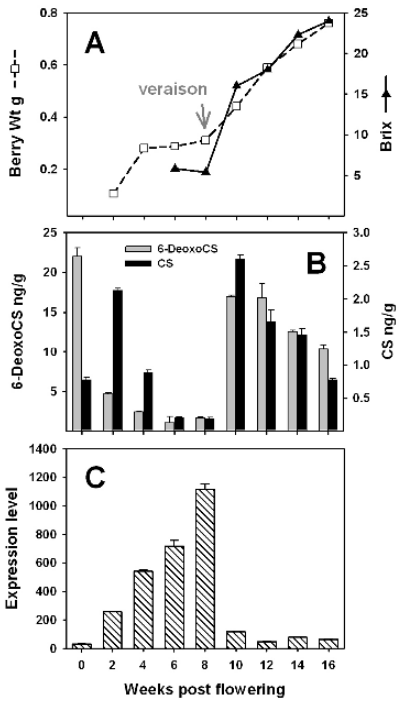

Fig. 3. Changes in BR levels and *VvBR6OX1* transcript levels during grape berry development. A, The pattern of grape berry development as described by berry weight and total soluble solids (^{o}Brix), véraison is indicated with an arrow. B, Levels (ng/g) of 6-DeoxoCS and CS in developing grape berries. C, Relative transcript levels of *VvBR6OX1* were determined by real-time PCR analysis. Adapted from Symons et al. (2006).

tana flowers/berries early in development with GA_3 increased invertase activity and hexose accumulation on a per berry basis (Dreier et al. 1998, Perez and Gomez 2000) but not on a concentration basis (Dreier et al. 1998). However, while Perez and Gomez (2000) suggested a correlation between berry size, invertase activity and hexose content Dreier et al. (1998) found no direct relationship between invertase activity and the rate of assimilate import. The above effects refer to the treatment of seedless berries with GA. The effects on seeded berries have been less extensively studied but they don't appear to respond greatly to exogenous GAs probably because of already having adequate levels in their tissues. In addition the developmental response of seedless fruit to GA application differs from the development of seeded fruit (Shiozaki et al. 1997).

In summary, it seems that there is evidence that supports a role for GAs during the early stages of berry development (including an important role in seed development) but there is little evidence to suggest that GAs are directly involved in the control of berry ripening.

3. INHIBITORS OF RIPENING

3.1. Auxins

3.1.1. The accumulation of auxins in grape berries

The role of auxins in many aspects of plant development has been intensively studied (Boutte et al. 2007, Cheng et al. 2007, De Smet and Jurgens 2007, Delker et al. 2008). In grapes there are a number of reports indicating that auxin levels are high early in development, after which they decline steadily to be very low at véraison (Inaba et al. 1976, Cawthon and Morris 1982, Zhang et al. 2003, Baydar and Harmankaya 2005). Detieux-Belleau et al. (2007) reported a small auxin peak at around the time of véraison and just before the increase in ABA levels. More recently, some doubt was cast on this accepted view of a decline in auxin levels approaching véraison. Symons et al. (2006) analysed Cabernet Sauvignon berries using GC-MS and suggested that IAA contents were low throughout development with no change before véraison; the reason for these conflicting observations is unknown.

3.1.2. The effect on berry ripening of exogenous auxins and auxin transport inhibitors

In the non-climacteric fruit strawberry, auxins have been shown to delay ripening (Given et al. 1988). There are a number of experiments where the application of auxins to grape berries before véraison has also resulted in a delay in ripening or some part of the ripening process, such as the accumulation of

sugars and anthocyanins and the reduction of acidity and chlorophyll levels. A range of auxins and auxin-like compounds have been used, including indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), benzothiazole-2-oxyacetic acid (BTOA) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Weaver 1962, Hale 1968, Hale et al. 1970, Coombe and Hale 1973, Hale and Coombe 1974, Davies et al. 1997, Yakushiji et al. 2001, Ban et al. 2003, Jeong et al. 2004, Fujita et al. 2006, Deytieux-Belleau et al. 2007). Many experiments have been carried out using the auxin-like compound BTOA.

The application of BTOA to Shiraz berries prior to véraison delayed ripening and altered gene expression (Davies et al. 1997). Dipping bunches twice, at 2 and 0 weeks before véraison, delayed softening and sugar accumulation by approximately two weeks. Anthocyanin accumulation was also considerably retarded as was the usual increase in *CHS* and *UFGT* transcript levels that occurs at véraison (Fig. 4 and Davies et al. 1997). The expression of other ripening-related genes was also delayed. The usual, véraison-associated increase in the transcript level of *GRIP4,* which encodes a proline–rich protein putatively involved in cell wall structure (Davies and Robinson 2000), occurred approximately two weeks later than in the control. Curiously, once induced, the transcripts were de-

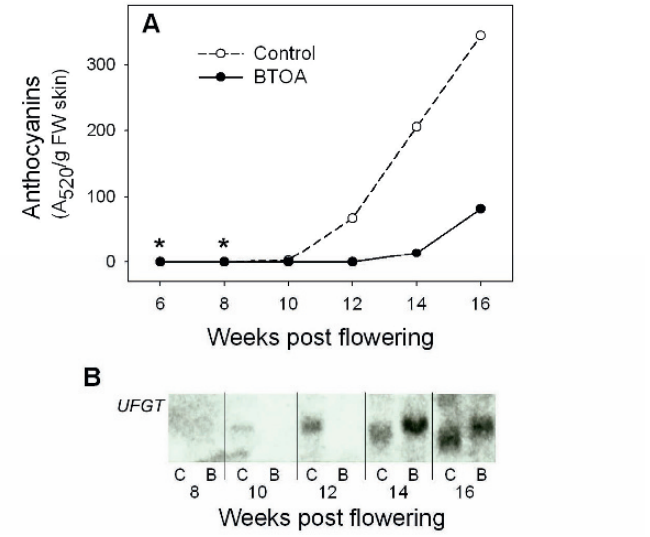

Fig. 4. Effect of BTOA treatment on (A) berry skin anthocyanin content and (B) expression of *UFGT*. Anthocyanin content was determined by measuring the A520 of berry skin samples taken at two weekly intervals. *UFGT* gene expression was determined by Northern-blot analysis. FW, fresh weight; C, Control; B, BTOA-treated. Asterisks indicate the timing of the two treatments with BTOA. Adapted from Davies et al. (1997).

tectable for longer than might be expected. The expression of *CHI4,* a chitinase induced at véraison (Robinson et al. 1997), was also delayed. The gene *GIN1* encodes a vacuolar invertase (Davies et al. 1996) whose expression is normally down-regulated shortly after véraison. BTOA treatment maintained the transcripts of *GIN1* at higher levels for at least four weeks longer than in the control (Fig. 5). The change in invertase activity did not align with transcript levels indicating that other control mechanisms were involved. The usual increase in ABA levels at véraison was also later in BTOA-treated berries. The results indicate that BTOA can delay berry ripening through maintaining fruit in the pre-véraison state.

The suppression of colour development by auxin-like plant growth regulators has been extensively studied. NAA treatment reduced anthocyanin accumulation in Kyoho berries, when applied at véraison (Kataoka et al. 1984). These authors also showed that NAA delayed the increase in ABA in berry skins that usually occurs at véraison and found that PAL activity, which is also usually elevated after véraison, increased much less than in control fruit. Ban et

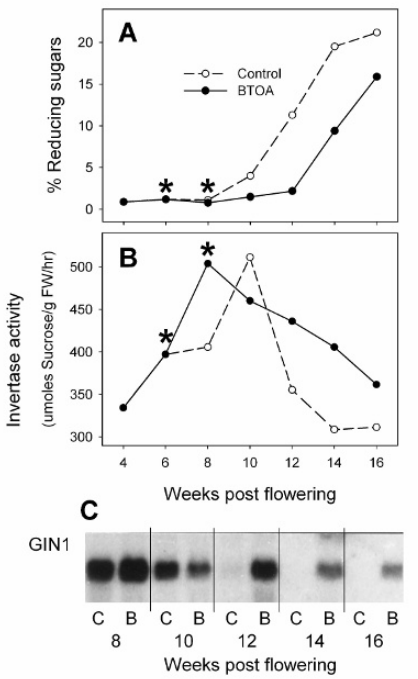

Fig. 5. Effect of BTOA treatment on reducing sugar accumulation, invertase activity, and invertase gene expression. A, Reducing sugars. B, Invertase activity was measured on a per gram basis fresh weight (FW) basis. C, Invertase mRNA expression was assayed by Northern-blot analysis using the *GIN1* cDNA (Davies et al. 1996) as the probe. Asterisks indicate the timing of the two treatments with BTOA. C, Control; B, BTOA-treated. Adapted from Davies et al. (1997).

al. (2003) applied 2,4-D at véraison to Kyoho berries and showed that anthocyanin accumulation was much decreased. The transcript levels of *PAL*, *CHS*, *CHI*, *F3H*, *DFR*, *LDOX* and *UFGT* were all reduced by the 2,4-D treatment and the effect became more apparent as development progressed. NAA application at véraison inhibited anthocyanin accumulation in Cabernet Sauvignon berries. Real time Q-PCR was used to show that the transcript levels of *CHS*, *CHI*, *F3H*, *DFR*, *LDOX*, *UFGT*, and *MYBA* were all lower in NAA-treated fruit (Jeong et al. 2004). The accumulation of flavonols, which arise from dihydro-flavonols, precursors common to the anthocyanin pathway branch, was also reduced by auxin treatment. When applied at véraison, NAA caused a reduction in quercetin levels in Cabernet Sauvignon and Merlot berries, particularly at five weeks after treatment in Merlot (Fujita et al. 2006). The transcript levels of a putative flavonol synthase gene (*FLS4*) were decreased by NAA treatment, particularly in Cabernet Sauvignon. The reduction in quercetin accumulation may well be due to a lack of substrate for FLS as the expression of genes in the flavonoid and general phenylpropanoid pathways were all down-regulated by NAA.

One other aspect of ripening is delayed by auxin treatment. Chlorophylls are usually broken down as berries progress through the early stages of ripening (Coombe 1976). Detieux-Belleau et al. (2007) showed that this breakdown was reduced (or slowed) in Merlot fruit treated with IAA at véraison; they also showed that a lower pH was maintained and that the increase in soluble solids was delayed. Auxin treatment can be seen as delaying véraison by maintaining berries in the non-ripening state, i.e. maintaining the *status quo*. Presumably, berries only go into the ripening state once metabolism of the auxins has occurred and the inhibition is released.

3.1.3. Putative auxin-related gene expression during berry ripening

Microarray analysis of Pinot noir berries has shown that a putative amidase involved in auxin synthesis and an auxin binding protein (ABP19a), thought to be involved in auxin perception, were both expressed more highly before véraison (Pilati et al. 2007). This is consistent with a role for auxins during the first part of berry development. Of the numerous other putatively auxin-regulated genes identified many were expressed similarly to the two genes described above. A more complex situation was reported by Deluc et al. (2007), who assayed seven time-points during development rather than the three time-points used by Piltai et al. (2007). Twenty nine genes were identified as being involved in auxin response. They were distributed between 10 out of 21 gene expression clusters indicating a wide diversity of expression patterns consistent with the role of auxins in a number of developmental processes throughout development, not just during the pre-véraison stage. Many auxin-induced or auxin-responsive genes were preferentially expressed in the skin and flesh but

some were more highly expressed in seeds (Grimplet et al. 2007). Not surprisingly, given the role of auxins in growth, a number of auxin-associated genes were down-regulated with water stress.

In summary, exogenous auxins can suppress berry ripening but the role of endogenous auxin in ripening is largely unexplained. An increased understanding of auxin biosynthesis, perception and signalling in grapevine is required to fully elucidate its role during berry development.

3.2. Cytokinins

Cytokinins are thought to be involved in berry set and in growth promotion. The concentrations of zeatin and zeatin riboside are high early in grape berry development but decrease rapidly to be low at around the time of véraison (Zhang et al. 2003). The decrease in cytokinin levels approaching véraison may be due to the expression of a putative cytokinin oxidase, whose transcript levels are high early in berry development but steadily decrease to be at low levels when berry colour is developing (Deluc et al. 2007). N-(2-Chloro-4-pyridinyl)-N'-phenylurea (CPPU) is a synthetic cytokinin, that has been widely used to manipulate berry development. CPPU applied before véraison increases berry mass but often results in reduced TSS levels and anthocyanin accumulation and increased acid levels (Reynolds et al. 1992, Retamales et al. 1995, Han and Lee 2004, Zabadal and Bukovac 2006, Peppi and Fidelibus 2008). It seems that cytokinins may act somewhat like auxins in that they can promote berry growth but tend to inhibit ripening. This seems in line with the long held view that fruit ripening can be seen as a senescence process which can be inhibited by cytokinins and auxins.

3.3. Salicylic acid

Salicylic acid (SA) and methyl-SA are involved in signalling in plants, particularly in the induction of defence responses. No reports could be found of the levels of these compounds during grape berry development. However, injection of SA into Shiraz berries at two to three weeks before véraison delayed skin colour development and perhaps delayed ripening in general (Kraeva et al. 1998). PAL mRNA levels and PAL enzyme and activity levels were increased when Cabernet Sauvignon berry slices (from fruit at véraison) were incubated with SA (Wen et al. 2005). SA does not appear to play developmental roles in other plants and so while these observations may be interesting, a role for salicylates in the control of ripening remains unproven.

4. INTERACTION BETWEEN HORMONES

The evidence for complex interactions between various signalling pathways in plants is compelling (Gazzarrini and McCourt 2003, Vandenbussche and Van Der Straeten 2007). Due to the difficulty in doing genetic research in grapevines because of their long generation time, the relatively small number of mutants and the difficulty of producing transgenic plants, the state of understanding of these interactions in grapevine is poor. There is no evidence for any direct interactions between hormone pathways during berry development. However, there are reports where the application of one hormone has altered the accumulation of another. This obviously complicates the interpretation of results from experiments using hormone application. For example, BTOA applied to berries during the lag phase caused a transient increase in ethylene evolution (Coombe and Hale 1973). As we have seen earlier (Davies et al. 1997), the application of BTOA delayed ripening and delayed the increase in ABA which normally occurs at véraison (Coombe and Hale 1973, Hale and Coombe 1974). CEPA applied 18 days pre-véraison did not greatly affect ABA accumulation but when used three days after véraison it enhanced ABA levels.

The application of combinations of plant growth regulators and hormones also suggest that interactions are possible. Combined treatment of Tempranillo berries with CEPA and ABA during the early ripening phase had a synergistic effect on the total polyphenol content (Delgado et al. 2004). Various combinations of gibberellic acid (GA3), CCPU and ABA have been used to increase the size and colour in table grapes (Dokoozlian et al. 1994, Retamales et al. 1995, Han and Lee 2004) but it is not possible to speculate on any interactions based on these experiments.

5. CONCLUDING REMARKS

While details of their roles in ripening and the manner in which they interact with each other are still unclear, hormones can be divided into those that promote and those that delay grape berry ripening. The apparent exception to this is ethylene, which appears to both delay and advance ripening depending on the time of application. During ‘normal’ berry development ABA, BRs and ethylene appear to promote the timing of ripening or some of the ripening associated processes. Their application prior to véraison can induce ripening to a greater or lesser extent depending on a range of factors. The more detailed molecular studies have involved ABA and ethylene and are summarised in Fig. 6. This diagram shows both ethylene and ABA having positive effects on sugar and anthocyanin accumulation. So far the only involvement of ethylene in sugar

accumulation appears to be that it positively affects sucrose transport but ABA appears to participate in a number of activities including influencing hexose transport, invertase activity and the strength of proton gradients across membranes. Both hormones appear to be regulators of the anthocyanin biosynthesis pathway.

During the earlier phases of berry development auxin and cytokinins may act to delay ripening. Again their application (and the application of ethylene at the appropriate time) can be used to alter the timing of ripening, this time by delaying its inception. The application of hormones or inhibitors of their synthesis or perception has been used in a range of horticultural industries (including grapes) to control fruit size, colour and maturity. From a scientific point of view, the use of inhibitors and the application of the hormones themselves are useful in defining the role of hormones during ripening. However, some caution must be exercised in the interpretation of results from these experiments. Inhibitors are not always specific in their action, and any applied hormones may be delivered at developmental stages, concentrations, or locations within the fruit, that are not biologically relevant.

One way of altering ripening through changing the biosynthesis or response to hormones is to produce grapevines with altered genomes either through breeding or transgenesis. It seems certain that the proof of hormone function will come through the use of transgenic grapevines over- or under-expressing genes involved in hormone biosynthesis, perception/signalling and action. When these roles are defined it will be possible to better target ways of manipulating both the timing and course of ripening. A better understanding of the biology of hormone action in grapevine can only lead to better biotechnological outcomes.

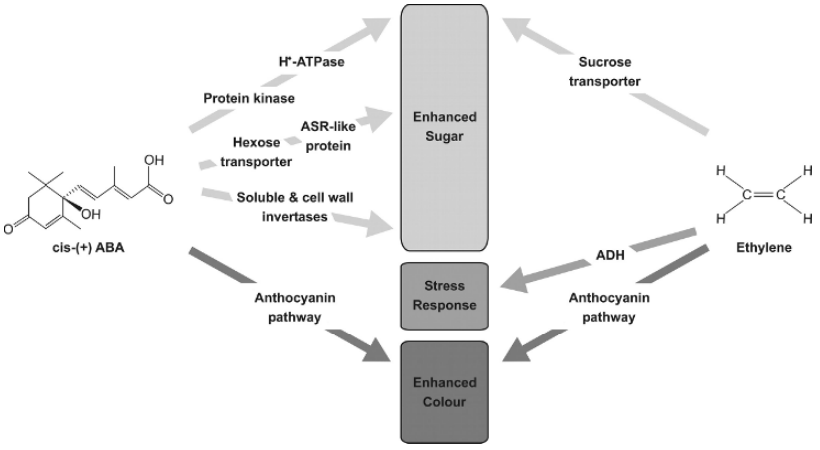

Fig. 6. Schematic detailing the possible pathways by which ABA and ethylene influence sugar metabolism, stress response and anthocyanin accumulation.

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MOLECULAR BIOLOGY OF ANTHOCYANIN ACCUMULATION IN GRAPE BERRIES

P.K. Boss & C. Davies

Commonwealth Scientific and Industrial Research Organisation, Plant Industry, P.O. Box 350, Glen Osmond, South Australia 5064, AUSTRALIA

1. INTRODUCTION

As grape berries develop they change in both size and composition. The most dramatic change in berry development occurs as the fruit enter into the ripening phase. During ripening, berries change from being small, firm and acidic with little sugar, desirable flavours or aroma into larger, softened, sweet, highly flavoured, less acidic and, in the case of some varieties, highly coloured fruit. The development of these characteristics determines the quality of the final product. One of the important and certainly the most visible change is the change in colouration of red and black skinned varieties as they ripen. Berry colour results from the synthesis and accumulation of a group of coloured secondary metabolites called anthocyanins.

Much of the basic physiology of grape berry ripening has been described but less is known of the biochemical and molecular events involved. Grapevines provide particular challenges for these studies as their tissues are rich in phenolic compounds, in particular, tannins. This makes the extraction of nucleic acids somewhat difficult and the extraction of proteins, especially active enzymes, even more problematic. In addition, the enzymes that produce the secondary metabolites responsible for quality factors, such as flavour may be present in low amounts further increasing the problems of studying them directly.

The striking changes in gene expression that occurs at véraison (Davies and Robinson 2000, Terrier et al. 2005, Deluc et al. 2007, Pilati et al. 2007) indicate that the transition into ripening is driven to a large extent by changes in gene transcription. Both structural genes and the controlling genes, that affect their transcription, are involved. Some studies have investigated the expression of proteins during berry development and have shown that the levels of many proteins also change during ripening (Deytieux et al. 2007, Giribaldi et al.

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2007). The use of molecular biology to study ripening related processes is appropriate for two reasons. First, molecular biology techniques can be adapted to deal with difficult tissues and have the sensitivity to detect small but important changes in gene expression. Second, changes in gene expression are a crucial component of the berry ripening process. The understanding of ripening gained using these techniques offers the potential to improve grape quality and productivity *via* modified cultural practices or plant breeding which includes the use of molecular markers and molecular breeding. The current state of knowledge of the molecular biology of anthocyanin accumulation in berries will be outlined in this chapter.

2. THE MOLECULAR BIOLOGY OF ANTHOCYANIN ACCUMULATION IN GRAPE

2.1. Introduction

The colour of red and black grapes is due to the accumulation of anthocyanins, which are usually only localized in the skin of the berry. Chemically, anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium salts. These compounds strongly absorb visible light and are responsible for many of the colours seen in plant tissues ranging from red through to blue. The different anthocyanins are distinguished by (i) the number and position of hydroxyl groups attached to the rings, (ii) the degree and position of methylation of the hydroxy groups, (iii) the nature and the number of sugars attached, and the position of their attachment, and (iv) the nature and the number of aliphatic or aromatic acids attached to these sugars (acylation). The basic chemical 'backbones' that make up the anthocyanins are called anthocyanidins and have no sugars attached to the aromatic rings. There are six common anthocyanidins in plants; pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. Each of these anthocyanidins can be glycosylated and acylated at different sites and with different sugars and acyl groups, and so there are many more anthocyanins than anthocyanidins.

2.2. Grape anthocyanins

The quantity and composition of anthocyanins in grape berries at harvest is often seen as one indicator of fruit quality. Each species or variety of grapes has a distinctive set of anthocyanins, and the anthocyanin profiles of many *Vitis* species and varieties have been described (for reviews, see Mazza and Miniati 1993, Mazza 1995). *Vitis vinifera* varieties usually produce 3-monoglucoside,

3-acetylglucoside and 3-*p*-coumaroylglucoside derivatives of the anthocyanidins delphinidin, cyanidin, peonidin, petunidin and malvidin. However, there are exceptions, Pinot noir being notable as it only produces non-acylated (3-monoglucoside) anthocyanins (Fong et al. 1971). There are no reports of any pelargonidin derivatives isolated from grape berry skins.

The anthocyanin content of grapes increases during ripening, and the accumulation begins at véraison. The extent of this accumulation is influenced by several variables including differences in cultivar, season, growing region and viticultural practices. Measurements of the final anthocyanin content of ripe grapes can vary considerably. For example, in a review published by Mazza and Miniati (1993), Pinot noir was recorded as containing 33 mg of anthocyanins/100 gfw of berries, whereas the cultivar Vincent contained 439 mg/100 gfw. These differences will be due to a number of variables and may not reflect simply the varietal differences. How these variables affect anthocyanin accumulation and composition at harvest involves an understanding of how they affect the control of the anthocyanin pathway, which provides the synthesis of the compounds, and the influence these variables have on anthocyanin degradation. This review focuses on the molecular genetics of the anthocyanin pathway in grape berries.

2.3. Structural genes of the anthocyanin biosynthesis pathway

2.3.1. The anthocyanin biosynthesis pathway

The pathway leading to the production of flavonoids has a branching nature, with the early steps producing intermediates that act as precursors for many types of related compounds (Fig. 1). In fact, recent evidence suggests that in some plant species only the last enzyme in the anthocyanin biosynthesis pathway acts specifically for the production of anthocyanins. Phenylalanine ammonia lyase (PAL) is the first enzyme involved in anthocyanin production: it catalyses the synthesis of cinnamic acid from phenylalanine (Hanson and Havir 1981). The cinnamic acid produced by the action of PAL is then converted to *p*-coumaric acid by cinnamate 4-hydroxylase (C4H; Nair and Vining 1965). The enzyme 4-coumarate CoA ligase (4CL) then adds CoA to *p*-coumaric acid to produce a *p*-coumaroyl-CoA ester (Heller and Forkmann 1988). The first flavonoid produced is a chalcone, and the enzyme involved is chalcone synthase (CHS). Chalcone is produced by the condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA (Kreuzaler and Hahlbrock 1972). Chalcones are then converted to flavanones by chalcone isomerase (CHI), which catalyzes a stereo-specific ring closure (Moustafa and Wong 1967). This reaction can also occur spontaneously, although at a slower rate than that catalysed by CHI (Kuhn et al. 1978), making *chi* mutants leaky. Flavanone 3-hydroxylase (F3H)

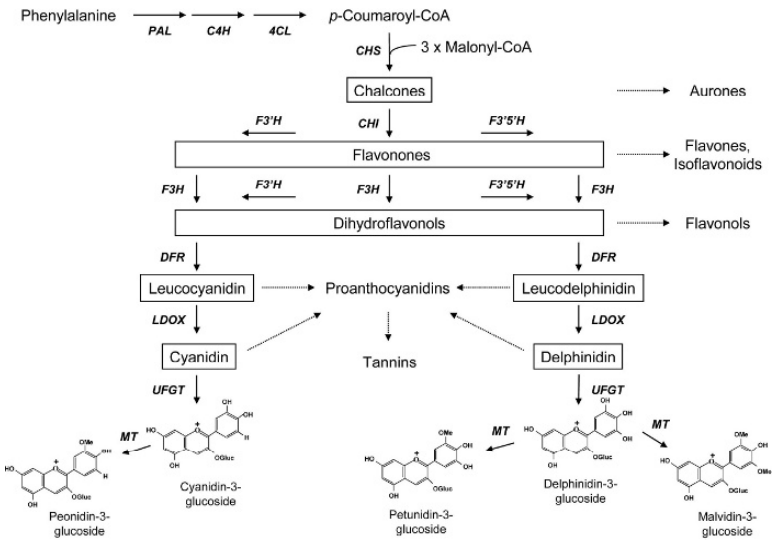

Fig. 1. The anthocyanin biosynthesis pathway. This diagram of the anthocyanin biosynthesis pathway has been modified to account for the major products found in grapes. The enzymes catalysing each reaction are indicated besides the solid arrows. The exact points of action of F3´H and F3´5´H have not been determined for grapes. The fine dotted lines indicate that some of the intermediates are precursors for other biosynthesis pathways with the final products indicated. Key:PAL phenylalanine ammonia lyase; C4H cinnamate 4-hydroxylase; 4CL 4-coumarate CoA ligase; CHS chalcone synthase; CHI chalcone isomerase; F3´H flavonoid 3´-hydroxylase; F3´5´H flavonoid 3´,5´-hydroxylase; F3H flavonone 3-hydroxylase; DFR dihydroflavonol 4-reductase; LDOX leucoanthocyanidin dioxygenase; UFGT UDP-glucose flavonoid 3-*O*-glucosyl transferase; MT methyltransferase.

then hydroxylates flavanones to form dihydroflavonols (Forkmann et al. 1980).

Dihydroflavonol 4-reductase (DFR) catalyses the first step in the conversion of dihydroflavonols to anthocyanins. It causes a reduction at the 4 position of the C ring to give leucoanthocyanidin (Stafford and Lester 1982). The exact chemical nature of the subsequent conversion of leucoanthocyanidins to anthocyanidins is somewhat uncertain. It is widely accepted that the enzyme responsible for the next step in anthocyanin synthesis is leucoanthocyanidin dioxygenase [LDOX; also called anthocyanidin synthase (ANS)]. Initially, it was thought that anthocyanidin production from leucoanthocyanidins requires a dehydratase in addition to LDOX activity (Heller and Forkmann 1988). However, studies using recombinant LDOX from *Perilla frutescens* showed that, under acidic conditions, anthocyanidins were formed from leucoanthocyanidins using just this enzyme (Saito et al. 1999). It is speculated that the primary product of LDOX activity is a colourless 2-flaven-3,4-diol, that can be converted to the coloured anthocyanidin by removal of a hydroxyl anion under acidic conditions (Saito et al. 1999). If this can occur *in vivo*, it means that no dehydratase en-

zyme is required for anthocyanidin synthesis, but does not rule out the possibility that one is present to catalyze a reaction that may occur at a low rate in plants. Anthocyanidins can then be stabilized through the addition of a glucose residue at the 3 position of the C ring. This reaction is catalysed by UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT; Larson and Coe 1977), and the product is believed to be able to be transported through the vacuolar membrane.

However, the exact nature of latter stages of the pathway and the role of LDOX has recently been questioned as the recombinant enzyme from *Arabidopsis* showed predominantly flavonol synthase activity with little LDOX activity (Turnbull et al. 2003). Wellmann et al. (2006) subsequently showed that recombinant LDOX from *Gerbera hybrida* can use catechin as a substrate and will produce cyanidin 3-*O*-glucoside catalyzed by UFGT. This implies that leucoanthocyanin reductase (LAR) activity is important for anthocyanin biosynthesis, in addition to its role in producing catechin for tannins. However, analysis of *lar* mutants in *Arabidopsis* does not support this (Devic et al. 1999) and so the biochemistry of the final steps of anthocyanin production remains uncertain.

2.3.2. Modification and transport of anthocyanins

The outline of the anthocyanin biosynthesis pathway in the previous section describes a linear pathway that begins with phenylalanine and malonyl-CoA and results in the production of a glycosylated anthocyanin. If no modification of the B ring of the anthocyanin molecule occurs, the anthocyanin produced is pelargonidin, but none of this type of anthocyanin is produced in grape berries. This is because grape anthocyanin species undergo hydroxylation of the B ring of the molecule. Enzymes, which catalyse hydroxylation of the B rings, have been shown to act on flavanones or dihydroflavonols, the substrates and products of F3H (Stolz et al. 1985, Menting et al. 1994) and are called flavonoid 3´-hydroxylases (F3´H) and flavonoid 3´5´-hydroxylases (F3´5´H). F3´H activity results in the accumulation of cyanidin-like anthocyanins rather than pelargonidin-like species in plant tissues. F3´5´H activity produces delphinidin species, which produce pigments that are more towards the blue spectrum.

Other modifications of the anthocyanins include methylation, acylation and further glycosylation. Non-*vinifera* species of grape have been characterised by their ability to produce anthocyanin 3,5-diglucosides indicating the presence of anthocyanin 5-*O*-glucosyltransferase activity. This enzyme activity could be encoded by a specific anthocyanin 5-*O*-glucosyltransferase gene in these species, or may result from functional change in the *UFGT* gene that allows for the sequential addition of glucose groups to anthocyanidins as has been described for roses (Ogata et al. 2005). The methylation of anthocyanins is undoubtly an important aspect of grape anthocyanin production given the predominance of

malvidin species in some of the most widely grown black-berried cultivars. Methyl groups may be added to the 3′ position of cyanidin to produce peonidin, or to the 3′ position or 3′ and 5′ position of delphinidin to yield petunidin and malvidin respectively. Bailly et al. (1997) partially purified a methyltransferase (MT) enzyme from anthocyanin-producing grape cell suspensions that had activity against cyanidin 3-*O*-glucoside. The enzyme lacked activity against cyanidin 3-*p*-coumaroyl-*O*-glucoside, suggesting that methylation occurs before acylation, and the lack of MT activity when dephinidin was used as the substrate for the partial purified enzyme also suggests that more than one MT may be involved in grape anthocyanin biosynthesis (Bailly et al. 1997).

Acylation of anthocyanins occurs in grapes by the addition of an aliphatic acetyl group or an aromatic *p*-coumaroyl (or occasionally a caffeoyl) group to the 6′ position of the 3-*O*-glucoside (Mazzuca et al. 2005). It is proposed that these modifications increase the stability of anthocyanins and change their water solubility (Strack and Wary 1994). Anthocyanin and flavonoid acyltransferases have been cloned from several plant species (Nakayama et al. 2003, D'Auria 2006, Luo et al. 2007). The enzymes show specificity for the acyl donor, but tend to be more promiscuous with regards to the acyl acceptor (Nakayama et al. 2003), suggesting that at least two anthocyanin acyltransferases will be responsible for the acylation of grape anthocyanins.

Anthocyanins are synthesised in the cytosol, but they accumulate in vacuoles. The transport of anthocyanins into the vacuole is thought to be mediated *via* a system that is used by plants to recognize, transport and metabolize herbicides and xenobiotics (Marrs 1996). This is presumably because anthocyanins are toxic in the cytoplasm. Evidence that this detoxification pathway is involved in anthocyanin transport first came from the cloning of the gene responsible for the *bronze2* mutant in maize, which does not accumulate anthocyanins in the vacuoles (Marrs et al. 1995). The mutated gene encoded a glutathione *S*-transferase (GST) and it was predicted that it is responsible for producing anthocyanin-glutathione conjugates that are then imported into the vacuole *via* an ABC-type transporter (Marrs et al. 1995). However, the lack of any evidence for the presence of anthocyanin-glutathione conjugates in anthocyanin producing plants, and the discovery that BRONZE2 protein lacking GST activity can still compliment the *bronze2* mutant, has lead to the development of other models for the vacuolar transport of anthocyanins (Mueller and Walbot 2001).

It is now thought that the GSTs act as carrier proteins that can bind anthocyanins and escort them to appropriate transporters for movement into the vacuole (Mueller et al. 2000). Given the important role of anthocyanin sequestration in the accumulation of these compounds in grape berries, this area of research has been given surprisingly little attention.

2.3.3. The identification of grapevine anthocyanin biosynthesis genes

The first grape cDNA clones coding for anthocyanin structural genes were isolated by Sparvoli et al. (1994). They used heterologous probes from maize and snapdragon to screen cDNA produced from 14-day-old grape seedlings grown in continuous light for 48 hours. Full-length cDNA clones that showed homology to *CHS*, *CHI*, *F3H*, *DFR* and *LDOX* genes were obtained and partial clones of *PAL* and *UFGT* were also isolated (Sparvoli et al. 1994). The activity of the enzyme encoded by the grapevine *DFR* gene has been confirmed *in vitro* (Petit et al. 2007). The activity of the enzymes encoded by *PAL*, *CHS*, *CHI*, *F3H*, and *LDOX* has been inferred from sequence homology to genes whose function had been characterised in other plant species.

For most of the core structural genes of the anthocyanin pathway sequence homology is a good indication of gene function due to; i) the high homology of the grape protein sequences to those of known function, ii) the conservation of these protein sequences across multiple species, and iii) the substrate specificity of these enzymes. However, enzymes involved in modifying activities tend to have broader substrate specificities than the core enzymes and they can lack interspecific sequence conservation. They are also often encoded by multigene families. A good example of this is the gene encoding *UFGT* in grapevine, which shows only 42% identity with the equivalent gene from maize (Boss 1998, Ford et al. 1998). Ford et al. (1998) showed that recombinant protein made from this cDNA could function as an anthocyanidin 3-*O*-glycosyltransferase *in vitro*. It was also demonstrated that the presence of this protein correlated strongly with the quantitative ability of the berries to glucosylate anthocyanidins but not with the ability to glucosylate the flavonol quercetin (Ford et al. 1998). Such characterization is essential for the assignment of function to putative modifying enzyme genes.

Two groups have reported the cloning of both *F3´H* and *F3´5´H* genes from grape (Bogs et al. 2006, Castellarin et al. 2006). It was shown that grapes possess two copies of *F3´H* and there are several copies of *F3´5´H* clustered on a single chromosome (Castellarin et al. 2006). Functional characterization of *F3´H* and *F3´5´H* genes from grape was conducted in a petunia mutant and confirmed the activities assigned to these genes based on sequence homology to genes from other plant species (Bogs et al. 2006). Microarray profiling of red and white grapes has lead to the identification of an *MT* and a *GST* gene that have expression patterns that correlate with anthocyanin production in developing berries (Terrier et al. 2005, Ageorges et al. 2006). However, confirmation of a role for these genes in anthocyanin modification and transport awaits functional characterisation.

The completion of the genome sequence for grapevine (Jaillon et al. 2007) will enable a complete genetic picture of the pathway leading to antho-

cyanin production in grapes to be generated. This will allow the copy number of genes from the pathway to be determined and facilitate the association of isogenes with the anthocyanin pathway and/or the synthesis of other flavonoids and phenolic compounds. The genome sequence will also aid the identification of genes encoding *C4H* and *4CL* from the general phenylpropanoid pathway that, to date, have not been extensively studied, and the identification of genes encoding putative acyl transferases, MTs, GSTs and membrane transporters responsible for the modification and transport of anthocyanins into vacuoles.

2.3.4. Anthocyanin gene expression in berry skins during development

The isolation of grapevine homologues of anthocyanin pathway genes has enabled the use of various techniques to study the transcriptional activity of the pathway in berries. Northern analysis of the expression of the genes from the anthocyanin pathway in berry skins during development revealed two distinct patterns of expression (Boss et al. 1996a, Fig. 2). Early in development all the anthocyanin pathway genes, except *UFGT*, are highly expressed and this is followed by a reduction in the expression of all the genes during the lag phase of grape berry development (Fig. 2). After véraison, there is a coordinate induction of all the genes from the pathway including *UFGT* and this coincides with the accumulation of anthocyanins in the skin. These results suggest that *UFGT* is under a different regulatory regime than the genes from the rest of the anthocyanin pathway. The link between *UFGT* expression and anthocyanin biosynthesis in berry skins was further demonstrated by retarding the ripening process through the application of the synthetic auxin-like compound, BTOA, to grape berries (Davies et al. 1997). When expression of *UFGT* was analysed in the treated and untreated samples it was found that *UFGT* transcripts were only present in those berries producing anthocyanins. In the BTOA-treated samples the onset of *UFGT* expression was delayed by four weeks, which paralleled the delay in other ripening parameters caused by the treatment (Davies et al. 1997). Several subsequent studies have been conducted where the expression of the anthocyanin structural genes during berry development has been determined using Northern analysis (Kobayashi et al. 2001), quantitative PCR (Jeong et al. 2004, Castellarin et al. 2007a) or microarray technology (Pilati et al. 2007). All confirmed that *UFGT* expression correlates with the accumulation of anthocyanins in grape berry skins.

A conundrum from the early studies on the anthocyanin pathway was that *LDOX* is expressed early in berry development in the absence of *UFGT*. The expression of the genes from *CHS* to *DFR* could be explained by the accumulation of tannins in the young berries (Boss 1998), as the production of these compounds was assumed to branch from the main flavonoid pathway at

this point. However, it has subsequently been shown that LDOX activity is required in plants for the production of certain tannin subunits (Xie et al. 2003)

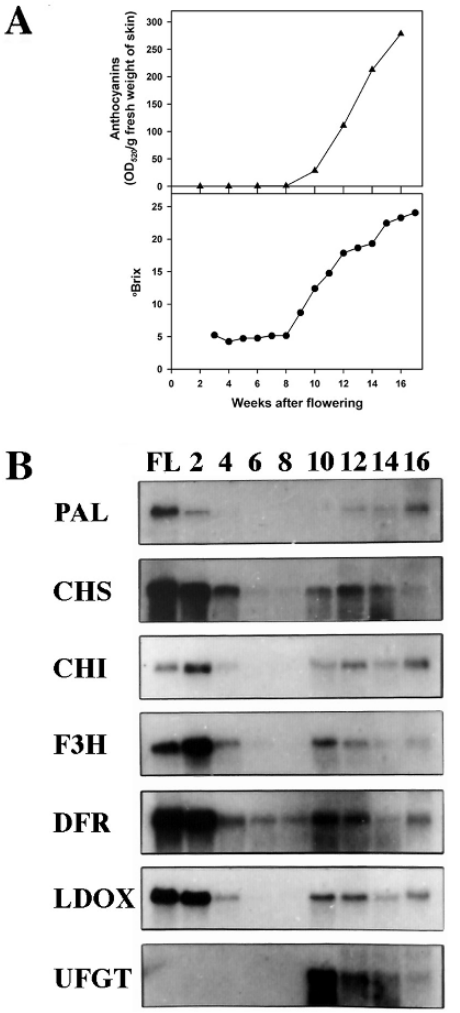

Fig. 2. Total anthocyanin accumulation and Northern analysis of anthocyanin gene expression during the development of grape berry skins. A) Changes in total anthocyanins per gram fresh weight of berry skin (top) and changes in total soluble solids in the berry juice, measured as °Brix (bottom) during the development and ripening of Shiraz grape berries. The vertical, dotted line represents véraison. B) Northern blots of total RNA from grape flowers (FL) and grape berry skin samples taken at fortnightly intervals throughout development, probed with grape cDNA clones for *PAL*, *CHS*, *CHI*, *F3H*, *DFR*, *LDOX* and *UFGT*. The numbers indicate weeks post-flowering at which the RNA was extracted from berry skins. Reproduced from Boss et al. (1996) Plant Physiol 111:1059-1066, with permission from the American Society of Plant Physiologists.

and this explains the need for *LDOX* expression during early berry development.

The expression patterns of genes encoding anthocyanin modifying enzymes have also been studied. Developmentally, the *F3´H* and *F3´5´H* genes are expressed in a similar manner to the genes encoding enzymes from the early part of the pathway (Terrier et al. 2005, Bogs et al. 2006). The expression of *F3´H* and *F3'5'H* during stages of berry development when anthocyanins are not being produced implies a role for these enzymes in the modification of other flavonoid pathway products such as flavonols or proanthocyanidins (Bogs et al. 2006). Additional functions outside of anthocyanin biosynthesis is also likely for the genes encoding putative anthocyanin-related MT and GST proteins, as they clustered with neither the early pathway genes nor *UFGT* when assigned to gene groups based on developmental expression patterns (Terrier et al. 2005). Flavonols that are methylated in the B ring of the molecule have been identified in grape berries (Castillo-Munoz et al. 2007) and the similar structure of anthocyanins and flavonols may allow the same MT to act on both. It is also possible that the *GST* described by Terrier et al. (2005) may encode a protein that is involved in the transport of many flavonoids. It has been shown in *Arabidopsis* that the mutation of a GST-encoding gene, *TT19* reduces the accumulation of anthocyanins in the vegetative organs, and proanthocyanidins in the seed (Kitamura et al. 2004).

Nevertheless, the confirmation of the proposed function of these grape genes is required before conclusions can be drawn about how their expression pattern may influence anthocyanin production in grape berries. The developmental studies described in this section have shown that there is a coordinate induction of the anthocyanin pathway genes after véraison. This suggests that the anthocyanin pathway in grapevines is tightly controlled by regulatory genes as has been observed in other plant species. The two patterns of expression seen in berry skins pre- and post-véraison (i.e. with and without *UFGT*) result in the tissue either producing anthocyanins or not. Thus, the production of anthocyanins appears to be controlled beyond the step catalysed by LDOX, later in the pathway than seen in other plant species including maize, petunia and snapdragon (Davies and Schwinn 2003).

2.3.5. Anthocyanin gene expression in pigmented *versus* non-pigmented tissues

White grape varieties are thought to have arisen from red- or black-skinned varieties (Slinkard and Singleton 1984). Before the identification of regulatory genes responsible for anthocyanin production in berry skins, it was unclear how many times the white phenotype had arisen. To investigate the possible nature of the mutations leading to the white-skinned phenotype, expression

of the anthocyanin pathway genes was compared in a number of white and black varieties (Boss et al. 1996b). Transcripts of all of the anthocyanin structural genes below *CHS* were detected in the red or black varieties studied (Fig. 3, Boss et al. 1996b). Most of the white grape varieties tested showed moderate levels of expression of all the genes except *UFGT*, which could not be detected in any of the white berry skin samples (Fig. 3). Bud mutations that restore anthocyanin production in the skins of white berries have also been examined for changes in anthocyanin gene expression (Boss et al. 1996c, Kobayashi et al. 2001).

These revertant sports are interesting as they suggest that, if white-skinned varieties are derived from pigmented varieties, it is possible for grapes

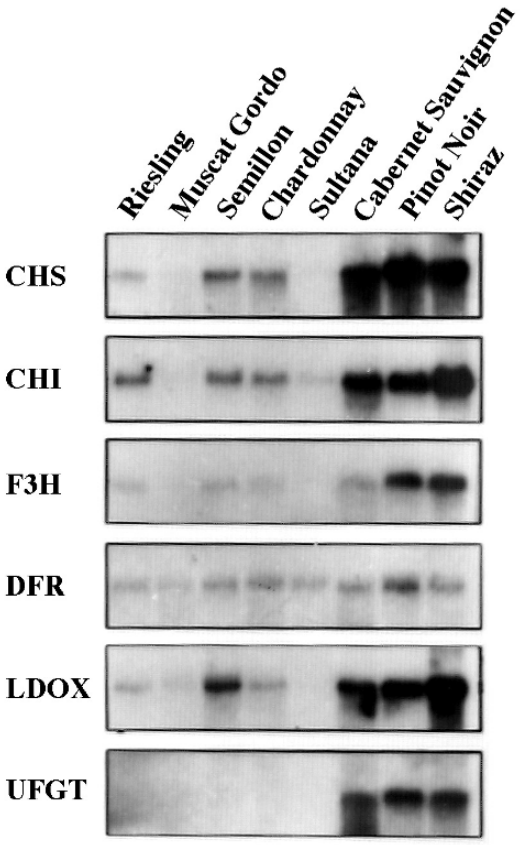

Fig. 3. Northern analysis of anthocyanin gene expression in the skins of various grapevine varieties. Total RNA was isolated from the skins of ripe berries from the varieties indicated at the top of the figure. Northern blots of the RNA were probed with grape cDNA clones for *CHS*, *CHI*, *F3H*, *DFR*, *LDOX* and *UFGT*. Reprinted from Boss et al. (1996b) with permission.

to regain the ability to produce anthocyanins. *UFGT* was again the only anthocyanin gene differentially expressed between these red-skinned varieties and their white-skinned progenitors (Boss et al. 1996c, Kobayashi et al. 2001). However, the anthocyanin profiles of these 'revertant' varieties often lack acylated anthocyanins, possess a high cyanidin:malvidin ratio, and have less total anthocyanins (Boss et al. 1996c). As a result, their colour is generally lighter (red rather than black) than that of the usual red wine varieties. These findings suggest that the flux down the anthocyain pathway is compromised in the revertants or that they are chimeric and the control of genes encoding some of the enzymes that modify anthocyanins may be altered in such bud sports.

The consistent difference between the white and red or black grapes was that the expression of *UFGT* was not detected in any of the white grapes (Boss et al. 1996b, Boss et al. 1996c). Southern analysis showed that all the white varieties tested contain at least part of the *UFGT* gene (Boss 1998). Thus the lack of *UFGT* gene expression in the white-berried varieties is due to a lack of transcription of the gene and not simply an absence of *UFGT* in the genome. Sequence analysis of the promoter and coding regions of grapevine *UFGT* genes from white cultivars and their red-berried sports showed no differences (Kobayashi et al. 2001). These results suggested that the loss of *UFGT* gene expression in the white-skinned varieties is due to the absence or mutation of a regulatory gene product.

Researchers have also looked at the expression of the structural genes from the anthocyanin biosynthesis pathway in grapevine tissues other than berry skins. Sparvoli et al. (1994) exposed etiolated grapevine seedlings to continuous light, which induced the production of anthocyanins. It was found that *PAL* was constitutively expressed in seedlings, whereas the mRNA levels of *CHS*, *CHI*, *F3H*, *DFR*, *LDOX* and *UFGT* were dramatically increased upon exposure to light. Dark grown seedlings had a low basal level expression of the genes in the anthocyanin pathway between *PAL* and *LDOX*, which is thought to drive the biosynthesis of other flavonoids in these seedlings (Sparvoli et al. 1994).

Using Northern analyses, Boss et al. (1996b) could detect expression of genes from the anthocyanin pathway down to *LDOX* in several tissues and organs from Shiraz vines, that did not accumulate anthocyanins, but *UFGT* was not expressed in these tissues. Castellarin et al. (2006) demonstrated that *UFGT* expression was associated with tissues other than berry skins that were producing anthocyanins, and that the genes encoding the early enzymes of the pathway down to *LDOX* were expressed in non-pigmented tissues. These results confirm the importance of *UFGT* expression to anthocyanin accumulation in all grape tissues.

In a study using RT-PCR and microarrays, Ageorges et al. (2006) identified nine genes whose expression patterns were consistently and significantly

correlated with the production of anthocyanins in grape berries independent of cultivar, developmental stage and tissue. The presence of *UFGT* in this list acted as a good internal control. An isogene of *CHS* (*CHS3*) was also in this group of genes, and these findings confirmed those of Goto-Yamamoto et al. (2002), who found that *CHS3* was expressed only in the anthocyanin producing tissues they tested. It was suggested that *CHS3* is the grape *CHS* isogene responsible for anthocyanin production in berries (Ageorges et al. 2006).

However, Real Time-PCR analysis of gene expression in red and white grape tissues and organs revealed that *CHS3* did not have the same expression pattern as *UFGT* in the pigmented samples (Castellarin et al. 2006). Also, *CHS1* and *CHS2* are expressed in ripe grape berry skins along with *CHS3*, meaning all could be involved in anthocyanin production in this tissue regardless of whether they are expressed in non-pigmented tissue as well. Interestingly, two other genes with expression patterns that correlate with anthocyanin production are homologs of genes involved in anthocyanin production and transport in other species (Ageorges et al. 2006). One encodes an MT and the other a GST. The transcript levels of both of these genes have been shown in other studies to increase in berries after véraison (Castellarin et al. 2006, 2007a).

However, unlike the structural genes that make up the main branch of the anthocyanin biosynthesis pathway, MTs and GSTs perform multiple functions in plant cells and are encoded by diverse multigene families. Whilst expression analysis provides good evidence that these grapevine genes are responsible for the methylation and transport of anthocyanins into the vacuole, unequivocal proof of function awaits characterization *in vitro* or *in planta*.

2.4. Regulatory genes of the anthocyanin biosynthesis pathway

2.4.1. Introduction

Some anthocyanin mutants were found to be caused by the change in expression of more than one structural gene, or they could not be mapped to specific structural genes. Such mutants have lesions in genes that regulate the expression of the structural genes. These regulatory genes were first studied extensively in maize, petunia and snapdragon (Davies and Schwinn 2003) and have subsequently been characterized in *Arabidopsis* (Broun 2005). The conclusion from this work is that three types of transcription factors (TFs) appear to play key roles in regulation of the structural genes, MYB, bHLH and WD40 type.

Current models suggest that MYB and bHLH partners interact as activators of the biosynthetic genes, and the WD40 proteins somehow assist in this process (Davies and Schwinn 2003, Broun 2005). The MYB TFs appear to be specific for the regulation of certain flavonoid structural genes, whereas there is

redundancy in the bHLH proteins that the MYB TFs can interact with to regulate gene expression (Broun 2005). The control of the flavonoid pathway in different plant species is influenced by both the promoter regions of the anthocyanin structural genes and the expression profiles and properties of the regulatory genes. This interaction determines the tissue specificity of anthocyanin synthesis and which genes of the pathway are upregulated upon the induction of anthocyanin synthesis.

2.4.2. *VvMYBA* genes

The identification of grapevine genes encoding MYB TFs responsible for anthocyanin production was first reported by Kobayashi et al. (2002). *VlMYBA1* and *VlMYBA2* were isolated from Kyoho grapes and were shown to induce anthocyanin formation when bombarded onto somatic embryos (Kobayashi et al. 2002). Kobayashi et al. (2004) then demonstrated that the white-skinned varieties Italia and Muscat of Alexandria have a *GRET1* retrotransposon inserted upstream of the coding sequence of *VvMYBA1*, the *V. vinifera* homologue of *VlMYBA1*. In red-skinned bud mutants derived from these varieties, *GRET1* is excised allowing expression of *VvMYBA1*. This suggests that the *GRET1* transposon-related disruption of *VvMYBA1* expression is the molecular basis for white coloration in Italia and Muscat of Alexandria berries (Kobayashi et al. 2004).

Confirmation that the *GRET1* retrotransposon affects *VvMYBA1* expression was obtained when *VvMYBA1* expression was compared in white-skinned Italia berries and its red-skinned sport Ruby Okuyama (Kobayashi et al. 2005). No *VvMYBA1* expression was examined in either cultivar before veraison, but expression was observed in Ruby Okuyama after véraison. Interestingly, *VvMYBA2* was expressed in both cultivars after veraison (along with another related gene *VvMYBA3*), suggesting that *VvMYBA2* does not encode an active protein or that a suitable bHLH partner is absent in post-véraison berries (Kobayashi et al. 2005). Lijavetzky et al. (2006) subsequently showed that *VvMYBA1* mapped to a locus previously shown to be responsible for berry colour (Doligez et al. 2002, Fischer et al. 2004) and that in 95% of cultivars they analyzed, molecular variation in *VvMYBA1* was associated with altered berry colour phenotypes. This et al. (2007) found that 81 of 84 white-skinned grape varieties they tested possessed the *GRET1* insertion in the *VvMYBA1* promoter. Both of these studies have shown that the *GRET1* insertion is present in most white-skinned cultivated grapes, but there is evidence that the white phenotype has been generated by other mutation events. For example, the genomic region around *VvMYBA1* was studied in the white-skinned variety Pinot blanc and its presumed progenitor, Pinot noir, to determine the nature of the mutation leading to the loss of berry pigmentation (Yakushiji et al. 2006). Pinot noir is heterozy-

gous at the *VvMYBA1* locus; it possesses one allele with *GRET1* in the promoter whereas the other allele contains neither the *GRET1* transposon nor the footprint of the transposon suggesting that it is the original active *VvMYBA1* allele (Yakushiji et al. 2006). Pinot blanc contains one copy of the *VvMYBA1* allele with the *GRET1* insertion, but the genomic region containing the functional *VvMYBA1* allele, present in Pinot noir, has been deleted resulting in white-skinned berries (Yakushiji et al. 2006). Similarly, Walker et al. (2006) suggested that a deletion in the region of the genome harbouring the functional *VvMYBA1* allele is responsible for the white-berried phenotype of Pinot blanc.

A model has been developed to explain the majority of phenotypic variation seen in grape berry skin colour in relation to the haplotype of *VvMYBA1* (This et al. 2007). Black or noir grapes possess at least one copy of the wild type allele of *VvMYBA1*. The grey or gris phenotype appears to be the result of a deletion of the wild type gene in the L2 cell layer, leading to the generation of a chimaera with lower levels of total anthocyanin in the outer tissues of the berry (Hocquigny et al. 2004, Walker et al. 2006). Red and pink (rouge and rose) coloured berries contain either an 829 bp or a 44 bp indel in the promoter region of *VvMYBA1* (This et al. 2007). Sequence comparisons suggest that the 829 bp indel is the footprint left after the excision of the *GRET1* transposon (Kobayashi et al. 2004). The 44 bp indel in other red/pink-skinned cultivars is always associated with two base pair polymorphisms in the first exon of *VvMYBA1* (This et al. 2007). The significance of these polymorphisms or the origin of these mutations is unknown.

In summary, the white berried phenotype is almost always associated with the presence of the *GRET1* retrotransposon in the promoter of *VvMYBA1*, although there are a small number of white varieties that do not have this insertion (This et al. 2007), and large deletions in the colour locus have also been shown to occur (Walker et al. 2006, Yakushiji et al. 2006). The identification and characterization of this locus will greatly assist the selection of progeny for berry colour phenotype in conventional breeding programs.

Interestingly, *VvMYBA2*, the *V. vinifera* homologue of *VlMYBA2*, is located adjacent to *VvMYBA1* in the berry colour locus (Walker et al. 2007). *VvMYBA2* is able to induce anthocyanin production in cell suspension cultures when co-bombarded with a construct containing a *bHLH* gene, and *VvMYBA2* is mutated in white-skinned varieties (Walker et al. 2007). It is predicted that both *VvMYBA1* and *VvMYBA2* need to be mutated in order to eliminate anthocyanin production in grape berry skins, as white cultivars tested have mutations in *VvMYBA2* as well as *VvMYBA1*. Although the experimental evidence suggests that *VvMYBA2* can also regulate anthocyanin production in the berry skin, definitive proof awaits the discovery of a red-skinned variety with the *VvMYBA1* gene mutated and the *VvMYBA2* gene still functional.

The first examination of *VvMYBA1* expression in grape berries was conducted on fruit that had been treated with hormones or shaded to alter anthocyanin accumulation (Jeong et al. 2004). The expression levels of *VvMYBA1* post-véraison mirrored that of not only *UFGT* but also the other structural genes from the anthocyanin pathway that were tested, leading to the suggestion that *VvMYBA1* regulates all the genes from the pathway during the ripening phase of berry development (Jeong et al. 2004). This would mean that other regulators control expression of genes from the pathway pre-véraison.

Ageorges et al. (2006) showed that the expression profile of *VvMYBA1* in various berry tissues of three cultivars matched the changes in expression seen for structural genes (*PAL*, *CHS3* and *F3H*). The study included two cultivars that had red-fleshed berries, suggesting that *VvMYBA1* expression drives the red-fleshed as well as the red-skinned phenotype. A role for *VvMYBA1* in the red-fleshed phenotype is supported by the findings of Jeong et al. (2006), that the red-fleshed cultivar Bailey Alicante A expresses *VvMYBA1* throughout the berry. In the same study *VvMYBA1* was not expressed in non-berry tissues accumulating anthocyanins, which suggests that *VvMYBA1* does not regulate the anthocyanin pathway in tissues other than the berry (Jeong et al. 2006).

2.4.3. Other regulatory genes

Another grapevine MYB-like transcription factor has been cloned from berry cDNA and it was functionally characterised in tobacco (Deluc et al. 2006). The gene, termed *VvMYB5a*, showed highest expression pre-véraison, but its mRNA could still be detected in berry skin post-véraison. Overexpression of *VvMYB5a* in tobacco using the 35S promoter significantly increased the levels of anthocyanins in the petals of the transgenic plants. The stamens of these transgenic plants were pigmented due to the accumulation of anthocyanins, whereas controls did not produce detectable levels of anthocyanins (Deluc et al. 2006). Definitive proof for a role for *VvMYB5a* in the accumulation of anthocyanins in berry skins awaits loss-of-function studies in a homologous system. Nevertheless, the data suggest that this MYB TF can regulate structural genes of the flavonoid pathway and may work in concert with other MYB regulators (e.g. VvMYBA1) to alter the levels of the different classes of compounds synthesised *via* this common pathway.

Questions remain over the role that grapevine bHLH and WD40 proteins play in the accumulation of anthocyanins in grape berries. Walker et al. (2007) found that VvMYBA1 and A2 proteins require the presence of a bHLH protein from *Matthiola incana* in order to induce anthocyanin production, when transiently expressed in grape cell cultures. However, Kobayashi et al. (2005) found that VvMYBA1 could induce anthocyanin production in both grape somatic embryos and berry skin tissue when transiently expressed. These conflicting

findings may reflect differences between the potential bHLH partners that may or may not be expressed in the cells or tissues used for these transient expression studies. As yet, no bHLH partners for VvMYBA1 have been isolated from grapevine. Similarly, no genes encoding WD40 proteins have been identified that regulate anthocyanin production in grape berries, although Ageorges et al. (2006) found a *WD40* gene that was only expressed in the pigmented berry tissues examined in their study. This would be an obvious target for further functional studies. The isolation and characterisation of genes coding for the bHLH and WD40 proteins predicted to complete the complex regulating the anthocyanin structural genes in grape berries will no doubt be accelerated by the sequencing of the grape genome (Jaillon et al. 2007).

2.5. Viticultural and environmental impacts on the anthocyanin pathway

2.5.1. Introduction

The cloning of the grapevine genes encoding enzymes from the anthocyanin biosynthesis pathway has enabled researchers to explore the effects of the environment and viticultural management on the transcriptional activity of the anthocyanin pathway. The effects of plant growth regulators on the anthocyanin pathway in grape berries has also been examined. More recently, the identification of the *VvMYBA1* regulatory gene, which controls the berry colour phenotype, has allowed researchers to study the effect of endogenous and exogenous stimuli on the regulation of the anthocyanin pathway. Furthermore, with the identification of genes encoding putative modifying enzymes, the effect of viticultural and environmental variables on the composition of anthocyanins in grapes has also been a target of molecular studies. The following sections summarize the research to date in this field.

2.5.2. Temperature

The accumulation of anthocyanins in grape berries is affected by temperature with higher temperatures generally decreasing overall anthocyanin levels. Studies have examined whether the reduction in anthocyanin production due to higher temperatures is related to transcriptional changes in expression of the anthocyanin pathway genes. The effects of different night temperatures on the anthocyanin pathway were studied by Mori et al. (2005). They found that an elevated night temperature (30°C cf 15°C) decreased anthocyanin accumulation in the table grape variety Darkridge. There was a marked increase in *UFGT* transcript levels and enzyme activity in the berries grown under cooler night temperatures suggesting there is a link between transcription of the anthocyanin structural genes and anthocyanin accumulation (Mori et al. 2005). Yamane et al.

(2006) studied the expression of the anthocyanin structural genes and *VvMYBA1* in the table grape variety Aki Queen in response to different growth temperatures. In these experiments the authors exposed potted vines to either 20°C or 30°C 24 hours a day for 2 weeks at 4 different stages of development. The only treatments to significantly alter anthocyanin levels in the berries at harvest were those conducted just after véraison; the 20°C treatment had significantly higher anthocyanins than the control and the 30°C treatment had significantly lower anthocyanins than the control (Yamane et al. 2006). The expression of the structural genes from the anthocyanin pathway were higher in the 20°C treated samples than in the berry skins from those vines exposed to the higher temperature. However, the 30°C samples still accumulated detectable levels of mRNA of the anthocyanin pathway genes, even though there was almost a complete inhibition of anthocyanin accumulation. This led the authors to suggest that the reduction in anthocyanins may be due to enzyme inhibition, anthocyanin degradation or a loss of substrate rather than a transcriptional effect (Yamane et al. 2006). Also, Mori et al. (2007b) found that the expression of anthocyanin structural genes and *VvMYBA1* was not significantly reduced when Cabernet Sauvignon grapes were exposed to 35°C day temperatures compared to the 25°C controls. However, anthocyanin levels were reduced by 50% due to the high temperature treatment, supporting a non-transcriptional basis for the affect of temperature on anthocyanin levels.

The influence of temperature on anthocyanin composition has been studied using potted Pinot noir vines grown in a phytotron (Mori et al. 2007a). The vines were subjected to either high morning and evening temperatures (30°C cf 20°C) or high day-time temperatures (35°C cf 25°C) and the anthocyanin composition was examined in relation to the expression of *F3´H* and *F3´5´H* genes in the berry skins. Total anthocyanin levels were not greatly affected by the treatments, but the amount of tri-hydroxylated anthocyanins was reduced by the higher temperatures. The expression of *F3´5´H* was reduced by temperature, suggesting the effect is due to changes in the steady state levels of *F3´5´H* mRNA. Whether this is due to changes in mRNA stability or transcription levels has not been determined.

One difficulty in drawing conclusions from the results of these experiments into the temperature affects on anthocyanin gene expression is the lack of uniformity between the studies. The use of different cultivars introduces genetic variables that need to be tested. For example, the presence of the *GRET1* footprint in red-skinned revertant cultivars may alter the response of *VvMYBA1* expression to elevated temperatures. The experiments have also been conducted with variable treatment lengths, different temperatures, during various stages of development and at different times of the day, which makes comparisons difficult. In general, it seems that elevated temperatures (above 30°C) do not greatly

affect the transcription of the anthocyanin pathway, but result in reduced anthocyanin accumulation in berry skins by increasing chemical degradation or a post-transcriptional mechanism that reduces pathway flux.

2.5.3. Light

The results of studies into the effects of light on the accumulation of anthocyanins are somewhat conflicting. However, this seems to be due to different research groups using different cultivars, and so there may be a genetic basis to the influence of light on anthocyanin accumulation. For example, Jeong et al. (2004) artificially reduced the light reaching Cabernet Sauvignon bunches to 18-20% of that of controls beginning at véraison, and found that anthocyanin levels were reduced in the berry skins. This was reflected in the reduction in expression of anthocyanin structural genes and *VvMYBA1* (Jeong et al. 2004). However, Downey et al. (2004), found that excluding light from Shiraz bunches only had a significant effect on anthocyanin levels in one out of three years of their study. The expression of *UFGT* was examined in the year in which significant differences in anthocyanins were observed, and expression was higher in the exposed fruit for the first four weeks after véraison (Downey et al. 2004). However, the fact that no significant differences were seen in anthocyanin accumulation in the other two years suggests that a variable other than light was responsible for the differences observed in the second year. Anthocyanin gene expression is induced by light in seedlings and cell cultures (Sparvoli et al. 1994, Gollop et al. 2001, 2002), but whether such experiments are relevant to the affect of light on the transcriptional activity of the anthocyanin pathway in berry skins is yet to be determined.

2.5.4. Irrigation

Specific irrigation regimes are often used by viticulturists to modify vine physiology and this in turn can affect grape composition. Castellarin et al. (2007b) employed a water irrigation treatment that created water stress in Merlot vines and compared the anthocyanin composition of treated vines to controls over two seasons. In both years the water-stressed vines had significantly higher levels of anthocyanins in the berry skins compared to the controls. *UFGT* expression was consistently higher in the water-stressed samples over the two years, whereas the expression levels of other structural genes tested was usually higher in the stressed plants just after véraison. The expression of these structural genes was not consistently higher in the deficit irrigated treatments throughout berry ripening. In the same study, the genes encoding the modifying enzymes *F3´H* and *F3´5´H* were examined for changes in expression in response to the irrigation treatment. It was found that *F3´5´H* was generally expressed to a greater degree in the water-stressed samples, and this was reflected

in an increase in the tri-hydroxylated anthocyanins in these berries (Castellarin et al. 2007b).

In the second season of the study, methylated anthocyanins accumulated to a greater degree in the berries from the water stressed plants and this correlated with an increase in expression of an *MT* gene putatively involved in anthocyanin modification (Castellarin et al. 2007b). The expression of five MYB-like transcription factors, including *VvMYBA1*, *B1*, and *5a*, was also examined in these samples. *VvMYBA1* expression was almost always higher in the water-stressed samples, although it was interesting to note that *VvMYB5a* was higher in the stressed samples just after véraison, and *VvMYBB1* was higher in samples immediately before harvest (Castellarin et al. 2007b). In another water stress experiment, Castellarin et al. (2007a) exposed Cabernet Sauvignon vines to either a pre- or post-veraison water deficit treatment. Both treatments significantly increased anthocyanins per gram fresh weight compared to the control, but on a per berry basis the difference was only significant in the early deficit treatment at three of seven time points tested after véraison. Some differences were observed in the expression of the anthocyanin genes, but there were no obvious trends (Castellarin et al. 2007a). It was observed that there was a greater ratio of trihydroxylated to dihydroxylated anthocyanins in the water–stressed plants through most of ripening, but this was not reflected in the relative expression of *F3´5´H* and *F3´H* genes throughout this period (Castellarin et al. 2007a).

There is often much difficulty in interpreting the results of irrigation studies. Changes in water status can modify the physiology of grapevine in many ways and often change berry size, crop load, vigour and canopy architecture, which in turn alters light interception. Furthermore, genetic differences between cultivars, involving their responses to water stress, will confound the uncertainty over which physiological affects can influence anthocyanin synthesis. It will be interesting to see if any trends become obvious in these types of experiments as more data are collected in the years ahead.

2.5.5. The effects of plant growth regulators on anthocyanin gene expression

2.5.5.1. ABA

ABA has been implicated in the regulation of ripening processes in grape berries (see Chapter by Davies and Böttcher, this edition). Kyoho grapes treated with ABA at the onset of berry softening showed a marked increase in anthocyanin accumulation compared to untreated controls (Ban et al. 2003). Analysis of anthocyanin gene expression seven days after the treatment showed an increase in the expression of all the anthocyanin structural genes except *F3H* and *LDOX* in the ABA-treated samples compared to the control. At 21 days after

ABA treatment, there was no obvious difference in expression of any of the anthocyanin structural genes (Ban et al. 2003).

Cabernet Sauvignon berries treated with ABA at véraison showed an increased accumulation of anthocyanins that was associated with an increase in the expression of all the anthocyanin structural genes tested, as well as *VvMYBA1* (Jeong et al. 2004). Peppi et al. (2008) reported a 6-fold increase in *UFGT* gene expression one week after application of ABA in Crimson Seedless berries. However, in two subsequent time points, three and nine weeks after ABA treatment, the expression of *UFGT* in the treated samples was only slightly higher than in the control samples (Peppi et al. 2008). It appears that ABA induces a transient increase in the expression of *VvMYBA1* and the anthocyanin structural genes, and this could be due to an earlier onset of the ripening process due to the ABA treatment.

2.5.5.2. Auxins

Auxins appear to have the opposite effect to ABA, in that they delay the onset of ripening in grape berries (see chapter by Davies and Böttcher, this edition). For example, the auxin-like compound BTOA was shown to retard ripening when sprayed onto Shiraz grape berries just prior to véraison (Davies et al. 1997). This treatment also delayed anthocyanin accumulation which was reflected in a delay in the upregulation of *CHS* and *UFGT* in the BTOA-treated berries (Davies et al. 1997). Ban et al. (2003) treated Kyoho grapes with the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) at véraison, which greatly reduced anthocyanin levels in the berries. The 2,4-D treatment significantly reduced expression of all the anthocyanin pathway genes up to 21 days after the treatment (Ban et al. 2003). Similar results were seen when NAA was sprayed onto Cabernet Sauvignon berries at véraison (Jeong et al. 2004). The upregulation of *CHS*, *CHI*, *F3H*, *DFR*, *LDOX*, and *UFGT* was delayed by NAA-treatment compared to the controls, as was the expression of *VvMYBA1* (Jeong et al. 2004). These studies showed that auxins suppress the anthocyanin pathway when applied at véraison and this is probably due to the inhibitory effect this class of plant growth regulators has on the ripening process in grape berries.

2.5.5.3. Ethylene

Historically, grapes have been considered as a non-climacteric fruits implying that ethylene is not important for triggering ripening (see chapter by Davies and Böttcher, this edition). However, there is recent evidence that ethylene may in fact have a role in grape ripening and may influence anthocyanin accumulation (El-Kereamy et al. 2003, Chervin et al. 2004, Tira-Umphon et al. 2007). For example, application of the ethylene releasing compound 2-CEPA to berries at véraison resulted in higher anthocyanin levels throughout ripening compared to untreated controls (El-Kereamy et al. 2003). *CHS*, *F3H* and *LDOX*

expression levels were found to be significantly higher in the 2-CEPA treated berries than in the controls at certain time points after véraison, but *UFGT* expression was not significantly increased by 2-CEPA. However, the authors did show that UFGT protein levels were higher in the treated berries than in the controls up to 10 days after véraison (El-Kereamy et al. 2003).

In a subsequent study, the application of ethylene itself increased *UFGT* gene expression in Cabernet Sauvignon berries and 2-CEPA was shown to have the same effect on Gamay cell suspensions (Tira-Umphon et al. 2007). However, this increase in *UFGT* expression does not seem to be caused by an increase in the expression of *VvMYBA1* in these systems (Tira-Umphon et al. 2007). It is possible that another regulatory gene, such as *VvMYBA2*, may mediate the ethylene induction of *UFGT* expression, although increased *UFGT* mRNA or VvMYBA1 protein stability may result in the observed increase in *UFGT* expression.

2.6. Manipulation of grapevine anthocyanins

2.6.1. Total anthocyanins

Total colour is a measure of grape quality, so any means of increasing the amount of anthocyanins in the berries would be an advantage to both the grape grower and the winemaker. Viticultural practices have been developed to try and increase the amount of colour in berries. Nevertheless, it may be possible to genetically manipulate grapevines to increase the total anthocyanins that accumulate in berries. The cumulative expression levels of *F3H* and *UFGT* during berry ripening have been shown to correlate strongly with total anthocyanin content in nine different cultivars (Castellarin and Di Gaspero 2007) suggesting that increasing flux through the whole pathway is essential to increase berry anthocyanin levels.

Interestingly, the same authors found that cumulative *VvMYBA1* expression did not show a strong correlation with anthocyanin accumulation (Castellarin and Di Gaspero 2007). Nevertheless, it could be speculated that the best way to achieve increased flux down the whole anthocyanin pathway would be by manipulating the expression of regulatory genes.

However, many attempts to increase the expression of an endogenous gene in plants have been hindered by the phenomenon of co-suppression, and so the grapevine regulatory genes would probably not be suitable tools for overexpression strategies. The use of regulatory genes from one plant species to increase anthocyanin accumulation in another has been reported (Lloyd et al. 1992, Mooney et al. 1995, Bradley et al. 1998) and perhaps would be the most promising approach. The berry flesh may also be a target for these genes, thus increasing overall anthocyanin levels by inducing the whole grape berry to pro-

duce anthocyanins. Grape berry flesh has the capacity to produce anthocyanins as red-fleshed varieties exist suggesting that this approach is feasible.

The branching nature of the anthocyanin/flavonoid biosynthesis pathway means that there is competition between enzymes for the same substrate (Holton and Cornish 1995). For example, DFR, F3´H, F3´5´H and flavonol synthase (FLS) can all use dihydroflavonols as substrates. It may be possible to increase the flux down the anthocyanin pathway by suppressing genes involved in the formation of other flavonoid compounds produced from anthocyanin precursors. Grape berry skins produce flavonols and proanthocyanidins both of which are generated from intermediates of anthocyanin biosynthesis. Holton et al. (1993) have shown that anthocyanin accumulation can be increased in tobacco flowers by cosuppressing the *FLS* gene.

Therefore, reducing the expression of *FLS* and/or the genes involved in proanthocyanidin production in grape berry skins may increase anthocyanin accumulation. However, the importance of tannins and other phenolics to the colour (as co-pigments) and mouthfeel of red wine may mean that increasing anthocyanins at the expense of these compounds will not enhance wine colour or quality. Perhaps strategies to improve colour should aim to increase the activity of all the enzymes of the pathway or the flux down the pathway by increasing the concentration of early intermediates. This will maintain a balance between anthocyanins and other flavonoids, both of which are reported to contribute to wine colour. This may ultimately involve the manipulation of the shikimate and aromatic amino acid synthesis pathways that lead to the production of phenylalanine, the substrate of PAL.

The transport of anthocyanins into the vacuole will also influence the amount of anthocyanins that accumulate in the berry skins. Anthocyanins are secondary metabolites that are believed to be toxic to the plant cells and are recognised and transported to the vacuole by a similar mechanism to other xenobiotics (Kitamura 2006). The sequestering of anthocyanins in the vacuole is an important process for the survival of those cells producing anthocyanins. The efficiency of this transport system will be important in determining the amount of anthocyanins that accumulate in these cells, as the toxicity of these compounds would suggest that a feedback inhibition would protect these cells if their levels increase in the cytoplasm. The isolation of grape genes encoding putative homologues of the transport machinery will enable a better understanding of how this might influence anthocyanin accumulation and may help devise strategies for enhancing anthocyanin sequestration.

2.6.2. Specific anthocyanins

The type of anthocyanins produced by a grape variety will influence the colour of the grape product. Grape berry skins produce at least 16 different spe-

cies of anthocyanins, all of which derive from either the dihydroxylated anthocyanidin cyanidin or the trihydroxylated anthocyanidin delphinidin. The various anthocyanin species have different colour spectra and so the relative amounts of each will contribute to the final colour of the fruit. Furthermore, in the case of wine production, the anthocyanin species have different properties with regard to extractability, stability and the reactions they undergo during winemaking and the subsequent storage of the wine (Mazza and Miniati 1993, Mazza 1995). If the colour of grape berries can be altered this may provide products that can be marketed as being novel, especially in the table grape industry.

The *F3 H* and *F3 5 H* genes are targets for altering anthocyanin composition, as they are involved in producing anthocyanin structures that provide pigment at the different regions of the red-blue colour spectrum (Holton and Cornish 1995). Gene silencing strategies could be used to alter the activity of these enzymes in the grape berry skins and thus force the anthocyanin pathway down one or other of the branches of the pathway (Fig. 1), altering the anthocyanin species produced. Similarly, MT activity appears to be important in determining the types of anthocyanins that accumulate in the skins of wine grapes. Often the major species found in wine grapes are malvidin-based, and to a lesser extent peonidin-based, both of which are methylated in the B-ring of the molecule (Fig. 1). The functional confirmation of putative grapevine anthocyanin *MT* genes will enable silencing or overexpression strategies to be developed in order to alter the percentage of methylated anthocyanins produced. Finally, acylation of anthocyanins could be modified by transgenesis. Recently, anthocyanin and flavonoid acyltransferases have been cloned from several plant species (Nakayama et al. 2003, D'Auria 2006, Luo et al. 2007) and this should allow the identification of grape homologues that may be targeted to alter anthocyanin acylation patterns.

Conventional breeding strategies could also be employed to alter anthocyanin composition. In most grapes that are known to produce wine with stable colour there are significant amounts of acylated anthocyanins (e.g. Shiraz and Cabernet Sauvignon). However, Pinot noir does not produce any acylated anthocyanins, but still accumulates high levels of anthocyanins in the vacuole (Merlin et al. 1985). Understanding the inheritance of this trait will provide a means to include the selection for this character in breeding programs.

It has also been shown that anthocyanin composition can be linked to the expression levels of *F3´5´H* and *MT*-like genes in cultivars with different levels of methylated or hydroxylated anthocyanins (Castellarin and Di Gaspero 2007). Linking such traits to molecular markers will again enable these phenotypes to be traced during conventional grapevine breeding. Such tools will become increasingly available to researchers in the future as the completion of the grape-

vine genome sequence means that linking molecular genetics to grape berry traits has become a more realistic goal.

3. CONCLUDING REMARKS

We now have a much better understanding of the accumulation of anthocyanins a process that is of prime importance to the grape industries. Our knowledge concerning both the function of genes involved in these processes and the manner in which they are controlled has improved considerably, as a result of use of molecular techniques. In particular, the cloning of key genes and their expression in both homologous and heterologous systems have proven to be powerful tools for understanding grape anthocyanin accumulation. The sequencing of the grapevine genome (Jaillon et al. 2007) and the development of grape microarrays will enable rapid progress on determining the effects of genotype, viticultural management and the environment on anthocyanin production in berries. We have seen, since the previous edition of this book, the flavonoid biosynthesis pathways and their control are much better understood and the interactions between the various pathways and processes are also becoming more evident. Our understanding of these interactions and how these processes are influenced by, and respond to, environmental influences is where much of the research effort will be focused. These aspects of berry development have increased importance given the changes in environmental conditions predicted as a result of climate change.

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11

THE PRODUCTION OF FLAVOUR & AROMA COMPOUNDS IN GRAPE BERRIES

J.D. Dunlevy[1], C.M. Kalua[2], R.A. Keyzers[2] & P.K. Boss[2]

[1]School of Biological Sciences, Flinders University, GPO Box 2100, Adelaide, South Australia 5001, AUSTRALIA

[2]Commonwealth Scientific and Industrial Research Organisation, Food Futures Flagship and Plant Industry, PO Box 350, Glen Osmond, South Australia 5064, AUSTRALIA

1. INTRODUCTION

1.1. Grape-derived compounds contribute to wine flavour and aroma

Wine is a complex mixture where the flavour and aroma compounds have multiple origins. The odour active components of wine can be traced to the direct contribution of compounds from grapes, oak and fermentation microbes. However, this complexity is multiplied by molecular transformations that can be catalysed by enzymes during the wine-making process or spontaneous chemical transitions that can occur in the wine matrix. Nevertheless, there is evidence that compounds derived from the grape berries are important contributors to the sensory attributes of wine. First, wines from different varieties have a fundamentally different array of flavour/aroma compounds which give them their typical character. This effect is determined solely by the difference in the genomes of the varieties. Second, seasonal and regional differences can be observed in the sensory properties of wines made from grapes of the same variety. These viticultural and environmental variables could alter grape composition by altering gene expression, but also may impact on compound stability.

Flavour and aroma are difficult properties to quantify and qualify in any food product. In grape berries, there are hundreds of compounds that could potentially contribute to the flavour and aroma of wine. Some grape components have been linked to wine volatiles, because they undergo no or minimal alteration during the fermentation process, good examples being monoterpenes and methoxypyrazines. However, wine flavour compounds may be produced by the

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action of yeast on grape berry precursors. Furthermore, flavour development during fermentation may be influenced by compounds in the grape berry, that do not contribute directly to aroma and flavour, but which may change yeast metabolism and associated yeast-derived or yeast-altered aroma compounds. This review focuses on those grape components which undergo minimal changes during the fermentation process and can be defined as grape-derived wine flavour and aroma compounds.

1.2. Aroma compounds exist in berries as both free and glycosidically-bound volatile compounds

Volatile compounds are low molecular weight compounds (usually < 300 Da) which vaporise readily at room temperature. These compounds give an odour sensation when they reach the olfactory epithelium, dissolve into the mucus and bind with olfactory receptors (Angerosa 2002). The aroma of grapes is attributed to aldehydes, alcohols, esters, hydrocarbons, ketones, furans and, probably, other yet unidentified volatile compounds. Free volatile compounds in grapes are minor constituents that are predominantly formed through the action of endogenous enzymes, when the grape is crushed. In addition to free flavour and aroma compounds that are formed and released when cell walls are damaged, grapes contain glycosidically-bound compounds that do not readily contribute to flavour and aroma. Glycosidically-bound volatile compounds constitute a latent pool of aglycones that can be a source of wine flavour and aroma. These flavourless and non-volatile glycosidically-bound volatile compounds consist of simple single glucosides and disaccharide glycosides, with sugars α-*L*-arabinofuranose, α-*L*-rhamnopyranose, and β-*D*-apiofuranose linked to the glucose (Francis et al. 2005). In grapes, the disaccharide glycosides are dominant. The disaccharides are linked to different aglycones of monoterpenes, norisoprenoids, volatile phenols and other benzene derivatives (Francis et al. 2005, Swiegers et al. 2005).

These glycosidically-bound volatile compounds are studied by analysing the aglycones released from the non-volatile precursors through either enzymatic or acid hydrolysis. Enzyme hydrolysis is an efficient method for releasing bound volatile compounds, unlike acid hydrolysis, which may result in unwanted artefacts from rearrangements of the aglycones, complicating the identification of the volatiles (Swiegers et al. 2005). Enzyme hydrolysis has therefore found wide application in studies of glycosidically-bound volatiles since many of the compounds released can be directly linked back to the aglycones (Williams et al. 1989). It should be noted that although bound volatiles have been released from grapes and wines in controlled studies, their release during vinification is dependent on grape variety and in some cases has been reported to be insignifi-

cant in terms of flavour perception (Rocha et al. 2005, Esti et al. 2006). Nevertheless, it has been shown that some of these glycosidically-bound compounds can be released during wine maturation and storage (e.g. Simpson 1978), and so their impact can not be discounted.

The presence of both free and glycosidically-bound forms of compounds and families of compounds in grapes makes the chemical analysis of grape-derived flavour compounds difficult. Established methods utilise deuterated standards, solid-phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS) (Siebert et al. 2005) to accurately determine levels of volatile compounds. When measuring the metabolites we also need to remember that there may be glycosylated pools of the compounds, as well as pools of the free form and different techniques are required to measure both. A simpler way of studying the production of these compounds in grapes may be to target the biosynthetic pathways that produce the aglycones.

1.3. Biosynthesis of flavour and aroma compounds

The simplest way to predict the likely activity level of a biosynthetic pathway is to study the expression of the genes that encode the enzymes responsible for the metabolic transformations, if they are known. Although this does not take into account the effects of translation or enzyme activity on the flux through the pathway, it is often found that secondary metabolism is linked to the transcription of biosynthesis genes. If we determine when the genes encoding these pathway enzymes are expressed, we can be confident that the berry is actively producing the metabolites at that time. We can also infer that a higher level of gene expression relates to a greater flux down the biosynthetic pathway. Thus, we will have some indication of the timing and level of synthesis of the metabolite that is occurring in the berry.

The origin of grape-derived flavour compounds and their precursors are from multiple biosynthesis pathways, as are outlined in the following sections of this chapter. The final mixture of secondary metabolites in ripe grapes depends on multiple variables including the grape variety used, the environmental conditions during the growing season, management of the vineyard and harvest date. In many cases, these variables will alter the expression of genes in the biosynthesis pathway thus altering flux. However, the initial challenge is to identify and functionally characterize grape genes encoding for the enzymes responsible for the chemical transformations, important for the biosynthesis of flavour and aroma compounds. In the following sections of this chapter we review the current knowledge of the biosynthesis of the major groups of flavour compounds found in grapes (terpenoids, norisoprenopids, aromatics and aliphatics) and also discuss two groups of compounds that have a major impact in certain

varieties, organo-sulfur compounds and methoxypyrazines.

2. TERPENOIDS

2.1. Introduction

Terpenoids are produced by organisms in all kingdoms of life (Davis et al. 2000) and make up the largest class of plant secondary metabolites (Dudareva et al. 2006). They are derived from the universal C_5 precursor, isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) and are classified into groups, based on the number of carbons in the molecule. Volatile hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), homoterpenes (C_{11} and C_{16}), and diterpenes (C_{20}) exist in plants (Dudareva et al. 2006). With regards to fruit aromatic composition, the monoterpenes and the sesquiterpenes appear to be the most important terpenes. Their diversity is immense with there being approximately 1000 monoterpene and more than 7000 sesquiterpene structures possible (Bohlmann et al. 1998). Given this diversity, it is not surprising that the aroma perception and aroma thresholds of these compounds also vary widely. Furthermore, the presence of enantiomers can alter sensory properties. For example, the monoterpene carvone exists as two optical isomers (-)-carvone and (+)-carvone, the (-)-carvone isomer has a spearmint odour, whereas the (+)- enantiomer is described as caraway-like (Leitereg et al. 1971).

The terpenoids have been recruited to a number of ecological roles in plants. They are often associated with floral scent which presumably is designed to attract appropriate pollinators and ensure reproductive success. Furthermore, terpenoids contribute to the volatile emissions from fruit, which may enable seed dispersers to both locate the fruit and assess its ripeness. However, the ecological role of specific compounds in pollination and seed dispersal in natural conditions has rarely been obtained (Gershenzon et al. 2007). They also play a role in plant defence (see review by Arimura et al. 2005). Monoterpenes and sesquiterpenes have been shown to have antimicrobial activities and may deter herbivores both above the ground and in the root zone.

One of the more remarkable discoveries concerning the function of volatiles in recent years has been the demonstration that plants can signal to attract carnivores, which prey on herbivores that are attacking them, a so-called tritrophic interaction. Also, plants that are being attacked by herbivores may also signal to other plants around them *via* the emission of volatiles. This enables the surrounding plants to upregulate defense pathways readying them against potential attack. These processes involve specific signalling molecules some of which are terpenoids. Presumably the chemical diversity present in the terpenoid fam-

ily of compounds has allowed plants, through evolution, to adapt the production of these compounds thus improving the fitness of their species.

2.2. Odour description and impact

Approximately 70 monoterpenes have been identified in grapes and wine (Rapp 1998), but the free monoterpene alcohols most often found in grapes and must are citronellol, 3,6-dimethyl-1,5-octadien-1,7-diol, linalool, geraniol, nerol and α-terpineol (Fig. 1; Rapp 1998, Mateo and Jiminez 2000). Other predominant monoterpene compounds in grapes and wine include monoterpene ethers (e.g. rose oxide, nerol oxide) and polyhydroxylated monoterpenes, which are generated from oxygenation products of the monoterpene alcohols (Williams et al. 1980, Williams et al. 1985, Luan et al. 2004). Odour thresholds of the volatile monoterpenes are varied; for example, the monoterpene ether *cis*-rose oxide has an odour threshold of 0.2 µg/L in 10% ethanol, whereas citronellol has an odour threshold of 100 µg/L in the same solvent (Guth 1997). In addition to these 'free' forms of monoterpenes, several glycosidically bound forms of monoterpenes have been identified in grapes (Williams et al. 1982, Voirin et al. 1990) and these bound forms are usually in greater abundance than the free monoterpenes (Dimitriadis et al. 1984, Gunata et al. 1985).

Grape varieties have been classified based on the level of monoterpenes produced in the berries (Strauss et al. 1986b). Although this separation was based on limited data, it divided cultivars into muscat varieties that have high levels (up to 6 mg/L) of free monoterpenes, non-muscat aromatic varieties with

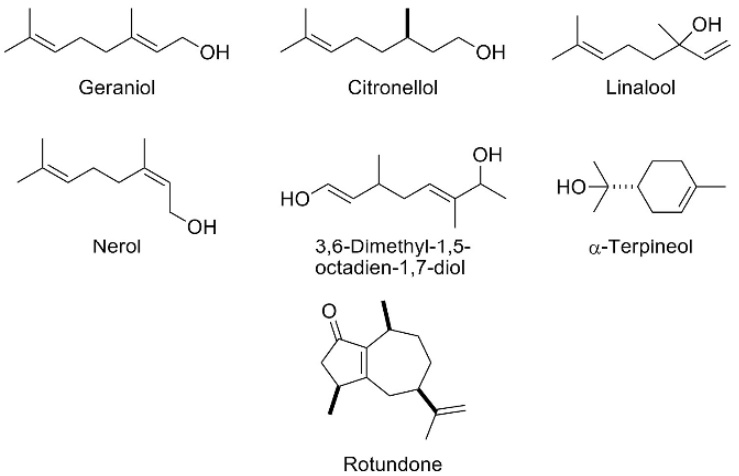

Fig. 1. Common grape monoterpene alcohols and the sesquiterpene rotundone.

between 1-4 mg/L free monoterpenes and, neutral varieties where monoterpenes do not appear to influence flavour (Strauss et al. 1986b). This separation, based solely on the overall levels of monoterpenes, appears to suggest the direct correlation of total monoterpenes with the level of 'muscat' character in the grapes and wine, which would suggest that monoterpenes are only associated with this flavour characteristic. However, a more qualitative comparison of the monoterpene profiles of grapes enabled a separation of 'Muscat-type', 'Riesling-type' and neutral varieties (reviewed in Rapp 1998). In general, it appears that high levels of grape monoterpenes tend to give rise to 'Muscat' character, whereas more moderate levels are associated with 'floral' or 'aromatic' varieties.

Sesquiterpenes have been studied less than monoterpenes, with regard to their role in grape-derived wine flavour and have rarely been reported as volatile components of grapes and wine. Nevertheless, several sesquiterpenes have been detected in the berries of some German wine varieties (Schreier et al. 1976), as well as the cultivars Baga (Coelho et al. 2006) and Shiraz (Parker et al. 2007). The first sesquiterpene shown to contribute to the flavour and aroma of a table wine was that of rotundone, which is responsible for the 'pepper' character of Shiraz wines (Wood et al. 2008). Rotundone is extremely potent and has an aroma threshold of 16 ng/L in red wine for tasters that are sensitive to the compound (Wood et al. 2008). Given the large number of sesquiterpenes identified in the varieties Baga and Shiraz and their potentially low odour thresholds, it would be surprising if they did not contribute to other wine flavour and aroma characteristics. This has been shown to be true for other alcoholic beverages, as the spicy character that hops impart on beer has been shown to be caused by trace levels of sesquiterpenes (Goiris et al. 2002). To date, there are no reports of sesquiterpenes being present in a bound glycosylated form as is common for the monoterpenes.

In general, it has been found that the levels of monoterpenes and sesquitepenes increases during berry ripening. Ebang-Oke et al. (2003) reported that free linalool begins to accumulate in grape berries 2 weeks after véraison in Muscat de Frontignan. Linalool levels then peaked 2 weeks later before decreasing until harvest (Ebang-Oke et al. 2003). A similar pattern had been previously reported for developmental changes in free and bound linalool in other Muscat varieties (reviewed by Strauss et al. 1986b). It was shown that monoterpenes are present in berries at fruit set, but the levels then decline until véraison, when accumulation of free and bound forms is reinitiated (Wilson et al. 1984). The levels of monoterpenes peaked in this study at about 17 weeks after flowering, but then declined, suggesting that extending the ripening period may result in loss of flavour compounds (Wilson et al. 1984). Monoterpenes have also been localised in berry tissues (reviewed by Strauss et al. 1986b). Interestingly, there are differences in the distribution of monoterpene alchols in berries. Nerol and ge-

raniol have been shown to be concentrated in the skin, whereas linalool appears to be more evenly distributed throughout the berry.

The production of monoterpenes and sesquiterpenes depends on the synthesis of C_{10} and C_{15} precursors. These are derived from the C_5 isoprene precursors IPP and DMAPP, which are produced *via* two independent pathways in plants. Extensive modification, including cyclization, oxygenation, reduction and isomerisation may also occur once a terpenoid alcohol has been produced. These biosynthetic steps are discussed in detail below.

2.3. Biosynthesis or monoterpenes and sesquiterpenes

2.3.1. The mevalonate (MVA) pathway

The MVA pathway is localised in the cytosol and produces IPP from three molecules of acetyl-CoA. Two acetyl-CoA molecules are first converted to acetoacetyl-CoA by the activity of acetoacetyl-CoA thiolase (AACT). Another acetyl-CoA is then coupled to acetoacetyl-CoA by 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) to yield hydroxy-3-methylglutaryl-CoA (HMG). HMG-CoA reductase (HMGR) then reduces HMG to produced mevalonate (MVA), which is subsequently phosphorylated twice by mevalonate kinase (MVK) and phosphomevalonate kinase (PMK) yielding mevalonate pyrophosphate (MVPP). Finally, MVPP is converted into IPP by mevalonate pyrophosphate decarboxylase (MVD). The IPP can be isomerised to the other important C_5 building block DMAPP by IPP isomerase (IPI).

Genes from the mevalonate pathway have been isolated from a number of plant species, but functional analysis is lacking for all but *HMGR* (Suzuki et al. 2004). In *Arabidopsis*, AACT, HMGR, MVD and IPI are encoded by more than one gene, suggesting that the pathway is controlled by multiple cues. For example, *hmg1 Arabidopsis* mutants, which are mutated in one of the two *HMGR* genes, have a severely dwarfed phenotype that could be rescued by the addition of squalene (Suzuki et al. 2004). Mutants in the other *Arabidopsis HMGR* gene, *hmg2*, did not display a visible phenotype (Suzuki et al. 2004). Expression of this gene is known to be localised to meristems and floral tissues (Enjuto et al. 1995), implicating that this gene may be involved in the production of substrate for floral sesquiterpenes in *Arabidopsis* (Tholl et al. 2005). The reaction catalysed by HMGR is generally regarded as the rate limiting step for isoprene unit production *via* the MVA pathway (Schaller et al. 1995, Suzuki et al. 2004).

The grapevine homologues for the MVA pathway genes have been identified in grape (Boss et al. unpublished), but determination of copy number awaits final annotation of the genome. Studies using cell cultures and jasmonates have shown that gene members of the MVA pathway can be upregulated in response to external stimuli (Boss et al. unpublished). The MVA

pathway is linked more to the production of sesquiterpenes than monoterpenes (see below), which in the past would have reduced the interest in this pathway with regard to grape and wine flavour and aroma. However, the association of the sesquiterpene rotundone with pepper flavour in Shiraz (Wood et al. 2008) means that products of the MVA pathway can influence the volatile profile of grapes and wine. An understanding of the control of the MVA pathway will be important in predicting genetic and environmental effects on the production of the grape metabolites that originate from the cytosolic pool of IPP.

2.3.2. The methyl-erythritol-phosphate (MEP) pathway

The recently discovered MEP pathway is an alternative route for IPP synthesis and it is found in most eubacteria, plants and the malaria parasite *Plasmodium falciparum* (for review, see Rodriguez-Concepcion et al. 2002). In plants, the enzymes of the MEP pathway are compartmentalized in plastids. The first reaction of the pathway involves the condensation of pyruvate and glyceraldehyde-3-phosphate to produce deoxyxylulose 5-phosphate (DXP). This is catalyzed by deoxyxylulose 5-phosphate synthase (DXS). DXP reductoisomerase (DXR) then catalyzes the formation of 2-C-methyl-*D*-erythritol-4-phosphate (MEP) before cytidine 5′-triphosphate is used as a substrate by 4-diphosphocytidyl-2-C-methyl-*D*-erythritol synthase (CMS) to produce 4-diphosphocytidyl-2-C-methyl-*D*-erythritol (CDP-ME). CDP-ME is in turn phosphorylated by CDP-ME kinase (CMK), before the CDP-ME-2-phosphate product is converted to ME-2,4-cyclodiphosphate (ME-cPP) by ME-cPP synthase (MCS). The cyclic ME-cPP undergoes a reductive ring opening by the action of hydroxymethylbutenyl-4-diphosphate (HMBPP) synthase (HDS) and HMBPP is then converted into a mixture of IPP and DMAPP by IPP/DMAPP synthase (IDS). This last step is interesting in that both IPP and DMAPP are produced by the same enzyme, whereas DMAPP is produced sequentially after IPP in the MVA pathway.

The *Arabidopsis* genes encoding for the enzymes of the MEP pathway have been identified from the genome sequence, and all but DXS are encoded by single genes. There are three *DXS* genes, but mutation of just one (*DXS* or *CLA1*) causes an albino phenotype, suggesting that the other two genes (*DXS2* and *DXS3*) are not functional or are not expressed widely enough to act redundantly in the MEP pathway (Rodriguez-Concepcion et al. 2002). Functional analysis has also been conducted for the genes encoding DXR, CMS and HDS in *Arabidopsis* (Schwender et al. 1999, Rohdich et al. 2000a, Querol et al. 2002) and CMK in tomato (Rohdich et al. 2000b). Work to date suggests that *DXS* has a general regulatory role in controlling flux through the MEP pathway (Lois et al. 2000, Estevez et al. 2001), although there is evidence that *DXR* and *IDS* may also contribute to the metabolic flux in some cases (Carretero-Paulet et al. 2002,

Botella-Pavia et al. 2004). Grapevine homologues to the MEP pathway genes are also identifiable in the grape genome sequence and some are also responsive to external stimuli in cell culture conditions (Boss et al. unpublished). The flux through this pathway will be an important determinant of the levels of not only monoterpenes but also norisoprenoids (see below).

2.3.3. Pathway compartmentalization

The production of monoterpenes and the sesquiterpenes is compartmentalised in plant cells due to both the localisation of the substrate and the enzymes responsible for terpenoid production. The C_{15} sesquiterpene substrate is farnesyl pyrophosphate (FPP) and it is produced in the cytosol by the consectutive condensation of IPP to DMAPP and then to the geranyl pyrophosphate (GPP) product by FPP synthase. FPP is essential to the production of many primary as well as secondary metabolites and so regulation of FPP synthases may not be important in determining flux to sesquiterpene production. In the plastid, geranyl pyrophosphate synthase (GPPS) condenses one molecule of IPP and one of DMAPP in a head-to-tail orientation to produce the C_{10} compound substrate (GPP) that is used for monoterpene production. GPPS represents a branch point in terpenoid production and there is evidence that its activity is important for determining levels of monoterpenes produced in plants (Tholl et al. 2004).

As outlined above, the MEP and MVA pathways are located in different subcellular compartments and it appears that they are independently regulated. However, there is evidence that there is some flow of substrates from one compartment to the other *via* metabolite transporters (Bick et al. 2003). Experiments in snapdragon and *Arabidopsis* suggest that this flow is unidirectional from the plastid to the cytosol (Laule et al. 2003, Dudareva et al. 2005). Precursor feeding studies with grape leaves and berries have shown that the products of the MVA pathway do not contribute to the pool of monoterpenes produced in these tissues (Luan et al. 2002, Hampel et al. 2005). However, sesquiterpenes have been shown to arise from both the MVA and MEP pathways in grape leaves, suggesting this unidirectional transfer of isoprenoid precursors is active in grape tissues (Hampel et al. 2005).

2.3.4. Terpenoid synthases and modifying enzymes

The final array of terpenes produced in plant tissues depends first on the presence of enzymes that catalyze the formation of terpenes, called terpenoid synthases (TSs). Generally, TSs produce cyclic products and are thus known as terpenoid cyclases, but enzymes that produce acyclic products are also known. It is also common for TSs to produce multiple products. For example, the range of over 20 sesquiterpenes produced in *Arabidopsis* flowers is due to the pres-

ence of two sesquiterpene cyclases (Tholl et al. 2005). TSs contain N-terminal transit peptides for the targeting of the protein to plastids, and these are absent from sesquiterpene synthases.

An important determinant for the production of terpenes in plants is the temporal and spatial control of *TS* gene expression. Often expression is spatially regulated in specialized organs or tissues, such as glandular trichomes, flowers or fruits, where the terpene products play a functional role in aspects of plant defense or attracting pollinators and seed dispersers. *TS* expression is also temporally regulated. Emission of terpenes from some tissues is induced upon herbivore damage, and is caused by the induction of *TS*s (e.g. Arimura et al. 2004). *TS*s in some species have day-night fluctuations due to control by the circadian clock (e.g. Dudareva et al. 2003). On a longer time scale, *TS*s may be developmentally controlled to coincide with reproductive competency or fruit ripeness, thus regulating timing of the interaction of the plant with its environment to improve reproductive success (e.g. Dudareva et al. 2003, Sharon-Asa et al. 2003).

Three *TS* genes have been characterized in grapevine to date. The first, which encoded an (-)-α-terpineol synthase, was isolated from Gewürztraminer and has been called *VvTer* (Martin et al. 2004). The authors reported the characterization of two cDNAs encoding enzymes with (-)-α-terpineol synthase activity, but with only two amino acid differences between them, it is likely they represent alleles of the same gene. When this protein was expressed in an *E. coli* system it was found to produce 14 monoterpenes. The major product was (-)-α-terpineol and accounted for 50 % of the total monoterpenes produced. The next two major peaks were 1,8-cineol (11.8 %) and (+)-β-pinene (8.5 %), with 11 other compounds making up the final 29.7 % of the total monoterpenes produced. It is interesting that 1,8-cineol (eucalyptol) has been identified as a product of a grape enzyme given that there is speculation that its presence in Australian wine is due to the proximity of Eucalyptus trees to vineyards.

In a subsequent study it was found that mRNA from this gene could not be detected in Gewürztraminer berries during development (Lucker et al. 2004). Two sesquiterpene synthase genes have also been cloned from Gewürztraminer (Lucker et al. 2004). One encoded a (+)-valencene synthase (*VvVal*) which produced two major products, valencene (49.5 % of total product) and (-)-7-*epi*-α-selinene (35.5 %), along with five minor products. The enzyme produced by the other cDNA was found to produce (-)-germacrene D (92 %) and a product thought to be δ-cadinene (8 %), and so the gene has been called *VvGerD*. *VvVal* was found to be expressed in berries in the later stages of ripening, whereas *VvGerD* was only detected in fruit at fruit set (Lucker et al. 2004). The expression of a linalool synthase gene (*Lis*) was found to be too low for detection by Northern analysis in grape berry RNA, but could be detected by RT-PCR (Ebang-Oke et al. 2003). The authors report that *Lis* is expressed in a pat-

tern that matches the accumulation of free linalool during berry development.

There is evidence that subsequent modification of the monoterpene alcohols is caused by enzymatic activity (Luan et al. 2004, Luan et al. 2005, Luan et al. 2006). The oxygenation and cyclization of these products produces monoterpene polyols, ethers and lactones, all of which have been found in free and bound form in grapes. Luan et al. (2004) showed that diols formed from linalool were enantiomerically pure, suggesting they resulted from enzymatic oxidation of linalool, and are probably not due to photooxygenation. Interestingly, the same authors found that the bound forms of diols derived from nerol and geraniol were present as almost racemic mixtures, suggesting that they may be formed by non-enzymatic means (Luan et al. 2004). In subsequent experiments, Luan et al. (2005) used *in vivo* feedling experiments with deuterium labelled geraniol to investigate its metabolism in grape berries. Labelled citronellol and nerol were found in these berries, suggesting there is enzymatic reduction and isomerisation occurring in the berries. They also found evidence of the oxidation and glycosylation of the monoterpene alcohols (Luan et al. 2005, 2006). After monoterpene alcohols are formed by TSs, secondary transformations of these alcohols therefore occur, that are catalyzed by enzymes and the activity of these will determine the final monoterpene composition of berries and the resulting wine.

3. NORISOPRENOIDS

3.1. Introduction

The norisoprenoids, also known as the apocarotenoids, are a family of compounds derived from the degradation of carotenoids (C_{40}). Norisoprenoids are common plant volatiles often found in flowers, where they are emitted as attractants to insect pollinators and in fruits, where they contribute desirable flavours and aromas. The norisoprenoids are variable in length and aroma compounds consisting of 18, 15, 13, 11, 10, 9 and 8 carbons have been reported as being derived from carotenoids (Wu et al. 1999, Lewinsohn et al. 2005). The oxidative degradation mechanism that leads to the production of these compounds has not been elucidated for each particular compound and so, it is possible that enzymatic and non-enzymatic mechanisms could be responsible in each case (Lewinsohn et al. 2005). In grapes, most research has focused on the C_{13}-norisoprenoids, as there seems to be a preponderance of norisoprenoids with this number of carbon atoms (Williams et al. 1992). However, there are few free norisoprenoids found in grape volatile fractions. Most accumulate as bound glycosylated conjugates, which renders them non-volatile and odourless until they are released by enzymatic or acid hydrolysis (Williams et al. 1992).

3.2. Odour description and impact

Many C_{13}-norisoprenoids have been identified in grape berries (Williams et al. 1992). However, only a few have been shown to be of importance to wine flavour, and these include β-damascenone, β-ionone, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) and (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB; Fig. 2). β-Damascenone is a key odorant in many fruits including grapes and is also an important contributor to the aroma of wine, beer and coffee. Described as having a 'fruity-flowery', 'honey-like' and 'stewed apple' aroma and having an extremely low odour detection threshold of 2 ng/L (Buttery et al. 1988), β-damascenone is not only important in natural products but is also commonly used in the perfume and flavouring industries. β-Ionone has an aroma often described as 'sweet' or 'violet' and is another important contributor to the flavor of many fruits. β-Ionone also has a low odour detection threshold of 7 ng/L (Buttery et al. 1990).

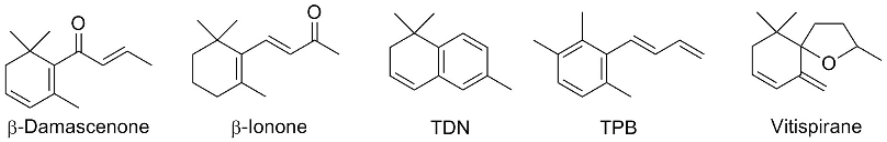

Fig. 2. Grape norisoprenoids that are important to wine flavour.

In grapes both of these compounds are commonly found at levels above their respective detection thresholds, and are therefore thought to be important contributors to the floral boutique of many wine varieties (e.g. Gomez-Miguez et al. 2007, Loscos et al. 2007). It has recently been suggested that even when β-damascenone is present below its odour detection threshold in wines, it may still enhance 'red fruit' aromas by altering detection thresholds of other compounds (Pineau et al. 2007).

While β-damascenone and β-ionone have a positive influence on wine flavour, the norisoprenoid TDN is generally considered to have detrimental effects as it has a 'kerosene-like' aroma. TDN has an odour detection threshold of 20 ng/L (Simpson 1978). In grapes and young Riesling wines TDN is present at concentrations well below its detection threshold and consequently has no sensory impact. However, as the wine goes through bottle aging, TDN levels can increase dramatically to levels above the detection threshold and impart negative attributes to the wine (Simpson 1978). TPB has been described as having a 'floral, geranium' character at low concentrations, but above 270 ng/L the descriptors suggest it is less desirable (e.g. 'insecticide', 'plastic'). It has been

found in white wines at levels above its odour detection threshold (40 ng/L in a neutral white wine matrix) but surprisingly is absent from red wines (Janusz et al. 2003).

3.3. Biosynthesis of norisoprenoids

3.3.1. Carotenoid biosynthesis

The biosynthesis of the norisoprenoids begins with the production of carotenoids, which are a class of ubiquitous pigmented compounds that have crucial functions in photosynthesis, membrane stability, nutrition, photo-protection and act as antioxidants, as well as functioning as precursors to various aroma compounds (Demmig Adams et al. 1996, Lewinsohn et al. 2005). Carotenoid synthesis begins the same as other isoprenoids, by the consecutive condensation of the C_5 isoprene units IPP and its isomer DMAPP. Carotenoids are synthesized in, and localized to, the plastids. Therefore the C_5 units involved in their synthesis are produced *via* the MEP pathway rather than the MVA pathway found in the cytosol (see section 2.3.). These subunits undergo consecutive enzymatic condensations to form the basic carotenoid phytoene that then undergoes a number of desaturation steps resulting in the addition of four double bonds to form lycopene. The conversion of the non-cyclic lycopene to cyclic carotene is a branch point in the pathway.

The action of different cyclase enzymes leads to the production of either α-carotene or β-carotene, which are essentially identical except for the positioning of a single double bond in one of the cyclic rings. Successive reactions catalyzed by hydroxylase enzymes convert α-carotene and β-carotene into lutein and zeaxanthin, respectively. Lutein is the end product of its respective branch pathway, whereas zeaxanthin can undergo an epoxidation step to form violaxanthin which is in turn converted to neoxanthin. The molecular genetics of the carotenoid biosynthesis pathway has been studied extensively in plants (see reviews by Cunningham 2002, Howitt et al. 2006). The genes coding for the enzymes that catalyze the reactions during carotenoid biosynthesis have been cloned from several plant species and found to be highly conserved amongst higher plants, but little work has been done on the genes of this pathway and its regulation in grapevines.

The carotenoid profile of grape berries is dependent on the cultivar, as well as environmental factors such as light and water status. However, in general, β-carotene and lutein comprise the majority of carotenoids present in the fruit, while neoxanthin, violaxanthin, zeaxanthin are present in lesser amounts (Bureau et al. 2000, Oliveira et al. 2004).

In ripening grape berries, carotenoid levels decrease after véraison and the levels of the C_{13} norisoprenoids increase (Razungles et al. 1993). This is

seen as evidence that the carotenoids are precursors of C_{13} norisoprenoids. Other evidence includes the maintainence of asymmetric centres found in carotenoids in the assumed C_{13} norisoprenoid products and the transfer of ^{13}C markers from carotenoids to norisoprenoids (Baumes et al. 2002). Although the generation of norisoprenoids from the breakdown of carotenoids can proceed *via* chemical, photochemical and oxidase-coupled degradation, the fact that the majority of norisoprenoids in grapes have 13 carbons suggests that it is regulated by enzymatic cleavage.

3.3.2. Carotenoid cleavage dioxygenases

Enzymes known as carotenoid cleavage dioxygenases (CCD) have been shown in various plant species to cleave carotenoids at certain double bond positions (for reviews see Bouvier et al. 2003, Kloer et al. 2006). One class of genes in this family (orthologues of the *Arabidopsis* gene *AtCCD1*) encode CCDs that have been shown to be able to cleave carotenoid substrates at the 9-10 and 9´-10´ double bonds to release volatile C_{13}-norisoprenoids (e.g. Schwartz et al. 2001, Simkin et al. 2004a, Simkin et al. 2004b). A list of common carotenoids and their theoretical C_{13}-norisopernoid products generated from CCD1 activity is summarized in Fig. 3. A *CCD1* homologue from grapevine (*VvCCD1*) has been cloned and characterised (Mathieu et al. 2005). Expression of *VvCCD1* in a bacterial strain engineered to accumulate zeaxanthin showed that the VvCCD1 enzyme has the ability to cleave zeaxanthin and release the C_{13}-norisopernoid 3-hydroxy-β-ionone. The authors also report that this enzyme has activity against lutein, but not β-carotene (Mathieu et al. 2005). This agrees with recent findings that CCD1 enzymes from some plant species have been shown to have high activity against a broad range of substrates, whereas others demonstrate a preference for certain carotenoids (Vogel et al. 2008). *VvCCD1* expression increases just prior to véraison and this expression level is maintained through to harvest (Mathieu et al. 2005). During this time, free and glycosylated C_{13}-norisoprenoids accumulate in the berries, further support that this gene plays a role in the production of these flavour compounds (Mathieu et al. 2005).

β-Ionone is a direct product from the 9-10 (9 -10) cleavage of both β-carotene and α-carotene (Fig. 3), whereas the formation of β-damascenone, TDN, TPB and vitispirane involves further chemical modification of C_{13}-norisoprenoids. Cleavage of neoxanthin at the 9,10 position results in the formation of a compound known as the grasshopper ketone, so called as it is regurgitated by some species of grasshopper as an unpleasant deterrent to predators (Meinwald et al. 1968). It has been shown that this compound can undergo a number of chemical modifications resulting in the formation of β-damascenone and other norisoprenoids (Puglisi et al. 2001, Puglisi et al. 2005).

Furthermore, the position of the glycosyl group in glycosylated precur-

sors may influence the ratio of various C_{13}-norisoprenoids produced by this acid-catalyzed hydrolysis (Skouroumounis et al. 2000). Studies have shown that a number of different C_{13}-norisoprenoid compounds can lead to the formation of TDN and vitispirane (e.g Strauss et al. 1986a, Winterhalter et al. 1994, Versini et al. 1996). The complexity introduced by the spontaneous chemical reactions that may occur to alter glycosylated norisoprenoids will make it difficult to discern the pathways that lead to the production of these important grape-derived flavour compounds. However, there is much to be gained from an understanding of the regulation of carotenoid synthesis and the enzymes that catalyze the targeted cleavage of the carotenoids. Furthermore, glycosyl transferases will also play a role in the accumulation of certain non-volatile precursors that may influence the nature of acid-catalysed chemical modifications.

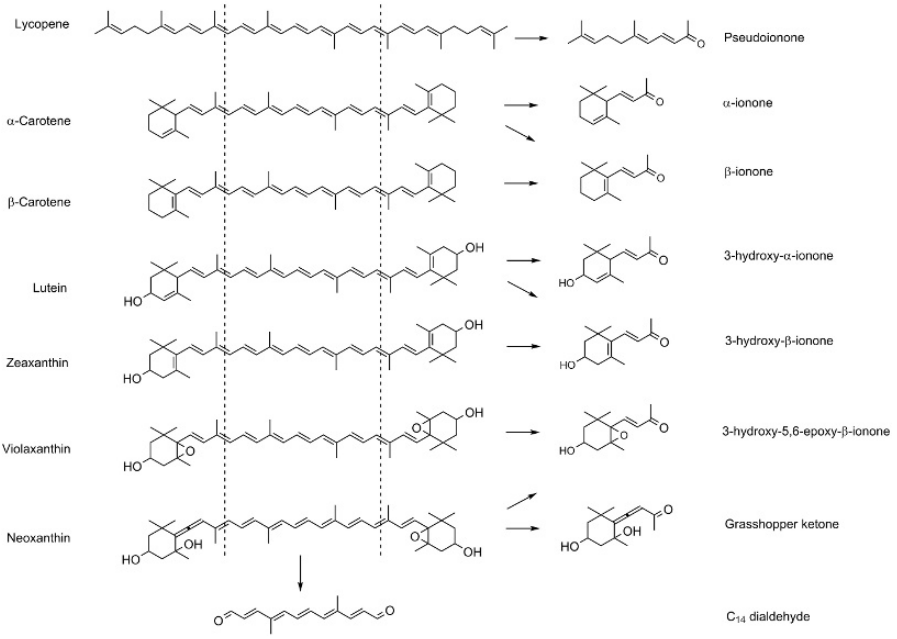

Fig. 3. C_{13}-norisopernoids released from the 9,10 (9′,10′) cleavage of carotenoids.

4. AROMATIC COMPOUNDS

4.1. Introduction

The term ‘aromatic’ in the context of flavour and aroma compounds can

be somewhat misleading and will be used exclusively in this section in its chemical sense *viz*. compounds that contain a planar, cyclic delocalized π-electron system, and not to imply compounds that have some detectable odour. Grouping the aromatic grape derived compounds together is also somewhat artificial, as the aromatics can be derived from a variety of biogenetic sources. For example, there are many aromatics generated by the terpenoid, polyketide and shikimate pathways in plant biochemistry (Pichersky et al. 2006). Aromatic terpenoids will not be mentioned in this section as they are discussed in the preceding sections.

The focus will rather be on aromatic grape flavour and odour compounds derived from other biosynthesis pathways, and in particular, those generated from shikimic acid. Volatiles in this family of molecules are important constituents of petunia, snapdragon and rose floral scent (Kolosova et al. 2001, Boatright et al. 2004, Guterman et al. 2006). Some may also work as plant defense molecules. An excellent example of this is eugenol, which has been shown to have excellent antibacterial and antifungal activities (Karapinar et al. 1987). It must be stressed that the genes involved in the latter stages of the biosynthesis of the aromatic odour compounds are not well characterized and therefore, the pathways that lead to their production are still unconfirmed (Dudareva et al. 2006). The difficulty in studying the biosynthesis of these molecules is compounded by the fact that they can be formed from a number of related substrates *via* different routes of biogenesis.

4.2. Odour description and impact

As with all other compounds that constitute the flavour and odour components of grape juice and any resulting wine, a number of factors will contribute to the overall organoleptic impact that each one will have. The odour thresholds of the aromatic compounds found in grapes tend to be higher than that of other classes of flavour and aroma compounds, for example the volatile organo-sulfur compounds or methoxypyrazines discussed elsewhere in this chapter, that are much more odour active. The detection limits of the aromatic compounds can vary from the low μg/L level up to over 10 mg/L in hydroalcoholic mixtures. A list of some representative and commonly found aromatic flavour and odour compounds are listed in Table 1 along with their corresponding odour description and detection limits. The chemical structures of those compounds are summarized in Fig. 4. Several of the compounds shown are of great importance not only to grape flavour and odour, but also to a wide range of industries and products. For example, vanillin and some of its derivatives are considered to be the main flavouring compounds used worldwide in a variety of

foodstuffs, as well as being of importance to perfumes and fragrances (Walton et al. 2000).

A series of seminal works have examined both the chemical composition and sensory contribution of several different wine grape cultivars, summarizing the levels of free volatiles and in some cases bound volatiles, in juices from these grapes (Williams et al. 1989, Francis et al. 1992, Sefton et al. 1993, 1994, Sefton et al. 1996, Sefton 1998). From these studies, it can be seen that the concentrations of volatile aromatic compounds, both free and hydrolytically released, can vary from trace levels to hundreds of micrograms per litre, indicating that many of these chemical species should contribute to the overall odour of juice from these grapes (Sefton et al. 1994, Sefton 1998), and also presumably any subsequent wine produced.

Table 1. Odours of some important volatile aromatic compounds.

Compound	Odour description	Odour threshold (μg/L)*	References
2-Phenylethanol	Honey, rose, spice, lilac	10000	1, 2
2-Phenylethyl acetate	Rose, honey, tobacco	250	1-3
2-Phenylacetaldehyde	Floral, honey	1	3
2-Phenylacetic acid	Honey, floral	1000	3, 4
4-Ethylphenol	Bitumen, leather, phenol, spice	440	1, 3, 5
Vanillin	Vanilla, honey	60-200	1, 2, 6
Ethyl vanillate	Vanilla, honey	990	3, 5
Guaiacol	Smokey, hospital, medicine, sweet	9.5	1, 3, 7
4-Ethylguaiacol	Leather, phenolic, spice, clove	33	1, 3, 7
4-Vinylguaiacol	Clove, curry	40	1, 2
Eugenol	Clove, honey	5	1, 2
(E)-Isoeugenol	Floral	6	3
m-Cresol	Leather, spicy	68	3
(E)-Ethylcinnamate	Honey, cinnamon	1.1	1, 7
Ethyl dihydrocinnamate	Floral, pollen, flowers	1.6	1, 3, 7

* Determined as hydroalcoholic mixtures or synthetic wines; see references.

Table references: 1 (Francis and Newton 2005), 2 (Guth 1997), 3 (Escudero et al. 2007), 4 (Maga et al. 1973), 5 (Lopez et al. 2002), 6 (Cullere et al. 2004), 7 (Ferreira et al. 2000).

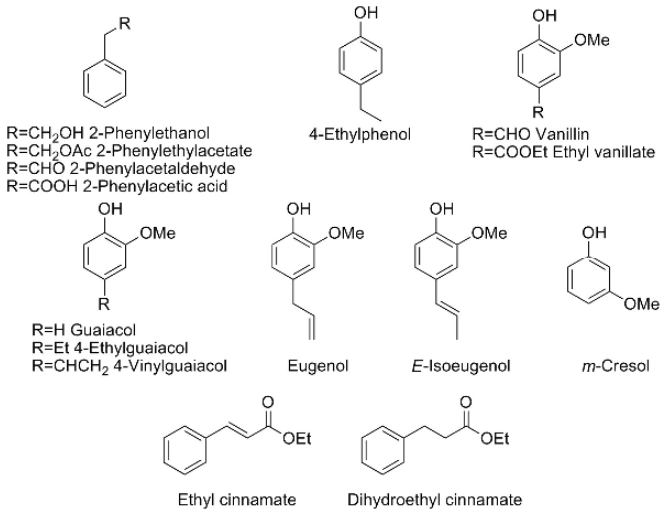

Fig. 4. Chemical structures of some important aromatic odour compounds.

4.3. Biosynthesis of aromatic compounds

4.3.1. The shikimate pathway and aromatic amino acid synthesis

Phenylpropanoid, meaning a three carbon chain attached to an aromatic six-membered ring, is a common term used to describe many volatile aromatic odour compounds such as eugenol (Dudareva et al. 2006). Other aromatic odour compounds are benzenoids, where there is one carbon attached to the central benzene core, or ethyl benzenes, where a two carbon chain is attached. The biosynthesis of most volatile aromatic plant odour compounds is thought to be derived from the shikimic acid pathway. Shikimate is formed from erythrose-4-phosphate (generated from *D*-glucose) and phosphoenol-pyruvate (PEP). Shikimate then undergoes addition of a second PEP followed by two eliminations of phosphoric acid to give chorismate, a precursor to many related aromatic compounds. This pathway has been the subject of many reviews (e.g. Dudareva et al. 2006), which provide detail on the individual steps within the pathway and the numerous enzymes involved. Chorismate can then undergo a series of different enzyme catalysed reactions. For example, elimination of pyruvic acid would yield *p*-hydroxybenzoic acid, while amination followed by elimination of pyruvic acid would yield the ubiquitous compound *p*-aminobenzoic acid (PABA).

Alternatively, isomerization to isochorismic acid followed by elimination of pyruvic acid yields salicylic acid, an extremely common metabolite and a precursor to the aromatic odour compound methyl salicylate, an aromatic with

peppermint characteristics, as shown in Fig. 5 (Seigler 1995). Note that salicylic acid can also be produced by direct hydroxylation of benzoic acid by way of benzoate hydroxylase (Seigler 1995). Moreover, in plants *L*-phenylalanine is formed from chorismate *via* prephenate and arogenate in turn, controlled by chorismate mutase, prephenate aminotransferase and arogenate dehydratase, respectively (Fig. 5) (Seigler 1995). It is *L*-phenylalanine that is the common intermediate in the formation of the various phenylpropanoids, benzenoids and other aromatic compounds (Dudareva et al. 2006).

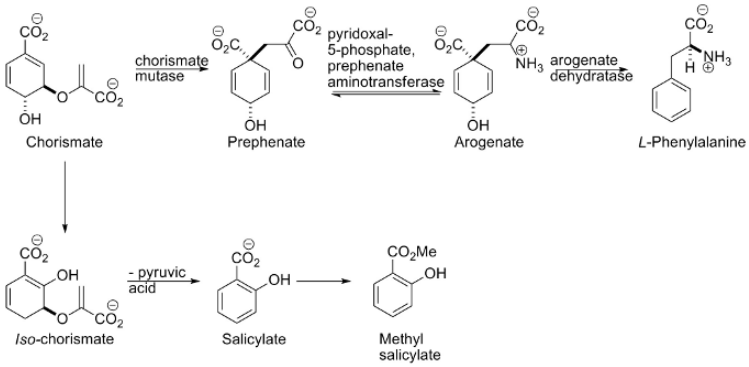

Fig. 5. Formation of *L*-phenylalanine and methyl salicylate.

4.3.2. Chemical modifications leading to the production of volatile aromatics

L-Phenylalanine is converted initially to *E*-cinnamic acid by way of the common enzyme *L*-phenylalanine ammonia lyase (PAL), to eliminate ammonia and generate a side chain olefin (Dudareva et al. 2006). Cinnamic acid can then be converted to *p*-coumaric acid in most plants, except grasses (Seigler 1995). Cinnamic acid is also the progenitor of the common metabolites caffeic, ferulic and sinapic acids (Humphreys et al. 2002). In many cases, these organic acids are found as their methyl esters, generated by the action of any number of methyltransferase enzymes in conjuction with *S*-adenosyl methionine. Activated ester analogues of the acids can also be generated by formation of their CoA derivatives. As well as the free acids and ester derivatives, ethers of these compounds are also found. In fact, many of the acids are found as a variety of (presumably non-volatile) glycosides (Seigler 1995). Any of these acids and their analogues can be considered as constituting part of the phenylpropanoid precursor pool.

Recently, it has been shown that eugenol and isoeugenol are generated from coniferyl acetate (Koeduka et al. 2006). This pathway is present in sweet basil and petunias and may be related to that responsible for the formation of

these compounds in grapes. Coniferyl acetate is generated from coniferol, which is itself formed by the conversion of ferulic acid to its CoA ester, followed by reduction to the alcohol. Coniferol is subsequently acetylated (Seigler 1995). Two possible mechanisms for the formation of eugenol and isoeugenol from the allylic acetate were suggested, either a direct reductive elimination or reduction of an intermediate quinone methide (Koeduka et al. 2006). The genes responsible for this conversion, *eugenol* (*EGS1*) and *isoeugenol* (*IGS1*) *synthase*, respectively, are related to the PIP family of NADPH-dependent reductases (Koeduka et al. 2006).

The other phenylpropanoids are considered to be made from the activated CoA esters of cinnamic, coumaric, caffeic, ferulic and sinapic acids. The esters are first reduced to the corresponding alcohols by a CoA reductase to form the corresponding aldehyde, followed by reduction catalysed by an alcohol dehydrogenase to form the alcohol (Humphreys et al. 2002).

Benzenoids can also be generated from *E*-cinnamic acid through the degradative loss of two carbon atoms from the side chain. Such compounds bear very close resemblance to those generated directly from the shikimate pathway (e.g. salicylic acid), or from variations of the mevalonate pathway (Seigler 1995). As with the other pathways involved in production of aromatic flavour and odour compounds, the genes and processes involved in their generation are not well understood (Dudareva et al. 2004). The current paradigm suggests that two pathways are possible, a CoA-dependent β-oxidation similar to that found in fatty acid biosynthesis, and a CoA-independent non-β-oxidative route. Stable isotope labelling studies have suggested that both pathways are active in petunias (Boatright et al. 2004).

The two routes diverge following deamination of phenylalanine by PAL to form *E*-cinnamic acid. In the CoA dependent β-oxidative route, the acid is esterified with CoA under the control of CoA-ligase. The ester is then hydrated to form the corresponding 3-hydroxy-3-phenylpropionyl ester by enol-CoA hydratase (Boatright et al. 2004). The 3-hydroxy ester is then oxidised by a NAD-dependent dehydrogenase to give a β-keto-thioester which is degraded by β-ketothiolase to liberate acetyl-CoA and benzoyl-CoA in a reverse Claisen reaction. Under the action of a thioesterase, benzoyl-CoA will then hydrolyse to benzoic acid (Fig. 6) (Hertweck et al. 2000, Hertweck et al. 2001, Boatright et al. 2004). The 3-hydroxy-3-phenylpropionyl-CoA precursor from *Nicotiana attenuata* has been shown to have the same absolute configuration as the corresponding alcohol found in fatty acid biosynthesis, while there is substantial sequence homology between cinnamoyl-CoA hydratase from the marine actinomycete *Streptomyces maritimus* and the fatty acid enoyl-CoA hydratases (Hertweck et al. 2001). These results suggest that the enzymes responsible for the β-oxidation pathway from phenylalanine to benzoic acid active in plants and

bacteria are related to those responsible for the β-oxidation of fatty acids (Hertweck et al. 2001).

The second possible route from *E*-cinnamic acid is *via* a retro-aldol cleavage. First, the olefin of the acid is hydrated to form 3-hydroxy-3-phenylpropionate. Following alcohol formation, a retro-aldol cleavage takes place that generates benzaldehyde. Benzaldehyde can then be oxidised to benzoic acid or reduced to benzyl alcohol as shown Fig. 6 (Boatright et al. 2004). A third pathway, a CoA-dependent retro-aldol route from the activated 3-hydroxy-3-phenylpropionic-CoA ester is also possible (Fig. 6). This pathway has shown to be active in tutsan (*Hypericum androsaemum)* (Ahmed et al. 2002). Once the basic benzenoid skeleton is generated, any number of hydroxylations and methylations in a variety of positions would generate the multitude of benzenoid aromatic compounds (e.g. vanillin). Note that benzenoids can also be produced by other mechanisms, such as the previously mentioned elimination of pyruvic acid from *iso*-chorismate to yield salicylic acid.

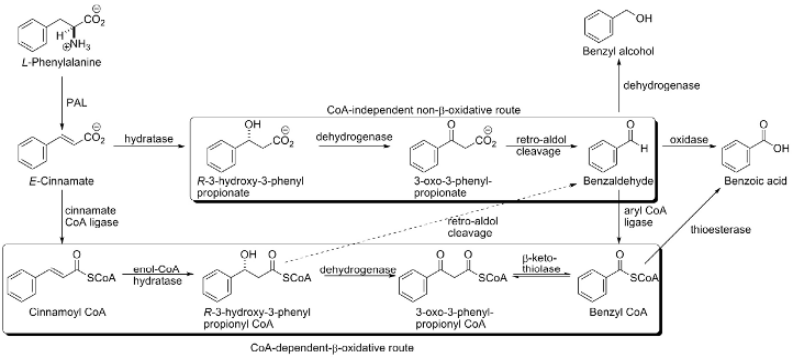

Fig. 6. Possible routes to benzenoid metabolites. CoA-dependent retro-aldol route shown by dotted arrow.

It appears that the volatile aromatic compounds with two carbon side chains such as 2-phenylethanol or 2-phenylacetaldehyde are not derived from cinnamic acid. Rather, they appear to come from phenylalanine in a process that is in direct competition with the cinnamate pathway to the benzenoids shown in Fig. 6 (Boatright et al. 2004). In tomatoes, phenylalanine is decarboxylated by tomato aromatic amino acid decarboxylase to give phenylethylamine that is then converted to 2-phenylacetaldehyde which itself can be reduced to 2-phenylethanol as shown in Fig. 7 (Tieman et al. 2006). A different pathway is active in petunias; this route goes through a series of quinoid and imine intermediates to finally give 2-phenylacetaldehyde, controlled by the enzyme phenylacetaldehyde synthase, a homotetrameric enzyme that has efficiently

coupled phenylalanine decarboxylation and oxidation reactions (Kaminaga et al. 2006).

Although the pathways to the aromatic compounds have not been explicitly explored in grape cultivars, all of the previously mentioned biosyntheses are active in various higher plants and therefore it can be hypothesized that similar processes are instrumental in the production of these important grape flavour and odour compounds. The identification of shikimate pathway genes and those concerned with the formation of aromatic amino acids should be achievable given the conservation of the genes in this pathway across plant species (Richards et al. 2006). However, many of the enzymes required for specific chemical modifications are encoded by large gene families that carry out similar reactions using distinct substrates. Some of these gene families, such as the oxidoreductases, methyltransferases and acyltransferases, show little sequence similarity amongst genes that act on substrates of similar structure (Pichersky et al. 2006). Therefore, the identification of relevant grape genes will require robust functional analyses to confirm targets identified by sequence similarity and gene expression analysis.

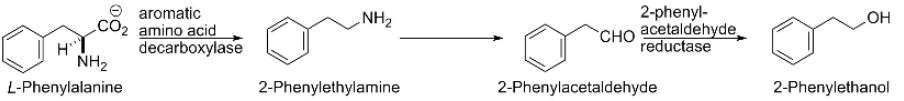

Fig. 7. Conversion of *L*-phenylalanine to 2-phenylethanol.

5. ALIPHATIC VOLATILE COMPOUNDS

5.1. Introduction

Aliphatic volatile compounds originate from fatty acid oxidation and amino acid degradation. The C_6 and C_9 compounds, produced by the action of the lipoxygenase pathway, are important contributors to the flavour of many fruits and vegetables (Schwab et al. 2008). The degradation of branched-chain amino acids produces aldehydes, alcohols and acids that may have a sensory impact themselves, or which serve as substrates for the production of esters that have large impacts on fruit odour (e.g. Aubert et al. 2005, Holland et al. 2005). The C_6 volatiles are considered to be typical wound signals (Hatanaka 1993), and are involved in direct defense against herbivores (De Moraes et al. 2001, Vancanneyt et al. 2001). Interestingly, they have also been implicated in plant-plant signalling to prime healthy plants against imminent attack from herbivores (Engelberth et al. 2004).

Grape aliphatic volatile compounds consist of predominantly short chain aldehydes and alcohols that are either straight or branched. Occasionally, esters are detected in grapes and if present, they are usually at low abundances with minimal flavour impact (Schreier 1979). The major aliphatic volatile compounds in grapes are the C_6 aldehydes and alcohols derived from fatty acid metabolism.

Apart from the C_6 compounds, methyl branched chain volatile compounds and C_5 and C_9 straight chain volatile compounds have been identified as minor constituents of the grape volatile profile. The C_9 volatile compounds are derived from fatty acids in a similar pathway to the C_6 straight chain aliphatic compounds. The other fraction of the minor aliphatic compounds in grapes, the C_5 straight chain compounds, are believed to be formed from the anaerobic action of lipoxygenase on polyunsaturated fatty acids (Gardner et al. 1996). The branched chain aliphatic aroma compounds are formed from reductive deamination of amino acids (Tressl et al. 1973, Nykanen 1986, Dudareva et al. 2006).

The common amino acid-derived branched chain aroma compounds detected in grapes and wines are 2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol derived from valine, isoleucine, and leucine, respectively (Nykanen 1986, Swiegers et al. 2005). These methyl-branched aroma alcohols are more abundant in wines than grapes where their respective aldehyde forms dominate. In wine, these compounds may originate from both grape and yeast sources and so the biosynthesis of amino-acid derived compounds will not be discussed in this review.

5.2. Odour description and impact

Odour impacts of aliphatic compounds based on grape juice or slurry are rarely explored, probably because most of these compounds are often not abundant in wines. For the few that have been investigated, the odour threshold values were measured in wines or model wines (Table 2), which might just be indicative of the actual odour impact in grapes due to their different matrices (Kalua et al. 2007). In general, most of the aliphatic aroma compounds in grapes are characterised by their green or leaf-like sensory descriptors (Table 2). In grapes, the aliphatic volatile compounds that have an undesirable flavour perception should not be ignored as they have a potential of contributing to positive sensory attributes during winemaking. During the vinification process, these grape aliphatic compounds are depleted and converted to alcohols and esters, usually with positive sensory attributes (Palomo et al. 2007).

5.3. Biosynthesis of aliphatic volatile compounds

5.3.1. The lipoxygenase pathway

The biosynthetic pathway for the formation of major aliphatic volatile compounds in grapes has not been specifically studied. It is assumed that biogenesis of aliphatic volatile compounds in grapes follow a similar pathway synonymous with fruits, commonly referred to as the lipoxygenase pathway (Gardner 1991, Salas et al. 2000). This pathway involves the enzymatic splitting of linoleic and linolenic acid into C_6 and C_9 aldehydes and C_9 and C_{12} oxo-acids (Fig. 8). Anecdotal evidence from grape volatile compounds show that C_9 volatile compounds from the degradation of 9-hydroperoxides are rare compared to the C_6 volatile compounds from 13-hydroperoxides which account for a major fraction of the volatile profiles of homogenized grape berries.

Table 2. Odours of common grape aliphatic volatile compounds.

Compound	Odour description	Odour threshold (μg/L)*	References
Hexanal	Grass	4500#	1,2
Z-3-hexenal	Grass	400	1,3
E-2-hexenal	Grass/lemon	17000#	1,2
Hexanol	Green/grass	8000	1,3,4
Z-3-hexenol	Green (cut grass)	606	1,4
E-2-hexenol	Green	8000^	5, 6
Hexyl acetate	Sweet, perfume, fruit	700	6
Z-3-hexenyl acetate	Green/banana	750^	5,6
E-2-hexenyl acetate	Green/banana	-	6
(E,Z)-2,6-nonadienal	Cucumber	0.02	1,6
(E,Z)-2,6-nonadienol	Cucumber	-	6
2-methyl-1-butanol	Malty	480^	6,7
3-methyl-1-butanol	Malty/whiskey/burnt, Nail Polish	30000	1,6,8
2-methyl-1-propanol	Nail polish	40000	1,8

* Determined as hydroalcoholic mixtures or synthetic wines except: # in water and ^ in oil.
Table references: 1 (Kotseridis and Baumes 2000), 2 (Buttery et al. 1971), 3 (Ferreira et al. 2000), 4 (Francis and Newton 2005), 5 (Aparicio and Morales 1998), 6 (Acree and Arn 2004), 7 (Morales et al. 2005), 8 (Guth 1997).

The lipoxygenase pathway involves a series of enzymes that oxidize (lipoxygenase) and cleave (hydroperoxide lyase) polyunsaturated fatty acids to yield aldehydes. These aldehydes may be reduced subsequently to alcohols (by alcohol dehydrogenase) and esterified (by alcohol acyltransferase) usually in the

presence of short chain carboxylic acids such as acetic acid. The different enzyme actions in the lipoxygenase pathway below (Fig. 8) are initiated when grapes are crushed and enzymes are in contact with fatty acids in the presence of oxygen (Chkaiban et al. 2007).

In the first step of grape aliphatic volatile formation (Fig. 8), acyl hydrolase (AH) hydrolyses bound fatty acids to release free fatty acids. Lipolytic AHs form a group of enzymes that include lipases, phospholipases and galactolipases. In grapes, bound fatty acids are usually in the form of phospholipids, fatty acyl-CoA esters, diglycerides, and triglycerides and exist either as fat globules in the cell or form part of the cell membranes (Costantini et al. 2006, Chkaiban et al. 2007). Therefore, the origin of free fatty acids in grapes may be diverse and involve a host of genes that encode various lipases. The free fatty acids released from the action of AHs are oxidized, forming hydroperoxides through the action of lipoxygenase (LOX). LOXs catalyze the dioxygenation of

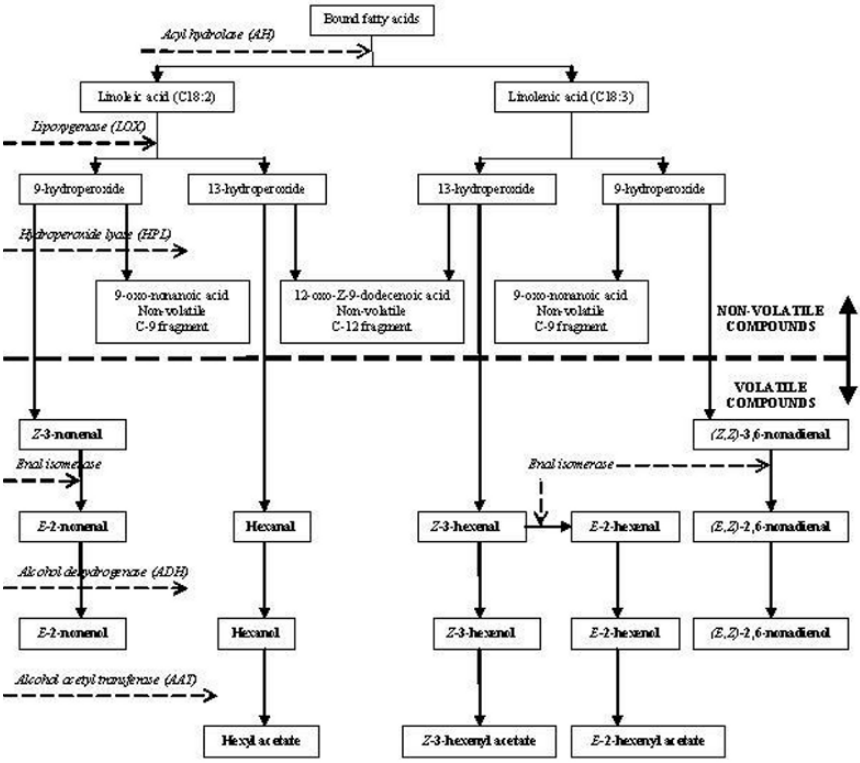

Fig. 8. Biogenesis of lipid derived volatile compounds in grapes. Volatile compounds are shown in bold; dashed arrows show enzymes.

polyunsaturated fatty acids, and are classified as either 9-LOX or 13-LOX depending on the positional specificity of the enzyme towards linoleic acid (Feussner et al. 2002). Based on sequence comparisons, *LOX* genes are divided into two subfamilies depending on the absence (type-1) or presence (type-2) of a chloroplast transit peptide (Feussner et al. 2002). Members of the type-2 subfamily are all 13-LOXs. Some substrate specificity has been noted, with free fatty acids being the preferred substrate for the majority of LOXs (Feussner et al. 2002).

Certain free fatty acids may also be preferred, for example the LOX enzyme in olives is more active with linolenic acid than linoleic acid by a factor of two (Salas et al. 1999, Salas et al. 2000). Not much is known about *LOX* genes or activity in grapes, although it may be similar to fruits like olives given their similar LOX-derived volatile profiles observed. Upon crushing, ripe grape berries produce more C_6 unsaturated volatile compounds than saturated C_6 compounds (Fig. 8), suggesting that the berries have a higher LOX activity for linolenic acid than linoleic acid, or a prevalence of this substrate. The understanding of the substrate specificity and regiospecificity of the LOX enzymes active in grapes will be important for the biotechnological engineering of flavour biogenesis for both grapes and wines.

Once the hydroperoxides are formed by the action of LOX, hydroperoxide lyase (HPL) catalyses the cleavage of fatty acid hydroperoxides – producing volatile aldehydes and oxo-acids (Fig. 8). The HPL enzyme yields C_6 aldehydes and C_{12} ω-oxo-acids from the 13-hydroperoxides of linolenic or linoleic acid, or C_9 aldehydes and C_9 ω-oxo-acids from the 9-hydroperoxide derivatives of the same fatty acids, depending on the substrate specificity of the enzyme.

Three HPL classes are found in plants. One cleaves the 13-hydroperoxides specifically (13-HPLs) and another class can cleave both 9- and 13-hydroperoxides with almost the same efficiency (9/13-HPLs; Matsui 2006). Recently, an HPL enzyme that cleaves the 9-hydroperoxides specifically (9-HPLs) was described in almond lipid bodies (Mita et al. 2005). The cleavage of 9-hydroperoxides is responsible for the characteristic cucumber-like odour of some fruits and vegetables, whereas cleavage of 13-hydroperoxides produces C_6 aldehydes responsible for 'green' aroma (Salas et al. 2000). Judging from the volatile profile of grapes, the HPLs that utilize 13-hydroperoxides seem to dominant. However, the reported detection of *(E,Z)*-2,6-nonadienal in grape juice (Kotseridis et al. 2000) indicates that the 9-hydroperoxide pathway (Fig. 8) is active to some extent. Silencing the 9-hydroperoxide pathway might minimise the negative sensory attributes associated with *(E,Z)*-2,6-nonadienal (Table 2).

The cleavage of 13-hydroperoxides forms C_6 aldehydes (Fig. 8) that include the saturated aldehyde hexanal from linoleic acid and the unsaturated al-

dehyde *Z*-3-hexenal from linolenic acid. The unsaturated aldehyde, *Z*-3-hexenal is unstable and undergoes rapid isomerisation to a stable compound, *E*-2-hexenal with the aid of the 3*Z*:2*E*-enal isomerase enzyme (Noordermeer et al. 1999). The aldehydes formed through HPL activity and isomerized by 3*Z*:2*E*-enal isomerase, are further reduced to alcohols. Alcohol dehydrogenases (ADHs) catalyse the reversible reduction of aliphatic aldehydes to alcohols (Fig. 8). *ADH* genes encode these enzymes that are responsible for the formation of volatile alcohols that contribute to the aroma of vegetable products. They are present as multigene families, with six *ADH* genes being present in the grape genome (Torregrosa et al. 2008; see also the chapter by Tesniere and Abbal, this edition). In grapes, stress conditions have been associated with an increase in ADH activity and *ADH* gene expression. It is presumed that ADH in grape berries can be activated at certain levels of water loss (Tesniere et al. 1993, Costantini et al. 2006). It can therefore be postulated that grapes grown under stress conditions could potentially produce more aliphatic alcohols provided enough substrate is produced from the lipoxygenase pathway. One of the grapevine *ADH* genes, *VvADH2* displays an increase in expression during berry ripening (Tesniere et al. 2000). However, overexpression of *VvADH2* in grapevines did not increase the levels of aliphatic alcohols in berries, and in fact reduced the levels of some aromatic alcohols (Torregrosa et al. 2008).

Therefore, the mechanism controlling the production of these aliphatic alcohols may not depend on ADH activity in the berries. Alcohols, produced by the action of ADH, can form volatile esters in the presence of carboxylic acids. Alcohol acetyl transferase (AAT) catalyzes the formation of acetate esters through acetyl-CoA derivatives. Acetate esters and esters of alcohols with other fatty acids are important constituents of many fruits. Anecdotal evidence shows low abundances of acetate esters in grapes, which might be an indication of low AAT activity. A decrease in *Z*-3-hexenyl acetate has been reported with an increase in grape ripeness (Callao et al. 1991). It is not clear whether this decrease is an indication of decrease in the activity of AAT or decrease in the concentration of its precursor, *Z*-3-hexenol (Fig. 8).

In general, esters are less abundant and investigated in grapes compared to other fruits such as bananas, strawberries, and apples (e.g. Perez et al. 1996, Dixon et al. 2000, Jayanty et al. 2002), a probable indicator of a lesser AAT activity. The potential of biotechnologically enhancing AAT activity in grapes for a consequent enhanced fruity flavour is an attractive option for the grape and wine industry.

6. ORGANO-SULFUR COMPOUNDS

6.1. Introduction

Sulfur containing organic compounds are common and often potent aroma components produced by many organisms. Organo-sulfur compounds are generally found as thiols, (previously referred to as mercaptans), thioesters, sulfides, polysulfides (e.g. disulfides, n=2), and heterocyclics (e.g. benzothioazole). Volatile thiols and their derivatives contribute to the flavours and aromas of many foods and beverages. For example, volatile thiols have been shown to contribute to the aromas of a variety of such diverse foodstuffs as meat (Mottram et al. 1995) to truffles (Pelusio et al. 1995) to coffee (Semmelroch et al. 1996, Charles-Bernard et al. 2005). Volatile thiols have also been shown to contribute strongly to the aroma profile of wine, both negatively as a sign of wine taint (e.g. hydrogen sulphide, H_2S, with a rotten egg aroma) but also positively as a distinctive and definitive component of varietal character (Moreira et al. 2002, Swiegers et al. 2007). In some cases, the source of the volatile can be assigned to yeast metabolism (e.g. H_2S) and in other cases the origin of the aroma compound is of some debate (e.g. 2-furanmethanethiol and 2-methyl-3-furanthiol). However, some volatile thiols have been shown to originate predominantly from the grape (e.g. 3-sulfanylhexan-1-ol) and are important constituents of the character of certain grape varieties (Schneider et al. 2006). The compounds in this final category are the focus of this section.

6.2. Odour description and impact

Volatile organo-sulfur compounds produce a large variety of aromas, whose organoleptic influence depend greatly on their relative concentration, as well as the composition of the matrix from whence they are detected (Francis et al. 2005). Organo-sulfur compounds can be present in wine in concentrations varying easily over seven orders of magnitude, ranging from below the lowest trace amounts (1 ng/L) to over 10 mg/L (Mestres et al. 2000). The perceived aroma quality of any compound varies greatly with concentration but this is especially true of organo-sulfur compounds; for example, the odour of dimethyl sulphide can vary from cooked asparagus through blackcurrant finally to cooked corn/tomato aromas as the concentration rises from low to high levels (Francis et al. 2005).

As well as greatly varying in concentration, the odour detection limits of various organo-sulfur compounds can range from well below 1 ng/L to the mg/L level (Mestres et al. 2000). Matrix effects can greatly enhance or suppress the aroma detection limits of these compounds (Francis et al. 2005). For exam-

ple, the detection limits of 3-sulfanylhexan-1-ol vary between 12-15 ng/L in water and 10-5000 ng/L in wine (Bouchilloux et al. 1998). It should be noted that many volatile organo-sulfur compounds that are important for final wine flavour and aroma are absent in their parent grapes (Swiegers et al. 2007). The current hypothesis is that volatile thiols are bound, as either cysteine (Tominaga et al. 1998a) or glutathione conjugates (des Gachons et al. 2002). In this chapter, the aromas of various volatile thiols, sulfides etc will be discussed although they are only found as bound-volatiles in their parent grapes.

The chemical structure of some important wine volatile organo-sulfur compounds are shown in Fig. 9, while their corresponding aromas are summarized in Table 3. Please note that there are multiple synonyms for several of the important compounds. In particular, the compounds 3-sulfanylhexan-1-ol, 3-sulfanylhexyl-*O*-acetate and 4-methyl-4-sulfanylpentan-2-one are commonly referred to as 3-mercaptohexanol (3MH), 3-mercaptohexyl-O-acetate (3MHA) and 4-mercapto-4-methylpentan-2-one (4MMP) respectively, in the literature.

It should be noted that 3-sulfanylhexan-1-ol can exist as two possible enantiomers, as can its acetate derivatives. This is also true for 4-methyl-4-sulfanylpentan-2-ol. This chirality could result in different interactions with biological aroma receptors and therefore the two possible enantiomers could in fact possess quite different aroma qualities and thresholds. This fact has been largely ignored in most studies, both of natural and synthetic aroma compounds, although some research groups are becoming more cognisant of the importance of chirality to flavour and aroma. A recent study showed that both enantiomers of 3-sulfanylhexan-1-ol and its acetates are present in Sauvignon blanc and Semillon wines (Tominaga et al. 2006). The authors found that the detection threshold for both the (*R*)- and (*S*)-forms of 3-sulfanylhexan-1-ol were similar (50 and 60 ng/L, respectively in model wine; racemic mixture 60 ng/L), while there was a distinct difference in the detection threshold for the corresponding acetates with the (*R*)-form being less detectable (9 ng/L) than the (*S*)-form (2.5 ng/L) or

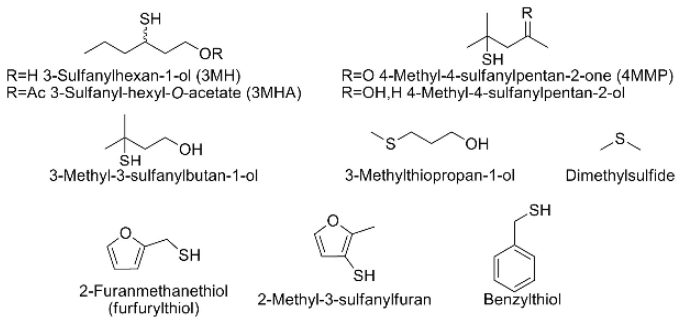

Fig. 9. Chemical structure of some important volatile organo-sulfur compounds**.**

Table 3. Odours of some important volatile organo-sulfur compounds.

Compound	Odour description	Odour threshold (µg/L)*	References
3-Sulfanylhexan-1-ol	Sulfur, passionfruit, cat urine, fruity, broom, box tree, rhurbarb, fruity	0.06	1-4
3-Sulfanylhexyl-1-acetate	Box tree, grapefruit, passion-fruit, cat urine, broom	0.004	1-3
4-Sulfanyl-4-methylpentan-2-one	Box tree, passionfruit, cat urine, guava	0.0008	1-3
4-Sulfanyl-4-methylpentan-2-ol	Citric zest, passionfruit, box tree, broom	0.055	1, 2
3-Sulfanyl-3-methylbutan-1-ol	Cooked leeks	1.5	2
3-Methylthiopropan-1-ol	Sweet, potato, cauliflower, soup or meat like	1000	1, 3, 5
Dimethylsulfide	Black currant, cabbage, sul-fur, gasoline, cooked aspara-gus, corn, tomato	10	1, 3, 6, 7
2-Furanmethanethiol	Roasted coffee, meat, bread, pop corn	0.0004	8, 9
2-Methyl-3-furanthiol	Meat	0.005	1, 3, 10
Benzylthiol	Struck match, struck flint, rubber	0.003	1, 3, 11

* Determined in hydroalcoholic mixtures; see references

References: 1 (Mestres et al. 2000), 2 (Tominaga et al. 1998b), 3 (Francis and Newton 2005), 4 (Bailly et al.2006), 5 (Ferreira et al. 2000), 6 (Guth 1997), 7 (Segurel et al. 2004), 8 (Tominaga et al. 2000), 9 (Tominaga and Dubourdieu. 2006),10 (Ferreira et al. 2002),11 (Ferreira et al. 2007).

Table 4. Odour threshold and quality for different enantiomers of 3-sulfanylhexan-1-ol and derivatives.

Compound		Odour threshold (ng/L)[1]	Odour description[1]
3-Sulfanylhexan-1-ol	*R*	50	grapefruit, citrus peels
	S	60	passion fruit
	racemic mixture	60	grapefruit, passion fruit
3-Sulfanylhexan-1-acetate	*R*	9	passion fruit
	S	2.5	boxwood
	racemic mixture	4	passion fruit, box wood

Reference: 1 (Tominaga et al. 2006)

a racemic mixture (4 ng/L) (Tominaga et al. 2006). A difference in aroma quality was also noted for the four compounds, which is summarized in Table 4 (Tominaga et al. 2006). Enantio-differentiation is also true for other chiral organosulfur compounds such as 3-sulfanyl-2-methylpropan-1-ol, which has been described as being present in some Bordeaux red wines. Initially described in wine for the first time in 1998, 3-sulfanyl-2-methylpropan-1-ol was ascribed a broth or sweat aroma with an odour threshold of 3 μg/L in water, and with concentrations ranging between 250 and 10000 ng/L in wine (Bouchilloux et al. 1998). Subsequent chiral analysis of synthetic 3-sulfanyl-2-methylpropan-1-ol showed that although the two enantiomers do in fact have similar aroma qualities, the (*R*)-enantiomer has an odour threshold approximately six- to ten-fold lower (water: 3-7 μg/L; model wine: 20-27 μg/L) than the corresponding (*S*)-enantiomer (water: 35-40 μg/L; model wine: 120-130 μg/L) (Bouchilloux et al. 2000). This study also revealed that only the (*R*)-form was present in Cabernet Sauvignon wine (Bouchilloux et al. 2000), unlike the case where both (*R*)- and (*S*)-forms of 3-sulfanylhexan-1-ol and its acetates were present in white wines (Tominaga et al. 2006).

6.3. Biosynthesis of organo-sulfur compounds

The mechanisms by which many volatile organo-sulfur compounds are produced in biological systems are not well understood. As well as possible enzymatic routes, other chemical and environmental aspects can influence the production of these compounds (Moreira et al. 2002). The production of volatile thiols, for example, can occur *via* several different mechanisms. The first, and most simple, is the simple addition of H_2S to an alkene. For example, addition of H_2S to 3-hexen-1-ol would generate 3-sulfanylhexan-1-ol. H_2S is a common bi-product of microbial metabolism, especially when certain nutrient sources are limited. This has been proposed as one source of 3-sulfanylhexan-1-ol in wine (Schneider et al. 2006). However, the formation of the simple acyclic thiols (e.g. 3-sulfanylhexan-1-ol) is not quite as simple as might be first envisaged. The enantio-differentiation mentioned in the previous section can give insight into the mechanism of formation of these compounds.

Both the (*R*)- and (*S*)-enantiomers of 3-sulfanylhexan-1-ol and its acetates were found to be present in Sauvignon blanc and Semillon wines. In dry wines made from these grapes, the ratio of enantiomers ranged from 50:50 *R*:*S* for 3-sulfanylhexan-1-ol to approximately 30:70 for the acetates. The ratio of the two enantiomers of the alcohol was also found to change during fermentation while the ratio for the acetates remained the same. The enantiomeric ratio of 3-sulfanylhexyl-*O*-acetate was independent of the strain of yeast used for fermentation (Tominaga et al. 2006). Finally and in contrast, a ratio of 30:70 *R*:*S* 3-

sulfanylhexan-1-ol was found when a sweet wine was made from white grapes infected with *Botrytis cinerea* (Noble rot) (Tominaga et al. 2006).

The 50:50 racemic mixture of both enantiomers of 3-sulfanylhexan-1-ol in dry white wines would tend to imply a chemical, non-enzymatic formation of the thiol by, for example, H_2S addition to an alkene. The higher proportion of (*S*)-3-sulfanylhexyl-*O*-acetate over the (*R*)-enantiomer could be explained by enzyme mediated esterification during yeast metabolism of the alcohol with a preference for one configurational isomer as a precursor over the other (Tominaga et al. 2006). A chemical biogenesis does not, however, adequately explain the non-racemic ratio of 3-sulfanylhexan-1-ol in botrytized wines, nor does it explain the changing ratios of enantiomers during fermentation. These observations imply that there is also an enzymatic biogenesis at play, in all likelihood in parallel with a chemical pathway.

The current paradigm for organo-sulfur compounds is that volatile thiols are found as non-volatile bound conjugates in the parent grape, either as cysteine or glutathione precursors. Glutathione is a tripeptide formed from glutamic acid, cysteine and glycine. Non-volatile conjugated precursors have been detected in grape musts. Both *S*-3-(hexan-1-ol)-glutathione (des Gachons et al. 2002) and the cysteine conjugates *S*-4-(4-methylpentan-2-one)-*L*-cysteine, *S*-4-(4-methylpentan-2-ol)-*L*-cysteine and *S*-3-(hexan-1-ol)-*L*-cysteine (Tominaga et al. 1998a) have been found in Sauvignon blanc musts.

The structures of these compounds are shown in Fig. 10. The non-volatile precursor conjugates are formed enzymatically and can therefore explain the apparent discrepancy in ratio of *R*:*S*-enantiomers present during fermentation of botrytized wines made from Sauvignon blanc and Semillon grapes (Tominaga et al. 2006). It is notable that β-lyases used to release 3-sulfanylhexan-1-ol from its cysteine conjugate show particular enantioselectivity, producing more of the (*S*)-enantiomer (Wakabayashi et al. 2004). This supports the premise of enzy-

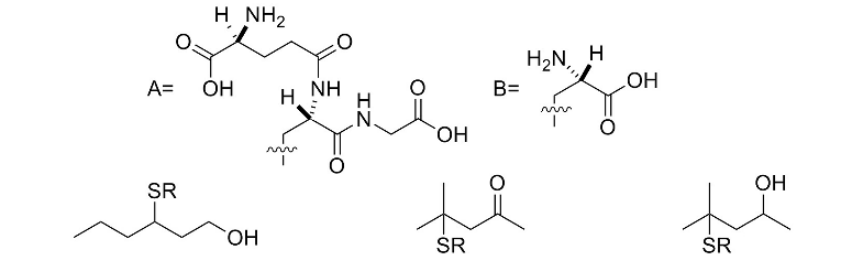

Fig. 10. Some common thiol conjugates from Sauvignon blanc musts.

matic enrichment of one enantiomer over the other in wines made from botrytized Sauvignon blanc and Semillon grapes.

A recent study has shown that several genes in yeast influence thiol release from *S*-4-(4-methylpentan-2-one)-*L*-cysteine (Howell et al. 2005). The liberation of the volatile free thiol would arise from the enzymatic degradation of the precursor conjugate. In the case of the cysteine conjugates, this involves simple C-S cleavage to yield the thiol. In the case of the glutathione derivatives, the cleavage could presumably proceed either by direct cleavage of the glutathione unit, or by sequential loss of the glutamic acid and glycine residues to form the corresponding cysteine conjugate which could then be further processed to the free thiol (Fig. 11). It has been noted, however, that only low amounts (< 5%) of the non-volatile precursors are actually liberated to the free volatile form (des Gachons et al. 2005).

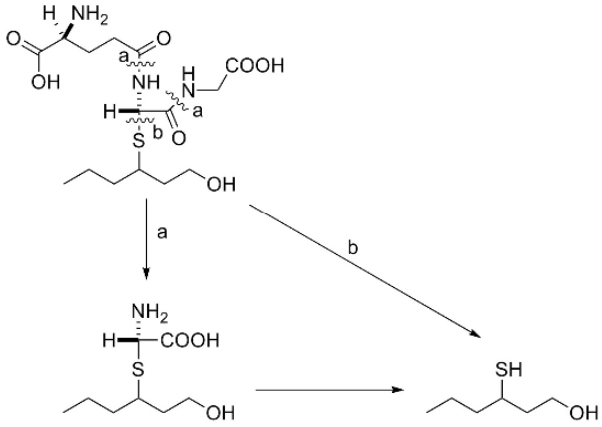

Fig. 11. Formation of 3-sulfanylhexano-1-ol from conjugated precursors

The biosynthesis pathway leading to the production of the cysteine-bound conjugates is at present unknown. Potential precursors for *S*-3-(hexan-1-ol)-*L*-cysteine include the unsaturated C_6 aldehydes and alcohols produced by the action of the lipoxygenase pathway described above. This would suggest that these cysteine conjugates are formed very soon after damage to the berry, or that this pathway is active in unwounded tissues. The compound 4-methylpent-3-en-2-one is potentially the precursor to *S*-4-(4-methylpentan-2-one)-*L*-cysteine production, and it is thought to be derived from fatty acids (Jorgensen et al. 2000). Glutathione addition to these precursors would occur by the action of a glutathione transferase (GST). GSTs form large gene families in plants, where their action is important for the detoxification of cytotoxic substrates (Basantani et al. 2007). They work by the addition of the tripeptide glutathione

to compounds, which are then transported to the vacuole or apoplast.

As the presumed precursors for the thiols are the cysteine conjugates, this requires the removal of the glutamic acid and glycine groups from the glutathione conjugate in the storage compartment. The current model is that the glutamic acid residue is cleaved by the action of a γ-glutamyl transpeptidase (GGT) and the glycine is then removed by dipeptidases (Ohkama-Ohtsu et al. 2007). The GGTs are encoded by a small gene family and little is known of the dipeptidases involved. As yeast are inefficient in their ability to convert the cysteine conjugates into volatile thiols, there is perhaps more to be gained by engineering yeast to release more of the precursors than increasing the amount of precursor in grapes. However, care must be taken not to release too much of these compounds as the descriptors for these compounds change from 'tropical fruit' to 'sweaty' when they are in high abundance. Silencing the pathways in grapes may be desirable if these characters are not sought in a wine style.

7. METHOXYPYRAZINES

7.1. Introduction

3-Alkyl-2-methoxypyrazines (MPs) are a family of volatile aroma compounds found in some vegetables, such as peas, beans, capsicum, asparagus and beetroot, where they are, in large, responsible for certain distinctive flavors (Murray et al. 1975, Ulrich et al. 2001). In grapes MPs are found at detectable levels in only some varieties including Sauvignon blanc, Cabernet Sauvignon and Merlot in which they are important contributors to the distinctive vegetative characters of these varieties (Chapman et al. 2004, Parr et al. 2007). The chemical structures of the three compounds belonging to this family that are reported to contribute to the flavour of grapes and wine, isopropyl methoxypyrazine (IPMP), isobutyl methoxypyrazine (IBMP) and *sec*-butyl methoxypyrazine (SBMP) are shown Fig. 12. Each of these MPs is extremely potent and they are amongst some of the most odour active aroma compounds known.

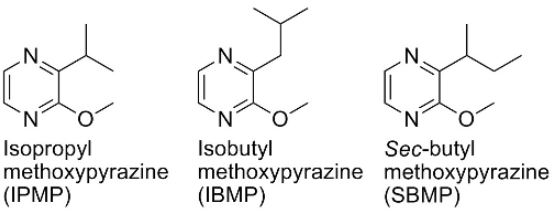

Fig. 12. Grape derived methoxypyrazines that contribute to wine flavour.

7.2. Odour description and impact

Each of the above mentioned MPs share a similar overall aroma in wine that is often described as vegetative, herbaceous, green, grassy or earthy. However, they have been shown to differ slightly in their perceived sensory description. IBMP has a distinct green capsicum (bell pepper) aroma (Buttery et al. 1969) while IPMP is often described as having an aroma reminiscent of asparagus or sweet peas (Murray et al. 1970) and the aroma of SBMP is described as being similar to galbanum oil (Murray et al. 1975, Mihara et al. 1990).

IBMP has an extremely low odour detection threshold of 2 ng/L in water (Seifert et al. 1970) and a slightly higher threshold of 10 ng/L and 16 ng/L in white and red wines respectively (Allen et al. 1991, de Boubee et al. 2000). Nevertheless IBMP is commonly found at concentrations above these thresholds ranging from <1-55 ng/L in wines of grape varieties that accumulate MPs, and therefore has a significant sensory impact on these varieties (Allen et al. 1994, Kotseridis et al. 1999, Belancic et al. 2007). IPMP has been found to have an odour detection threshold of 1-2 ng/L in water and both white and red wine (Pickering et al. 2007), however it is only occasionally found above this concentration, and is therefore considered of less importance to wine flavor than IBMP. Minimal sensory data is available on SBMP in wine. However, similar to IPMP, it has an aroma threshold of 1-2 ng/L in water (Murray et al. 1970). Like IPMP, SBMP very rarely reaches this threshold in wines and is thought to be of minor importance to wine flavour (Sala et al. 2004, 2005). Like most sensory characteristics of food products, it is debatable whether or not the flavours imparted by MPs are desirable in wine and it ultimately comes down to personal choice. However it is generally accepted that at high concentrations MPs become overpowering and are detrimental to wine quality (Parr et al. 2007).

As MPs, IBMP in particular, contribute to the distinct flavour of Sauvignon blanc and Cabernet Sauvignon wines, a large number of studies have been conducted investigating the influence of different environmental and viticultural factors on the level of MP in these varieties. Consequently, it has been shown that the accumulation of MPs reach a peak prior to véraison after which the levels of MP drop as the berry ripens (Boss et al. 2008). It has been shown that MPs are degraded by light and this is thought to be the major factor of why levels decrease following véraison, but data also exists suggesting that in immature grapes light actually promotes MP synthesis (Hashizume et al. 1999). Temperature has been shown to greatly influence the levels of MPs in grapes, with vines grown in cooler climates tending to produce higher amounts of MPs than vines grown in warmer climates (Lacey et al. 1991, Allen et al. 1994, Belancic et al. 2007). Other factors such as vine vigor, yield and irrigation have also been found to influence the amount of MPs present in grapes (de Boubee et al. 2000,

Chapman et al. 2004, Sala et al. 2005).

7.3. The biosynthesis of methoxypyrazines

The complete biosynthetic pathway leading to the formation of MPs is yet to be elucidated in not just grapes but any plant species. A number of pathways have been proposed and all agree that the pathway involves an amino acid and an unknown 1,2-dicarbonyl compound leading to the formation of a 3-alkyl-2-hydroxypyrazine (HP) intermediate, which is enzymatically methylated to form MP (Murray et al. 1970, Leete et al. 1992). It is thought that the amino acids valine, leucine and isoleucine are each precursors to IPMP, IBMP and SBMP, respectively because of similarities in the alkyl side chains (Murray et al. 1975). Feeding experiments in bacterial strains that accumulate IPMP have shown that the addition of ^{13}C-*L*-valine results in the production of ^{13}C containing IBMP, thus confirming that amino acids are a precursor to MPs (Gallois et al. 1988). What is unclear is the mechanism by which the amino acid is converted to the HP intermediate. It has been proposed that the respective amino acid gains a second nitrogen through an unknown amidation reaction and then undergoes a condensation reaction with a 1,2-dicarbonyl compound such as glyoxal to produce HP as shown in Fig. 13 (Murray et al. 1970). The final step in the pathway, involving the methylation of HP to MP, has been detected in grapes (Hashizume et al. 2001a) and a methyltransferase with this activity has been purified from grapes (Hashizume et al. 2001b). In unripe grapes the level of HPs was found to be between 2 and 20-fold higher than MP levels suggesting that this step might be rate-limiting (Hashizume et al. 2001a).

Functional enzyme assays showed that this enzyme is multifunctional and has the ability to methylate each of the respective HP precursors of IPMP, IBMP and SBMP, as well as a number of other hydroxyl containing molecules, such as caffeic acid (Hashizume et al. 2001b). Interestingly, the activity of this enzyme against caffeic acid was found to be >100-fold greater than for HPs, which may explain why MPs are found at extremely low concentrations. Sequencing of the N-terminus of the purified methyltransferase enzyme (Hashizume et al. 2001b) enabled the identification of a grape cDNA that encodes this enzyme (Boss et al. 2008). While this gene is yet to be functionally characterized, a number of results imply that this gene is involved in the pathway of MP synthesis. The peak of expression of this gene during development of Cabernet Sauvignon berries correlates well with the peak of IBMP accumulation.

It was also shown that the expression of this gene is higher in cooler conditions that in warmer conditions (Boss et al. 2008), which supports that vines grown in cool climates produce grapes with greater levels of MP than vines from warmer climates. An understanding of the biosynthesis of methoxypyrazi-

nes in grape berries will enable the development of biotechnological or conventional breeding strategies to manipulate this trait in grape varieties or to develop management regimes to control its accumulation in fruit.

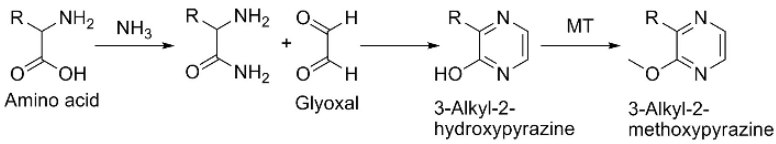

Fig. 13. Proposed biogenesis pathway of methoxypyrazines.

8. CONCLUDING REMARKS

This chapter has summarized our knowledge of four groups of grape-derived compounds found in wines made from most varieties, and two groups of compounds that are considered important to the sensory character of a few varieties. In general, our understanding of the biosynthesis of flavour and aroma compounds from grapes that contribute to wine flavour is minimal. Nevertheless, the progress that is being made in other plant species in the elucidation of pathways leading to the production of plant volatiles (Dudareva et al. 2006), combined with the availability of the grapevine genome sequence (Jaillon et al. 2007), will hasten grape flavour research in the future. However, in some cases convergent evolution has meant that different plant species produce the same compounds from different precursors and orthologous genes may not necessarily show sequence homology across species (Pichersky et al. 2006). Furthermore, many genes involved in secondary metabolism are members of large gene families. Therefore the major challenge facing pathway elucidation in grapes involves the need to functionally characterize candidate genes. The absence of an efficient grapevine transformation technique or rapid genetic system makes this difficult. While appropriate grapevine molecular genetic tools are developed, heterologous expression systems will be necessary, and although not perfect, they are often well established.

Our knowledge about the various impact odorants and interactions that occur in wine to produce the final sensory qualities the consumer enjoys is improving, but many questions remain unanswered. This is mainly due to the complexity of the system and the potential for hundreds of compounds to play a role in imparting a sensory impact. Silencing of certain pathways may eventually contribute to the understanding of the sensory attributes assigned to certain compounds if appropriate transgenic plants are produced. The discovery of

genes involved in the production of certain compounds will also aid our understanding of the effect that environmental, viticultural and genetic variables have on flavour development in berries. Again our current knowledge is limited and often leads to gross generalizations that may not be true in all varieties and for each important impact odorant. The combination of chemistry (both organic and analytical), biochemistry and molecular genetics will provide tools to develop strategies to manage wine flavour in the vineyard.

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12

PHYSIOLOGY & MOLECULAR BIOLOGY OF GRAPEVINE STILBENES: An Update

L. Bavaresco[1], C. Fregoni[1], M.I. van Zeller de Macedo Basto Gonçalves[1] & S. Vezzulli [2]

[1]Istituto di Frutti-Viticoltura, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84 29100 Piacenza, ITALY

[2]IASMA Research Center, Via E. Mach 1 38010 San Michele all'Adige (TN), ITALY

1. INTRODUCTION

Stilbenes are naturally-occurring compounds found in a large number of plant families including the *Vitaceae.* In the genus *Vitis* they are synthesized by several species, including *V. vinifera* L. which is of worldwide importance for the production of table grapes, raisins and wine. Stilbenes are low molecular weight phenolics with antifungal activity, thus enabling plants to cope with pathogen attack. Grapevine stilbenes (Fig. 1) include a number of substances, such as resveratrol (*trans*- and *cis*- isomers, 3,4',5-trihydroxystilbene, Langcake and Pryce 1977a), resveratrol glucosides including piceid (*trans*- and *cis*-resveratrol-3-O-β-D-glucopyranoside, Waterhouse and Lamuela Raventòs 1994, Mattivi et al. 1995, Romero-Pérez et al. 1999) and resveratroloside (resveratrol-4'-O-β-D-glucopyranoside), viniferins (Langcake and Pryce 1977b, Bavaresco et al. 1997, Pezet et al. 2003), pterostilbene (*trans* -3,5-dimethoxy-4'-hydroxy-stilbene, Langcake et al. 1979), piceatannol or astringinin (*trans*-3,3',4,5'-tetrahydroxystilbene, Bavaresco et al. 2002), astringin (piceatannol-3-O-β-D-glucopyranoside, Waffo Teguo et al. 1998), pallidol (*trans*-resveratrol dimmer, Landrault et al. 2002), and other resveratrol trimers and tetramers.

These compounds are induced (phytoalexins) in non-woody plant organs/tissues (leaves, flowers, berries and other non-woody organs), whereas they are constitutively synthesized in woody and hard ones (seeds). Resveratrol is the most studied and best known stilbene. It was first detected in 1940 as a root constituent in the white hellebore lily (*Veratrum grandiflorum* O. Loes) although the richest source is *Polygonum cuspidatum*, an Asian medicinal plant.

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Resveratrol is also present in wine (Siemann and Creasy 1992) and is claimed to be responsible for the positive health effects arising from wine drinking, including the reduction of risk from heart disease, from cancer and from other diseases (Aggarwal and Shishodia 2006).

An extensive review on grape stilbenes was published in the previous edition of this book (Bavaresco and Fregoni 2001). In this chapter, effort has been made to update the review, presenting the state-of the-art in the topic.

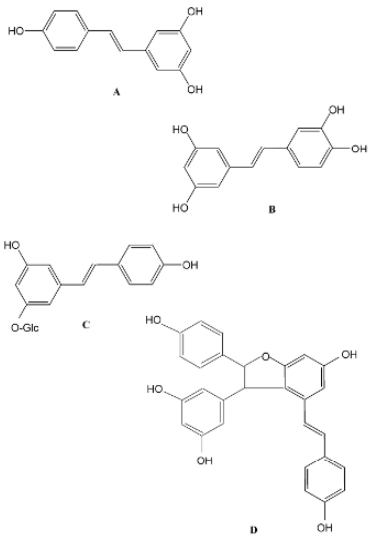

Fig. 1. Structure or *trans*-resveratrol (A), piceatannol (B), *trans*-piceid (C) and ε-viniferin (D).

2. GRAPEVINE INDUCED STILBENES (PHYTOALEXINS) AND THEIR BIOTIC AND ABIOTIC ELICITORS

The term phytoalexin (from the Greek *phyton*=plant and *alexein*=to defend) was first proposed by Müller and Börger (1940) who did an experiment on the interaction between *Phytophthora* infection and potato. A definition of phytoalexin was established by the NATO Advanced Study Institute on 'Active Defense Mechanisms in Plants' (Paxton 1981) as 'low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms'.

Subsequently, phytoalexins were defined as 'plant antibiotics synthesized *de novo* after the plant tissue has been exposed to microbial infection, and not preformed or released from pre-existing plant constituents that function as the basis of the resistance mechanism'. The new definition was necessary because

an important problem arises with the NATO concept: the obligatory role as part of the defense mechanism since in some cases this has been very difficult to prove in terms of *in vitro* activity.

To fully describe all compounds within the antibiotics classification, Mansfield (1999) introduced the term phytoanticipins, which is a term complementary to phytoalexins. The new term classifies 'low molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms, or are produced after infection solely from pre-existing precursors'. The molecules responsible for triggering plant responses are called elicitors, which can be either biotic or abiotic, according to the previous review of Bavaresco and Fregoni (2001).

It is well known that stilbenes are synthesized by a wide variety of higher-plant species, although the main human dietary sources of these compounds are grapes, wine and grape juices and, less importantly, peanuts (Sanders et al. 2000), cranberries, blueberries and other *Vaccinium* berries (Rimando et al. 2004). Review on stilbenes and their occurrence in the *Vitaceae* was presented by Bavaresco and Fregoni (2001) and updated in this Chapter.

2.1. Biotic elicitors

When grapevine is under biotic stress, stilbenes act as phytoalexins. Grey mould (*Botrytis cinerea* Pers.), downy mildew (*Plasmopara viticola* Berk. and Curt. Berl. and de Toni), powdery mildew (*Erysiphe necator* Schw. Burr.), berry rot (*Rhizopus stolonifer* Ehrenb. Fr. Lind) and some *Aspergilli* spp. are some of the main fungi that attack the grapevine and lead to the production of *trans*-resveratrol, ε-viniferin, α-viniferin, δ-viniferin, pterostilbene and piceatannol, in both the grape berries and the leaves (Table 1). Unlike disease-resistant genotypes, that can rapidly synthesize large amounts of phytoalexins, the susceptible ones synthesize these only slowly and at low concentrations.

2.1.1. *Botrytis cinerea*

The interaction between grey mould and stilbene synthesis/degradation was widely studied since 1976 (Bavaresco and Fregoni 2001). Recently Montero et al. (2003) assessed *trans*-resveratrol and ethylene levels after infection with *Botrytis cinerea*. In non-infected grapes the two compounds exhibited an inverse pattern, high content in *trans*-resveratrol and low content of ethylene; however, 48 h post-infection, ethylene increased whilst *trans*-resveratrol content decreased irreversibly.

2.1.2. *Plasmopara viticola*

Since 1981, the role of downy mildew infection on stilbene synthesis has been extensively investigated (Bavaresco and Fregoni 2001). A more recent study accounted for a resveratrol dehydrodimer, the *δ*-viniferin that along with *ε*-viniferin should be the major dimer to be synthesized under stress from *Plasmopara viticola* (Pezet et al. 2003, Gindro et al. 2006). According to Pezet et al. (2004b) resistance is associated with the conversion of resveratrol to viniferins, while susceptibility is associated with the formation of piceid from resveratrol. It seems therefore that host metabolism of resveratrol may play an important role in resistance or susceptibility.

Not all stilbenes are equally toxic to *Plasmopara viticola* zoospores. Pterostilbene and *δ*-viniferin were found to be the most fungitoxic stilbenic metabolites against zoospore mobility, with approximately the same potential even though pterostilbene is usually absent or in too low concentration to be effective (Pezet et al. 2004a). Resveratrol is not very toxic due to its hydrophilic characteristics and piceid has never been shown to exert any toxic activity against *Plasmopara viticola* zoospores (Pezet et al. 2004a, b).

Table 1. Stilbenic compounds induced in *Vitis* spp. organs by biotic elicitors (cited literature).

Organ	Elicitor	Stilbenic compound	Content (μg gfw^{-1})
Leaves	*Botrytis cinerea*	*trans*-Resveratrol	4.8 – 9.4
		α-Viniferin	26.7 – 72.4
		ε-Viniferin	9.6 – 15.8
	Plasmopara viticola	*trans*-Resveratrol	10.0
		δ-Viniferin	156.0
		ε-Viniferin	100.0
		trans-Pterostilbene	22.0
	Bacillus sp.	*trans*-Resveratrol	31.1 – 78.3
Berry skins	*Botrytis cinerea*	*trans*-Resveratrol	4.0 – 7.0
Fruit flesh	*Botrytis cinerea*	*trans*-Resveratrol	<0.10
Unseeded berries	*Botrytis cinerea*	*trans*-Resveratrol	1.3 – 6.6
		ε-Viniferin	2.3 – 10.3
		trans-Pterostilbene	0.1 – 0.2
	Aspergillus spp	*trans*-Resveratrol	1.4 – 4.3
		Piceatannol	< 0.3
Whole berries	*Rhizopus stolonifer*	*trans*-Resveratrol	18
		trans-Pterostilbene	15

2.1.3. *Erysiphe necator, Phomopsis viticola, Rhizopus stolonifer*

No recent findings are available in the literature, following the review by Bavaresco and Fregoni (2001).

2.1.4. *Trichoderma viride*

T. viride is not only a fungus but it also acts as a bio fungicide and so can be used to control other plant pathogenic fungi. Thus, in a suspension-cell culture trial, grapevine cells can respond to *T. viride* as an elicitor with the result that induction and accumulation of resveratrol occur (Calderon et al. 1993). In this trial, pterosilbene was not found and ε-viniferin was detected only in trace amounts.

2.1.5. *Aspergillus* spp.

Recent trials (Bavaresco et al. 2003a) have shown that fungi producing ochratoxin A such as *A. carbonarius, A. japonicus and A. ochraceus*, can elicit *trans*-resveratrol synthesis in grape berries, whereas *trans*-piceid is not affected. Only *A. ochraceus* elicits piceatannol significantly. A positive correlation between ochratoxin A and *trans*-resveratrol synthesis was also found. *Trans*-resveratrol and piceatannol both showed fungicidal activity against *A. carbonarius*, being able to completely inhibit fungal growth at concentrations of 300 μg gfw $^{-1}$ and 20 μg gfw $^{-1}$ respectively.

Novel experiments have coupled the study of *A. carbonarius* inoculation with soil lime content (see Paragraph 2.3.3. below).

2.1.6. Esca complex fungi

Esca is a complex disease of grapevines involving several pathogens, the main ones isolated from an esca necrosis are: *Phaeomoniella chlamydospora, Phaeoacremonium aleophilum, Phaeoacremonium angustius, Eutypa lata, Fomitiporia punctata and Stereum hirsutum*. In recent trials, resveratrol levels of up to 100 μg gfw^{-1} reduced mycelial growth of *S. hirsutum* and, to some extent also of *F. punctata* (Mazzullo et al. 2000). No effects were observed on the other pathogens. Pterostilbene inhibited mycelial growth of all the fungi tested and the inhibition increased with the amount of pterostilbene used. Mixtures of these compounds with phosphorous acid reduced mycelial growth in all types of fungi.

Other trials (Santos et al. 2006) conducted on *V. vinifera* plants inoculated with *Phaeoacremonium angustius* and *Phaeomoniella chlamydospora* to which resveratrol was added showed that resveratrol at concentrations equal to or greater than 867 mM inhibited colony growth of both species and lead to reduced chlorosis symptoms.

There is not yet any evidence that plants produce stilbenes as a defense reaction when attacked by the esca complex disease.

2.1.7. Bacteria

No recent findings are available in the literature following the review by Bavarersco and Fregoni (2001).

2.1.8. Laminarin

Laminarin is a polysaccharide that on hydrolysis yields only glucose. It is produced as an energy storage compound by *Laminarina* and other brown algae. When used as an elicitor in grapevine (cv Gamay) cells (Aziz et al. 2003) it leads to a peak of 65 μg gfw^{-1} of resveratrol in the extracellular medium after 8 h. After 20 h, there is a peak of *ε*-viniferin production that reaches 130 μg gfw^{-1} in the cells although a significant amount can also be found in the extracellular medium, peaking at 62 μg gfw^{-1} after 12 h.

When compared to the *Botrytis cinerea* infected controls, treatment with 1 g l^{-1} of laminarin reduced the diameter of the lesions by 55%. In the *Plasmopara viticola* (downy mildew) infected controls, treatment with 0.5 g l^{-1} of laminarin lead to a 75% reduction in lesion diameter.

2.2. Abiotic elicitors

In addition to biotic elicitors (elicitors caused or produced by living organisms), phytoalexin production in grapevines can also be triggered by abiotic elicitors, that is by chemical or physical factors (Table 2).

2.2.1. UV-irradiation

The positive role of UV-irradiation in triggering stilbene synthesis has been widely investigated in the past, from 1977 on, as reported by Bavaresco and Fregoni (2001). More recently stilbenes (especially *trans*-resveratrol and *trans*-*δ*-viniferin) have been identified in cv Chasselas leaves (Pezet et al. 2003), and *trans*-resveratrol has been detected in cv Cabernet Sauvignon berries (Larronde et al. 2003), UV-C irradiated.

The role of postharvest UV irradiation in enhancing the stilbene content of table and wine grapes has been widely investigated by Cantos et al. (2000, 2001, 2002, 2003). Trials conducted by Bonomelli et al. (2004) using Chardonnay and Rupestris du Lot, led to resveratrol accumulation after elicitation with UV-irradiation although the time courses and the accumulation levels were different. Chardonnay accumulated nearly 400 μg gfw^{-1} while Rupestris du Lot accumulated a maximum of 800 μg gfw^{-1}. Resveratrol disappeared three days after exposure in Chardonnay and six days after exposure in Rupestris du Lot. It is possible that in Chardonnay resveratrol is more rapidly glycosylated into piceid or oxidized into viniferins.

Table 2. Stilbenic compounds induced in *Vitis* spp.organs by abiotic elicitors (cited literature).

Organ	Elicitor	Stilbenic compound	Content (µg gfw^{-1} if not differently indicated)
Leaves	UV irradiation	*trans*-Resveratrol	50-800
		α-Viniferin	20
		ε- Viniferin	15
		β-Viniferin	9
		γ-Viniferin	80
		δ-Viniferin	166
		trans-Pterostilbene	9
	Aluminium chloride	*trans*-Resveratrol	170-350
	Fosetyl-Al	*trans*-Resveratrol	4.8
		ε-Viniferin	4.1-12.6
	MeJA	*trans*-Resveratrol	74-176 nmol gdw^{-1}
		trans-Piceid	244-1200 nmol gdw^{-1}
		cis-Piceid	106 nmol gdw^{-1}
		ε- Viniferin	80 nmol gdw^{-1}
		δ-Viniferin	8 nmol gdw^{-1}
		Pterostilbene	11 nmol gdw^{-1}
	Chitosan oligomers	*trans*-Resveratrol	1-15
		cis-Resveratrol	2-13
		trans –ε-Viniferin	4-38
		cis-ε-Viniferin	<8
		trans-Piceid	5-28
		cis-Piceid	2-14
Grape skins	UV irradiation	*trans*-Resveratrol	0.3-15.8 µg $.cm^{-2}$
		trans-Resveratrol	50-115
	Benzothiadiazole	*trans*-Resveratrol	0.5
		cis-Resveratrol	0.1
	Salicylic acid	Resveratrol	4.5-8.2
	Anoxic treatments	*trans*-Resveratrol	13- 37
	ABA	*trans*-Resveratrol	3.3
Fruit flesh	UV irradiation	*trans*-Resveratrol	1-3
Whole berries	UV irradiation	*trans*-Resveratrol	10-1165 nmol gdw^{-1}
		trans-Resveratrol	8-23
		trans-Piceid	1-3
		Piceatannol	0.5-1.7
		Total viniferins	1.2-11.0
	Ozone	*trans*-Resveratrol	13-22
		trans-Pterostilbene	1.5
		Total stilbenoids	29
	MeJA	*trans*-Resveratrol	26 nmol gdw^{-1}
Unseeded Berries	MeJA	*trans*-Resveratrol	<0.3
		ε-Viniferin	<0.2

Li et al. (2008) conducted a trial on the differences between UV-B (280-315 nm) and UV-C (<280 nm). Using detached berries of three grape varieties, Takasuma, Tano red and Carignane, analysis of the skins revealed that the effects of UV-C irradiation on resveratrol inducement were much stronger than

with UV-B.

Also, rapid methods are being developed to study stilbene synthesis. One of these (Poutaraud et al. 2007) is based on *in vivo* fluorescence using a commercial spectrofluorometer. In leaves of *V. vinifera* cv Muscat Ottonel, stilbene synthesis was induced through UV-irradiation; fluorescence was measured on both sides of the leaves and stilbene content was determined by HPLC. Highly significant regressions were found between HPLC measurements of stilbene and an induced blue fluorescence, indicating that *in vivo* fluorescence is probably an excellent method for the study of stilbene synthesis.

2.2.2. Aluminium chloride, ozone, fosetyl-Al

No recent findings concerning the positive effect of aluminium chloride on stilbene synthesis are available in the literature, after Bavaresco and Fregoni (2001). Previous trials (till 2001) on the effect of ozone and fosetyl-Al in promoting stilbene synthesis are reported by Bavarersco and Fregoni (2001).

More recently, other trials using post-harvest ozone treatments (Artés-Hernández et al. 2003) have revealed an increase after storage of all stilbenoids. Treatments involving 8 ppm ozone increased both the resveratrol and the piceid contents.

Other authors (González-Barrio et al. 2006) tested different post-harvest treatments with ozone concentrations ranging from 3.88 to 1.67 g h^{-1} and different exposure times of 1, 3 and 5 h. Stilbene production was enhanced during storage at 22°C and 95% relative humidity and a maximum level was reached two days later. Ozone treatments seem to be more effective than UV-treatments as they also lead to viniferin accumulation. A sequence in biosynthesis was observed, starting with the resveratrol monomer, continuing with ε-viniferin and δ-viniferin, and ending with four different resveratrol dehydrotrimers.

Other trials (Qi and Creasy 2005) showed that in detached leaves, the combined effect of fosetyl-Al (aluminium ethyl phosphite) and UV-irradiation increased the amount of phytoalexins produced, but that fosetyl-Al alone had no effect on resveratrol content. On the other hand, when treating leaves that were still attached to the vine, a direct effect could be seen on resveratrol concentration only with fosetyl-Al.

2.2.3. Methyl jasmonate

Some experiments (Larronde et al. 2003) have shown that very low levels of atmospheric methyl jasmonate (MeJA) can enhance stilbenes in leaves and berries, though there are differences in levels between these organs. In berries treated 15 days after véraison there was an increase in the content of *trans*-resveratrol whereas treatments imposed after 30 days had no effect. It seems that maturing grape berries lose their ability to respond to MeJA. In leaves,

trans-resveratrol enhancement was independent of the phenological phase but the main compound induced was *trans*-piceid. Moreover, UV treatments of leaves from MeJa pre-treated plants resulted in a greatly increased *trans*-resveratrol accumulation and to an increased *trans*-piceid content. This suggests that *trans*-resveratrol is produced by direct stimulation of stilbene synthase and not by piceid deglycosylation.

In other trials (Belhadj et al. 2006) with *V. vinifera* cv Cabernet Sauvignon, only *trans*-piceid could be found in control leaves while in leaves treated with MeJA all types of phytoalexins were found. Twelve hours post-treatment *trans*-piceid was induced and it reached a plateau of 244 nmol gdw^{-1} at about 48 h; a similar trend was found for *trans*-resveratrol but at lower concentrations, reaching 176 nmol gdw^{-1} at 48 h. ε-Viniferin started to accumulate after 18 h until it reached 80 nmol gdw^{-1} and decreased thereafter to 25 nmol gdw^{-1}. Pterosilbene and δ-viniferin both peaked at 18 h and later decreased to 3 nmol gdw^{-1}. In cuttings, and also in the vineyard (cv Merlot), pretreatment with MeJA induced a strong reduction (75% and 73%, respectively, compared to the control) in the severity of powdery mildew infection; MeJA also upregulates PAL and STS gene induction leading to increased production of stilbenes. The activation of the biosynthesis pathway is an important resistance reaction.

According to Tassoni et al. (2005) MeJA was highly effective in stimulating resveratrol endogenous accumulation and release into the culture medium in Barbera cell cultures.

Recently, Vezzulli et al. (2007a) sprayed with MeJA solution at fruit set, at véraison, and at ripening clusters of *V. vinifera* cv Barbera grafted on 3309 C rootstock. Cumulative MeJA treatments significantly increased berry resveratrol and -viniferin at ripening.

2.2.4. Benzothiadiazole

Benzothiadiazole (BTH) is a functional analogue of the endogenous hormone-like salicylic acid and along with its derivates it is completely translocated and degraded in plant tissues. Pre-harvest treatments enhanced both *trans*- and *cis*-resveratrol in grape berry skin post-harvest (Iriti et al. 2004). The *trans*-isomer concentration was higher than the *cis*-isomer and over 40% higher than in the controls. BTH simultaneously enhanced production of anthocyanins, proanthocyanidin and stilbenes probably due to a combined action of PAL induction and the timing of the treatments (Iriti et al. 2005). Its action seems to reduce substrate competition between CHS and STS and so the metabolic switch between the branches of the pathway seems to be avoided.

2.2.5. Chitosan oligomers

In a recent study, Aziz et al. (2006) reported that chitosan oligomers with

different molecular weights and degrees of acetylation could trigger accumulation of *trans*-resveratrol, *cis*-resveratrol, ε-viniferin and piceids in grapevine leaves. The amounts of the phytoalexins elicited were directly dependent on the molecular weight and degree of acetylation of the oligomers. Furthermore, not only the combination of the chitosan oligomers with $CuSO_4$ did increase phytoalexin production, but also $CuSO_4$ was, for the first time, shown to be able alone to induce their production acting. Concentrations above 10 μg ml $^{-1}$ strongly promoted the induction of *cis*-resveratrol and *cis*-ε-viniferin.

Moreover, the eliciting activity of chitosan oligomers in grapevine leaves is not associated with cell death and it can also prevent cell death from $CuSO_4$ treatment.

2.2.6. Salicylic acid

Recently, Li et al. (2008) tested salicylic acid (SA) as an elicitor of resveratrol synthesis. SA is a low molecular weight phenolic molecule that signals adversities to the plants. Results showed that spraying detached grape berries with 100 mg l $^{-1}$ SA enhanced their resveratrol contents even though its effects varied somewhat from variety to variety; the stronger effect was found in the Tano red variety when compared to cvs Takasuma or Carignane. After 24 h the content of resveratrol in Tano was 8.21 μg gfw^{-1}. This was 13.6-fold higher than in the controls. Control samples of cvs Takasuma and Carignane had higher values than Tano control samples, but 24 h after SA spraying, their contents were increased over the controls by only 1.62-fold in Takasuma and 2.77-fold in Carignane.

2.2.7. Anoxic treatments

Short anoxic treatments (berries placed in a vacuum chamber filled with nitrogen gas at room temperature for 6 to 24 h) led to differences in stilbene synthesis. Anoxic treatment for 6 and 15 h greatly enhanced the amounts of resveratrol in the berries and no organoleptic damage could be detected. Treatments for 24 to 48 h led to considerable berry damage and to a rapid decrease in stilbene levels, when normal oxygen levels (21%) were restored. No organoleptic differences could be detected in the wines produced with 6 to 15 h anoxic-treated berries. These anoxic treatments also improved post-harvest quality and berry health (Jiménez et al. 2007)

2.2.8. Other chemicals

Following the review of Bavaresco and Fregoni (2001), more recent experiments have shown that dimethyl-β-cyclodextrins induced high stilbene synthesis in cell cultures of Pinot noir and Merzling (Zamboni et al. 2006). Leaf

sprays with sucrose-phosphate enhanced resveratrol synthesis in cvs Barbera and Pinot noir, as also did a mixture of amino acids (Vercesi and Presutto 2004).

Abscisic acid (ABA) also exerted elicitor activity on Kyoho grapes, enhancing the resveratrol concentration over the controls by about 1.2-fold (Ban et al. 2000). Finally, metal ions such as lanthanum, europium, calcium, silver and cadmium can also induce phytoalexin biosynthesis in plant cell cultures (Radma et al. 2003).

2.3. Viticultural factors affecting stilbene synthesis

Stilbene synthesis in both the leaf or the berry can be triggered by several biotic and abiotic factors, which are directly related to stilbene accumulation, as discussed in the previous sections. There are also a number of viticultural practices, that interact with the elicitors, so as to result in a wide range of stilbene variations. Some of these are well documented in the literature. Viticultural factors include variety, rootstock, geographical location, meteorological conditions, fungal infection pressure and cultural practices. An understanding of the different roles of these factors is crucial because, only with this information, it will be possible to develop cultural practices aimed at improving stilbenic contents in the plants and in the derived products.

2.3.1. Grape variety and rootstock

The effect of the grape variety has not yet been thoroughly investigated because most literature refers to resveratrol concentrations in varietal wines of different vintages. If only the wines are considered, then the resveratrol values detected may have as much to do with enological practices as well with variety, cultural practices and growing environment.

According to some authors (Soleas et al. 1995b, Goldberg et al. 1995, 1996, Sato et al. 1997, Eder et al. 2001) the best resveratrol-producing cultivars are Pinot noir and Cabernet Sauvignon, but this is not always the case (Bavaresco 2003). Okuda and Yokotsuka (1996) studied 33 grape cultivars grown in Japan and found significant effect of genotype on resveratrol. Berry contents ranged from a low of 0.06 μg gfw^{-1} in Pizzutello bianco berries to a high of 1.76 μg gfw^{-1} in Müller Thurgau berries.

According to Ector et al. (1996), seeded berries of muscadine grapes (*Vitis rotundifolia*) contained resveratrol concentrations of 6.39 μg gfw^{-1} (in bronze-skinned fruit) and 12.26 μg gfw^{-1} (in dark-skinned fruit). Unlike other studies, these authors also found resveratrol in the fleshy part of the berries. According to Pezet and Pont (1988a, b) pterostilbene in grape berries ranged from 0.074 μg gfw^{-1} in Gamay to 0.530 μg gfw^{-1} in Pinot noir. Resveratrol and

piceid were found in berry skins of various *Vitis* genotypes (Waterhouse and Lamuela-Raventos 1994) ranging between 9.3 to 78.5 μg gdw^{-1} and 2.8 to 187 μg gdw^{-1} for resveratrol and piceid, respectively.

Studies conducted on 120 grape germplasm cultivars of *Vitis* for two consecutive years showed that berries of interspecific rootstock cultivars had very high levels of extractable resveratrol in their skins (<210 μg gfw^{-1}). The various genotypes of *V. riparia* tested usually contained high levels of extractable resveratrol, whereas most genotypes of *V. vinifera* and their hybrids with *V. labrusca* usually contained relatively low levels (<2 μg gfw^{-1} in skin). This study also found higher levels of extractable resveratrol in seeded varieties than in the seedless ones and higher levels in the flesh and skin of wine varieties than in table varieties (Li et al. 2006).

As a general rule, the red grape cultivars contain higher levels of stilbenes than the white cultivars. Thus, Romero-Pérez et al. (1999) found differences in piceid concentration in red and white commercial grape juices. The average concentration in the red juices was 3.38 mg l^{-1} for *trans*-piceid and 0.79 mg l^{-1} for *cis*-piceid, while in white juices the average was 0.18 mg l^{-1} for *trans*-piceid and 0.26 mg $l^{-1}$for *cis*-piceid. Also, Bavaresco et al. (2007b) found higher resveratrol and piceid concentrations in berries of Barbera and Croatina (red) than in Malvasia di Candia aromatica (white). Sun et al. (2006) compared three red cultivars, Castelão, Syrah and Tinta Roriz and showed that the skins did not have significantly different *trans*-resveratrol contents but did have very different levels of *trans*-piceid (67.2, 10.4 and 11.6 μg gdw^{-1}, respectively). *cis*-Piceid, was found only in Castelão at high levels (58.9 μg gdw^{-1}).

Furthermore, we would expect that grape berries from disease-resistant genotypes (interspecific hybrids and *Vitis* species other than *V. vinifera*) would have higher levels of stilbenes than the disease-susceptible ones. In some cases this has been confirmed (Creasy and Coffee 1988, Jeandet et al. 1991, Bavaresco et al. 1997a), while in others it has not (Soleas et al. 1995a, b). A number of grape juices pressed in south-eastern Ontario were analyzed for *trans*-resveratrol (Soleas et al. 1995a), but very low concentrations were found. Values ranged from 3 μg l^{-1} in Muscat and Seyval Blanc to 15 μg l^{-1} in Riesling, while much higher quantities were found in the skins of the same cultivars, ranging from 0.3 μg gfw^{-1} in Niagara to 5.64 μg gfw^{-1} in Merlot.

For wine, the literature is contradictory regarding the relationship between stilbene level and disease resistance/susceptibility. According to Romero-Perez et al. (1999), resveratrol and piceid levels in wine from the Spanish *V. vinifera* cultivars tested correlated well with their known resistance/susceptibility to fungal disease. Also, Lamikanra et al. (1996) detected higher resveratrol concentrations in wines from *V. rotundifolia* (a disease-resistant species) than in those from *V. vinifera* varieties (generally having low disease resistance). On the other hand, according to Soleas et al. (1995b) and Eder et al. (2001) resvera-

trol content was higher in the wines from *V. vinifera* cultivars than in those from interspecific hybrids. In some other cases, differences between resveratrol concentration of wines from *V. vinifera* cultivars and from interspecific hybrids were not significant (Korbuly et al. 1998). It is difficult to explain these contradictory results but most likely factors other than just the cultivar are important and these were not measured.

The effect of rootstock has not been much investigated and no data are available after the review of Bavaresco and Fregoni (2001).

2.3.2. Climate

The effect of climate seems to be crucial. Climate should take into account the geographical location of the vineyard (especially latitude and elevation) and the seasonal meteorological variability in the area.

For the latitude effect, only wine comparisons have been available and even for this, the literature does not give a clear or consistent indication. According to Goldberg et al. (1995, 1996), wines (especially Cabernet Sauvignon) produced in cool-climate regions contain higher levels of resveratrol than the same wines from warmer regions. However, in another survey by the same authors (Goldberg et al. 1999), this observation was not supported, suggesting inconsistency in the relationship of wine stilbene level to climate. This apparent contradiction can possibly be explained if other factors are considered that may interfere with the final result. Differences in enological practice are especially likely.

The seasonal meteorological conditions, especially temperature, rainfall and relative humidity during the last month before harvest, all affect stilbene synthesis, because they all relate to fungal-disease pressure (Jeandet et al. 1995, Martinez-Ortega et al. 2000). It is well known that grey mould (*Botrytis cinerea*) is an important stilbene elicitor. To trigger stilbene synthesis in the berry, it is necessary to have at least a low level of fungal pressure during ripening (perhaps undetectable to the naked eye) and this can occur under moderate humidities. Dry conditions during the last stages of ripening are generally unfavourable to stilbene synthesis, because there is little fungal (grey mould) pressure.

According to Li et al. (2006), rainfall during late berry development usually results in higher levels of extractable resveratrol in the skin, whereas the content in the seeds is not so strongly related to meteorological factors. Bavaresco et al. (2007b) carried out a trial in Piacenza (northern Italy) investigating the role of the season's meteorological conditions and vineyard elevation on the stilbene contents of ripe grapes. The concentration of *cis*-piceid was positively correlated to relative humidity during ripening and negatively to temperature (growing degree days). Elevation also seemed to affect resveratrol and piceid levels in the berries, increasing from 150 to 320 m a.s.l. and decreasing at

420 m a.s.l.

Drought stresses imposed on Barbera grapes did not induce any significant changes in stilbene concentrations in the ripe berries (Vezzulli et al. 2007a).

2.3.3. Soil

There is very little experimental evidence that relates to the effect of soil on stilbene synthesis. The soil could have an indirect role by affecting the mineral nutrition of the plant. Adrian et al. (2000) found no effect of "terroir" on stilbene concentration in Pinot noir from Burgundy. The effect of soil lime on berry stilbene concentration was investigated by Bavaresco et al. (2005) on Merlot grafted on 3309 C rootstock. Besides inducing the typical chlorosis symptoms in the leaves, the calcareous soil significantly enhanced the stilbene content in ripe berries compared to control vines growing in a neutral soil. The calcareous soil still showed a positive effect on berry stilbene content, even when grapes were infected by *A. carbonarius* (Bavaresco et al. 2008b).

According to Bavaresco et al. (2003b) a calcareous soil can increase the concentration of *trans*-piceid in young roots of various hybrid rootstocks (*V. berlandieri* x *V. riparia* 420 A, Kober 5BB and SO_4, *V. berlandieri* x *V. rupestris* 1103 P and 140 Ru, *V. vinifera* x *V. berlandieri* 41B). The effect of soil type on the stilbene content of wines was also studied in two cv Grenache vineyards (Andrés-de Prado et al. 2007). The vineyard having the most fertile soil and the greatest water-holding capacity produced wine with a lower total phenolic content and a lower color intensity but with a higher stilbene concentration.

2.3.4. Cultural practices

Fertilizer supply is an important factor interacting with the physiology of the plant, including its disease resistance. Nitrogen is the most reactive mineral element being easily taken up by the plant which reacts rapidly and strongly to changes in nitrogen nutrition and metabolism. According to Graham (1983), under nitrogen-limiting conditions, the balance between primary and secondary metabolic pathways is probably shifted to the shikimate pathway, thus enhancing polyphenol (including the stilbenes) and alkaloid synthesis.

Low, compared to high rates of soil nitrogen increase resveratrol concentrations in the berries of potted and of field-grown grapevines (Bavaresco et al. 2001, 2007a). The effects of potassium supply have been already described in the previous review (Bavaresco and Fregoni 2001).

Copper spray treatments against fungal diseases (e.g. downy mildew, grey mould) increased stilbene levels in grapes and wines compared with unsprayed plants (Coulomb et al. 1999). The comparison between the effects of copper and the alternative synthetic sprays against downy mildew gave contra-

dictory results. According to Albert et al. (2002) chemicals other than copper increased resveratrol concentrations in Mourvèdre wine compared with levels in the copper treatment. On the other hand, French organic wines (these allow only the use of copper sprays against downy mildew) showed higher resveratrol levels than did non-organic wines where less copper, or presumably other chemical spray products may have been used (Tinttunen and Lehtonen 2001).

The intensity of winter pruning did not affect resveratrol concentrations in grapes from the Valpolicella region in northeast Italy (Celotti et al. 1998).

According to Threlfall and Morris (1996) and Bertamini and Mattivi (1999), the training system had an effect on resveratrol levels in wines of *V. aestivalis* Michx. cv Cynthiana and of *V. vinifera* L. cv Cabernet Sauvignon. This is likely to be a cluster microclimate effect (different levels of cluster shading). However, cluster shading had no effect on the concentrations of resveratrol in Kyoho grapes (Ban et al. 2000). On the other hand, low cluster insulation was favourable to resveratrol synthesis in warm years but the opposite was true in cool years (Bertamini and Mattivi 1999).

Leaf removal at véraison increased *trans*-piceid grape concentration in cv Barbera and decreased *trans*-resveratrol and *cis*-piceid in the cvs Croatina and Malvasia di Candia aromatica, under cool meteorological conditions; leaf removal had no effect on grape stilbene content under warmer and drier climatic conditions, according to a 4-year trial carried out in Piacenza viticultural area (Bavaresco et al. 2008a). Finally, cluster thinning (crop load adjustment) on the interspecific hybrid Chambourcin increased the berry content of resveratrol (Prajitna et al. 2007).

3. GRAPEVINE CONSTITUTIVE STILBENES

Grapevine constitutive stilbenes are present in lignified organs, such as stems and canes, in seeds, in roots, and in ripe cluster stems; the concentrations of most of the above mentioned stilbenes are reported in Bavaresco and Fregoni (2001). Thereafter, Li et al. (2006) reported that the highest resveratrol concentration (28 μg gfw $^{-1}$) was recorded in the seeds of an interspecific rootstock.

4. MOLECULAR AND BIOTECHNOLOGICAL ASPECTS OF STILBENE SYNTHESIS IN GRAPEVINES

4.1. Grapevine stilbene synthesis

Following the data reviewed by Bavaresco and Fregoni (2001), Hou et al. (2002) obtained genomic clones of three different stilbene synthase (*StSy*) genes

and one pseudogene from the cv Norton, derived from *V. aestivalis* and also three new partial clones of *StSy* gene have been recently identified (Richter et al. 2006a). Many studies concerning *StSy* gene expression under different abiotic (Bais et al. 2000, Versari et al. 2001, Borie et al. 2004) and biotic (Kortekamp 2006, Vezzulli et al. 2007b) stress conditions have been undertaken. Moreover, recent genome-wide transcriptome analyses have reported on the expression modulation of *StSy* genes (Fung et al. 2008, Polesani et al. 2008).

Recently, two genome-sequencing projects resolved the entire DNA sequence of grapevine, the first fleshy fruit plant to have its genome deciphered. The analysis of the heterozygous Pinot noir (clone ENTAV 115) genome predicted at least 21 *StSy* copies (Velasco et al. 2007). This number agrees well with a recent *StSy* sequence analysis in infected grape leaves (Richter et al. 2006a), but it differs from the 43 predicted in the PN40024 grape genome sequence (Jaillon et al. 2007). In Velasco et al. (2007) most of these copies, as well as most *PAL* genes, were clustered in the chromosome 16. Furthermore, several peroxidase genes were predicted, some of which could participate in the formation of viniferins, as previously suggested (Jeandet et al. 2002). Recently, a resveratrol glucosyltransferase, putatively involved in piceid synthesis has been isolated and biochemically characterized from *V. labrusca* grape berries (Hall and De Luca 2007). The genome analysis revealed that its homolog in Pinot noir (99% sequence similarity) is present as a single gene, mapping on chromosome 3.

4.2. Transferring StSy genes

The enzyme StSy has been the target of several investigations aiming to transfer the gene encoding for this protein to plants lacking this genetic information, in order to confer increased disease resistance. The introduction of a single gene is sufficient to synthesize resveratrol in heterologous plant species because StSy uses as substrates, precursor molecules that are already present throughout the plant kingdom. Following the first transformation and regeneration of transgenic monocots and dicots, reviewed by Bavaresco and Fregoni (2001), other dicots followed such as alfalfa (Hipskind and Paiva 2000) and papaya (Zhu et al. 2004). In all cases, transgenic plants expressing the grapevine gene *StSy* exhibited increased resistance to pathogens. Other authors (Fettig and Hess 1999, Liang et al. 2000) reported on the transformation of wheat plants with the *Vst1* gene, resulting in an increased resistance to *Botrytis cinerea* and to *Oidium tuckeri*.

Recently, the stable inheritance of the antifungal genes in the transgenic plants was demonstrated. Richter et al. (2006b) transformed pea (*Pisum sativum* L.) plants with two different antifungal genes encoding for polygalacturonase-

inhibiting protein (PGIP) from raspberry (*Rubus idaeus* L.) driven by a double 35S promoter, or *VST1* from grape (*V. vinifera* L.) driven by its own elicitor-inducible promoter. Transgenic lines were established and the transgenics were combined *via* conventional crossing. Resveratrol, produced by *VST1* transgenic plants, was detected using HPLC and the PGIP expression was determined in functional inhibition assays against fungal polygalacturonases.

Moreover, transformation in a homologous system was described by Coutos-Thévenot et al. (2001). In this study a chimeric gene combining an alfalfa PR10 promoter and *VST1* gene was introduced into the genome of 41B rootstock. Trangenic plants were analysed for resveratrol production in leaves infected with *Botrytis cinerea* using an *in vitro* test. Among the 50 transgenic lines analysed, some exhibited a production of resveratrol that was lower than that in the non-transgenic control but others accumulated resveratrol at levels from 5- to 100-fold higher. Moreover, in the latter clones, disease symptoms were very much reduced in response to infection. These results are a good indication that the combination of a pathogen-inducible promoter and a defense gene may indeed increase tolerance against fungi in grapevines.

While all the cases described above concerned stable transformation *via Agrobacterium tumefaciens*, a transient expression of *VST1* has been recently performed in grapevine leaves before infection with *Plasmopara viticola* (Santos-Rosa et al. 2008). The agro-infiltration itself induced the synthesis of stilbenes in grapevine leaves, thus preventing the test of the effect of the *StSy* over-expression in defense. However, these results revealed that agro-infiltration before *Plasmopara viticola* inoculation had an effect on the development of the infection.

Experiments in which plants transformed with *StSy* genes did not show increased resistance to pathogens have also been reported. In particular, *StSy* gene over-expression in tobacco caused altered flower pigmentation and male sterility, probably due to the competition between the exogenous StSy and CHS for shared substrates (Fischer et al. 1997). Various studies, regarding the expression of an introduced *StSy* gene, also revealed the accumulation of the resveratrol-glycoside piceid in kiwifruit (Kobayashi et al. 2000), in apple (Szankowski et al. 2003) and in white poplar (Giorcelli et al. 2004). Even if kiwifruit and apple did not show an increased disease resistance, they may have some beneficial effects on human health.

4.3. Grapevine breeding and fingerprinting based on molecular aspects of stilbene synthesis

Conventional grapevine breeding deals with intraspecific and interspecific crosses and with clonal selection, aimed at achieving improvement in berry

quality and in resistance to biotic and abiotic stresses (Alleweldt and Possingham 1988).

An important contribution of molecular biology to plant breeding has been the use of molecular techniques such as MAS (Marker Assisted Selection) as diagnostic tools to assist with the conventional breeding process. The approach is very promising, but no data are so far available on the MAS for disease resistance based on stilbene synthesis. Regarding fingerprinting, following the results reported by Bavarersco and Fregoni (2001), clones of Touriga Nacional (Faria et al. 2004) have been identified on the basis of *StSy* genes.

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13

BIOCHEMICAL & MOLECULAR ASPECTS OF FLAVAN-3-OL SYNTHESIS DURING BERRY DEVELOPMENT

N. Terrier, D. Ollé, C. Verriès & V. Cheynier

Unité Mixte de Recherche Sciences pour l'Œnologie, INRA-Montpellier SupAgro 2, Place Pierre Viala, F-34060 Montpellier Cedex 1, FRANCE

1. INTRODUCTION

Flavan-3-ols make up a large group of flavonoid compounds, encountered in several tissues of plants and involved in reactions against various biotic aggressions, such microbial pathogens (bacteria and fungi), insects and larger herbivores (Dixon et al. 2005). They comprise monomers (often called catechins), and oligomers and polymers, called condensed tannins or proanthocyanidins (PAC). In grapevine they are present in wood, stems, leaves, and in fruits (Boukharta et al. 1988, Souquet et al. 2000, Bogs et al. 2005, Tesnière et al. 2006). They are quantitatively the most abundant secondary metabolites of grape berries. They are extracted during winemaking and are a major qualitative factor in red wines because of their implication in colour stability, astringency and bitterness.

All flavonoids are based on a common C6-C3-C6 skeleton (Fig. 1) which consists in two phenolic rings (A and B) linked *via* an heterocyclic pyran ring (C ring). This large group is subdivided in several families based on the oxidation state of their C-ring. In flavan-3-ols, which bear one hydroxyl group in the 3 position, the C-ring is saturated and thus shows two asymmetric carbons (in C2 and C3). This opens the possibility for different stereoisomers, as detailed below. The A-ring of flavan-3-ols is generally hydroxylated in C5 and C7 and the B-ring in C4 (Fig. 1: R, R′=H, afzelechin series), but further diversity arises from the substitution pattern of the B-ring. Thus the presence of an extra hydroxyl group in 3′ (R=OH, R′=H) or two extra hydroxyl group in 3′ and 5′ (R, R′=OH) give rise to the catechin and gallocatechin series, respectively. The most abundant natural isomers show 2R configuration, the 2R, 3S (2,3 *trans*-conformation) being called (+)-afzelechin, catechin, or gallocatechin, while the

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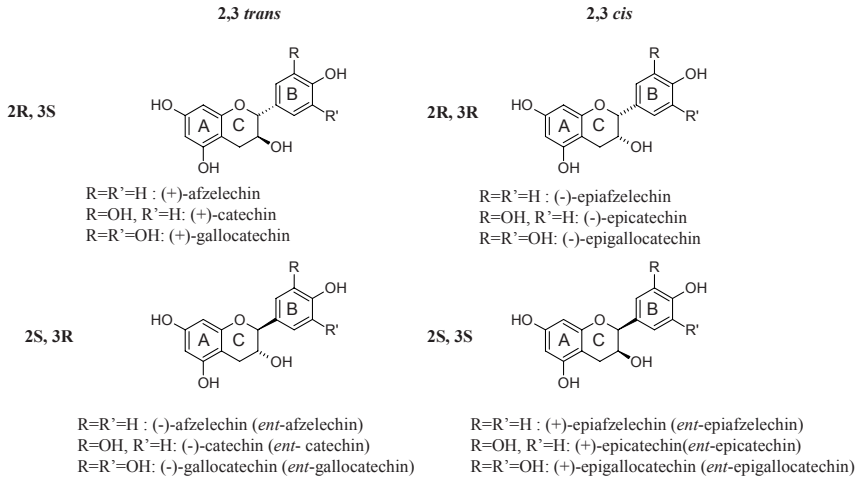


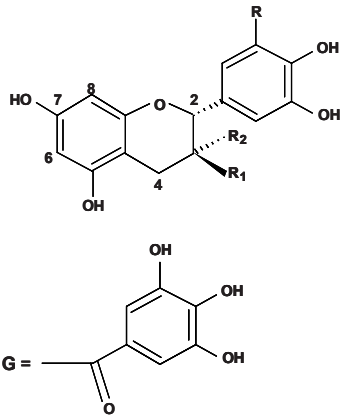
Fig. 1. Structures of major flavan-3-ol monomers.

2R,3R (2,3 *cis*-conformation) are called (-)-epiafzelechin, epicatechin, epigallocatechin. The corresponding 2S isomers, respectively (-)-afzelechin, catechin, or gallocatechin in the 2,3 *trans*- (2S,3R) series, (+)-epiafzelechin, epicatechin, epigallocatechin, in the 2,3 *cis* (2S,3S) series, can be designated with the prefix *ent*. In PAC structure, substitution in the 4 position gives rise to another asymmetric center on extension and upper units but the usual configuration is 3,4 *trans* (i.e. 3S,4S or 3R,4R).

Note that PACs are called propelargonidins, procyanidins or prodelphinidins, respectively, if their extension units (substituted in C4) are (epi)afzelechin, (epi)catechin, or (epi)gallocatechin units. Diversity is increased by the existence of gallic ester of the monomers (Fig. 2). Single linkages between constitutive flavan-3-ol units (i.e. C4-C6 or C4–C8 bonds) give rise to B-type PACs while A-type PACs show additional C2-O-C7 or C2-O-C5 bonds (Fig. 3). The trivial nomenclature often used to designate PACs (*e.g.* B1 through B8 for B-type procyanidin dimers) rapidly becomes impractical as the chain length increases. A more universal nomenclature has been proposed by Porter (1988). Using this system, B1 becomes epicatechin-(4β→8)-catechin, B2 epicatechin-(4β→8)-epicatechin, B3 catechin-(4α→8)-catechin, etc. Stereochemical differences and chiral intermediates in biosynthesis are discussed in the third section of this chapter.

Major flavan-3-ols monomers in grape are (+)-catechin, (-)-epicatechin and (-)-epicatechin 3-gallate (Fig. 2). All these units are also found in grape PACs, along with (-)-epigallocatechin and trace amounts of (+)-gallocatechin.

Grape PACs are essentially of B-type, C4-C8 linkages being much more abundant than C4-C6 bonds (Fig. 3). Beside flavan-3-ol oligomers (Ribéreau-Gayon 1964, Weinges and Pretti 1971, Cheynier and Rigaud 1986, Ricardo da Silva et al. 1991) a wide range of polymeric forms are found in grape berries and qualitative and quantitative differences depend on several factors.



(+)-catechin: R= H, R_1= OH, R_2= H
(-)-epicatechin: R= H, R_1= H, R_2= OH
(-)-epicatechin 3-gallate: R= H, R_1= H, R_2= O-G
(+)-gallocatechin: R= OH, R_1= OH, R_2= H
(-)-epigallocatechin: R= OH, R_1= H, R_2= OH
(+)-gallocatechin 3-gallate: R= OH, R_1= H, R_2= O-G
(-)-epigallocatechin 3-gallate: R= OH, R_1= O-G, R_2= H

Fig. 2. Structures of grape flavan-3-ol monomers

2. FACTORS AFFECTING FLAVAN-3-OL COMPOSITION IN GRAPEVINE

2.1. Compartment

Seed PACs consist of the above mentioned monomers except (-)-epigallocatechin and are thus partly galloylated procyanidins. B-type dimers and trimers are usually described (Ribéreau-Gayon 1964, Weinges and Pretti 1971, Cheynier and Rigaud 1986, Ricardo da Silva et al. 1991). A-type galloylated oligomers have been recently found in seeds of Chardonnay and of a mixture of red grape cultivars (Passos et al. 2007), but the reported mass signals may also correspond to fragments of larger molecular weight B-type polymers or oxidation products. In seed PACs, mean degree of polymerization from 3 (Mané et al. 2007) to 16 (Cheynier et al. 1998) have been reported while the percentage of galloylation can reach 22.6 (Verriès et al. 2008).

The content of PACs at harvest ranges from 0.5 to about 6.4 $mg.g^{-1}$ berry fresh weight in different cultivars (Cheynier et al. 1998, Souquet et al. 2006, Rodriguez et al. 2006, Mané et al. 2007). The content per berry varies from 0.33 $mg\ berry^{-1}$ in Centibel (Rodriguez et al. 2006), to 8.7 $mg.berry^{-1}$ in Muscat de Hambourg (Souquet et al. 2006). Harbertson et al. (2002) underlined that the

major factor contributing to the difference between Syrah and Cabernet Sauvignon is the number of seeds per berry.

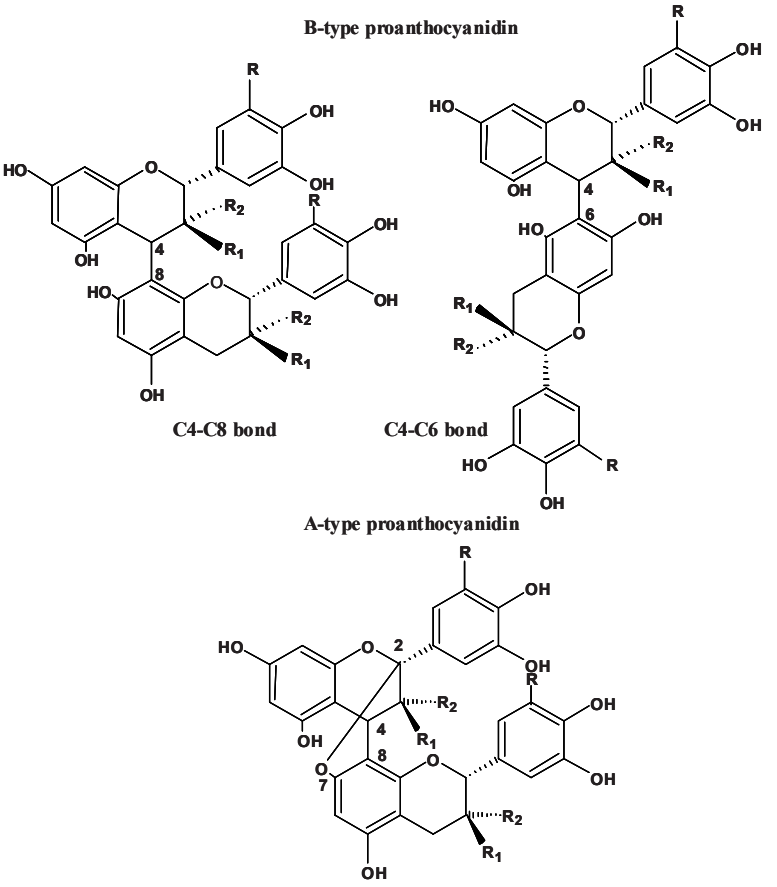


Fig. 3. Structures of B-type and A-type proanthocyanidins (for R, R1and R2 see Fig. 2).

Major constituents of skin PACs are (-)-epicatechin and (-)-epigallocatechin as extension units with a lower percentage of galloylated units (Table 1). Terminal subunits are mainly (+)-catechin and, to a lesser extent, (-)-epicatechin. The mDP values are around 20-30 but higher values (up to 50) have been found (Monagas et al. 2003, Souquet et al. 2006). Reported values of content in skin PACs at harvest are 0.58 mg.berry^{-1} for Cabernet Sauvignon, 0.54 and 0.28 mg.berry^{-1} for Pinot noir, 0.51 mg.berry^{-1} (Harbertson et al. 2002), 1.39 mg.berry^{-1} (Downey et al. 2003) and 3.3 mg.berry^{-1} for Syrah (Verriès et al. 2008). These large differences are probably due to many factors, such as culti-

var, soil, climate, etc. The influence of vintage has been pointed out by Gagné et al. (2006) who measured 6.5 and 1.5 mg.berry^{-1} at harvest in the same vineyard of Carbernet Sauvignon, in 2004 and 2006, respectively.

PACs have also been detected in the pulp of several cultivars at very low concentration and can represent a rather large proportion of the total berry content. Table 1 summarizes studies concerning PAC composition of two parts of the berry pericarp in several red cultivars. Except for Pinot noir and Pinot meunier, mDP values are higher in pulp than in skin but the percentage of epigallocatechin is lower in pulp than in skin for all cultivars.

Finally, PACs are also present in the vegetative parts of the plant. Souquet et al. (2000) found from 28 to 390 mg.(kg of fresh bunch of grape)$^{-1}$ of PACs in grape stems of different cultivars with mDP around 10 and percentage of galloylation intermediate between seeds and skins. Bogs et al. (2005) showed that PACs are present in noticeable amounts in Syrah leaves from the earlier stages of development, with calculated mDP around 10, presence of galloylated and epigallocatechin subunits and final concentration reaching 10 mg.g^{-1} of fresh weight. Tesnière et al. (2006) reported higher values of mDP, reaching 50 in Portan leaves.

Table 1. Flavan-3-ol composition of pulp and skin in several red cultivars at ripe stage.

Cultivar	Berry compartment	Quantitation			mDP[d]	EcG [e] (%)	Egc [f] (%)
		mg.berry^{-1}	mg (g of berry fw)$^{-1}$	Ratio Pulp or Skin /Pericarp (%)			
Syrah[a]	Pulp	1.0	0.6	31.6	34.3	3.4	15.3
	Skin	2.3	1.3	68.4	29.5	3.6	33.6
Mourvèdre[b]	Pulp	1.1	0.5	20.8	46.2	4.4	6.1
	Skin	4.0	1.9	79.2	26.4	1.8	15.6
Grenache[b]	Pulp	1.0	0.5	21.7	28.4	7.7	3.4
	Skin	3.8	1.8	78.3	25.5	4.9	10.1
Muscat de Hambourg[b]	Pulp	1.8	0.6	15.8	25.6	4.1	8.6
	Skin	9.9	3.2	84.2	16.0	2.5	12.1
Pinot noir[c]	Pulp	na[g]	0.4	11.8	21.0	5.2	1.7
	Skin	na	3.0	88.2	39.0	1.1	14.5
Meunier[c]	Pulp	na	0.2	4.3	18.0	4.3	1.9
	Skin	na	4.4	95.7	37.0	1.5	13.2

[a]Verriès et al. 2008. [b]Souquet et al. 2006. [c]Mané et al. 2007. [d]mDP : mean of degree of polymerization. [e](-)-epicatechin 3-gallate. [f] (-)-epigallocatechin. [g]Not available.

2.2. Berry development

Flavan-3-ols are synthesized at the earlier developmental stages after flowering (Downey et al. 2003, Kennedy et al. 2001) but Bogs et al. (2005) showed that an accumulation of PACs has already begun two weeks before flowering. Then, the pool of flavan-3-ols increases until véraison (Kennedy et al.

2001, Downey et al. 2003, Verriès et al. 2008).

PACs contents remained constant after véraison (Fournand et al. 2006) or decreased by 42% (Downey et al. 2003), 39% (Pastor del Rio and Kennedy 2006) and 24% (Verriès et al. 2008) from véraison to ripe stage. Even if some analyses has been performed on both extracted PACs and residues (Downey et al. 2003, Verriès et al. 2008), the apparent loss may be due to lower extraction rate as a result of increased adsorption of PACs in plant cell walls (Kennedy et al. 2001). *In vivo* conversion of PACs to yet uncharacterized brown oxidation products, catalysed by laccase, has also been reported in *Arabidopsis thaliana* seeds (Pourcel et al. 2005).

The evolution of mDP between véraison and ripe stage is unclear since an increase has been measured from 11.3 to 27 by Kennedy et al. (2001) and from 25.1 to 29.5 by Verriès et al. (2008) while Downey et al. (2003) reported a drop from 40 to 25. In seeds, the final pool of PACs is reached a few weeks after véraison, i.e. a bit later than in skin (Bogs et al. 2005).

2.3. Abiotic conditions

Vine vigor contributes significantly to grape quality. By selecting zones with different vigor within a vineyard and using tools of precision agriculture, Cortell et al. (2005) showed that seed PACs composition was slighlty different between zones but their total amount was not affected. On the other hand, total amount, (-)-epigallocatechin content and mDP values of skin PACs were increased in low vigor zones. On the contrary with anthocyanins or flavonols, shading seems to have little effect on skin PAC contents except in low vigor vines where shaded clusters contain less PACs (Cortell and Kennedy 2006).

Temperature effect was recently studied by Cohen et al. (2008) during green stage and maturation. At véraison, higher temperatures increased the PAC amount but had no effect on mDP values. Conversely, day-cooling and damping the diurnal temperature fluctuation decreased PAC amounts. The later treatment significantly reduced mDP but also berry enlargement. The effects of treatments applied on clusters from véraison to harvest were not significant, except for day-cooling that induced a decrease in PAC content. Although water deficiency strongly increased anthocyanin accumulation, its effect on PAC accumulation was very mild (Ojeda et al. 2002, Kennedy et al. 2002, Roby et al. 2004, Castellarin et al. 2006, Castellarin et al. 2007).

2.4. Biotic conditions

Few studies have been reported on the influence of pathogens or pest diseases on accumulation and composition of PACs. Induction of resistance in

Merlot grapevines with the plant activator benzothiadiazole against gray mold caused by *Botrytis cinerea* increased the PAC fraction up to 36% (Iriti et al. 2005). The use of this compound in stimulation of primary and secondary metabolites could be a way of limiting excessive use of fungicides.

Moreover, Kortekamp (2006) showed that (+)-catechin and related PACs may be implicated in the acquisition of the resistance of the sensitive cultivar Riesling against *Plasmopara viticola*. Their synthesis after inoculation of sensitive plant by the non-host pathogen *Pseudoperonospora cubensis* (the downy mildew pathogen of cucumber) was noticed, and the resistance of the vine seemed to be improved.

3. MOLECULAR ASPECTS OF PROANTHOCYANIDIN BIOSYNTHESIS

3.1. Synthesis of PAC units

3.1.1. The upstream biosynthetic pathway is common to anthocyanin and PACs

The PAC pathway has been well described, especially thanks to the analysis of mutants of *Arabidopsis thaliana* exhibiting a transparent testa (*tt*) phenotype as a result of genetic lesions in flavonoid/PAC biosynthesis (Lepiniec et al. 2006). This collection of mutants allowed the identification of several enzymes, transcriptional regulators of PAC biosynthesis and deposition, and transporters potentially involved in movement of PAC precursors to the cell vacuole. PACs share the same upstream biosynthetic pathway as the anthocyanin pigments (Fig. 4). This pathway is already described in Chapter 3.

The analysis of the complete grape genome sequence (Jaillon et al. 2007, Velasco et al. 2007) revealed that most of the enzymes from the upstream pathway, from PAL to hydroxylases, F3´H and F3´5´H, are encoded by genes belonging to multigenic families (Fig. 4). This is particularly emphasized in the cases of PAL with 13 members and F3´5´H with 10 members. On the other hand, enzymes from the downstream part are encoded by a single gene or small gene families.

3.1.2. Two genes of the downstream pathway are specific of PAC: leucoanthocyanidin reductase and anthocyanidin reductase

Two specific structural genes of PAC biosynthesis, leucoanthocyanidin reductase (*LAR*) and anthocyanidin reductase (*ANR*) have been recently identified.

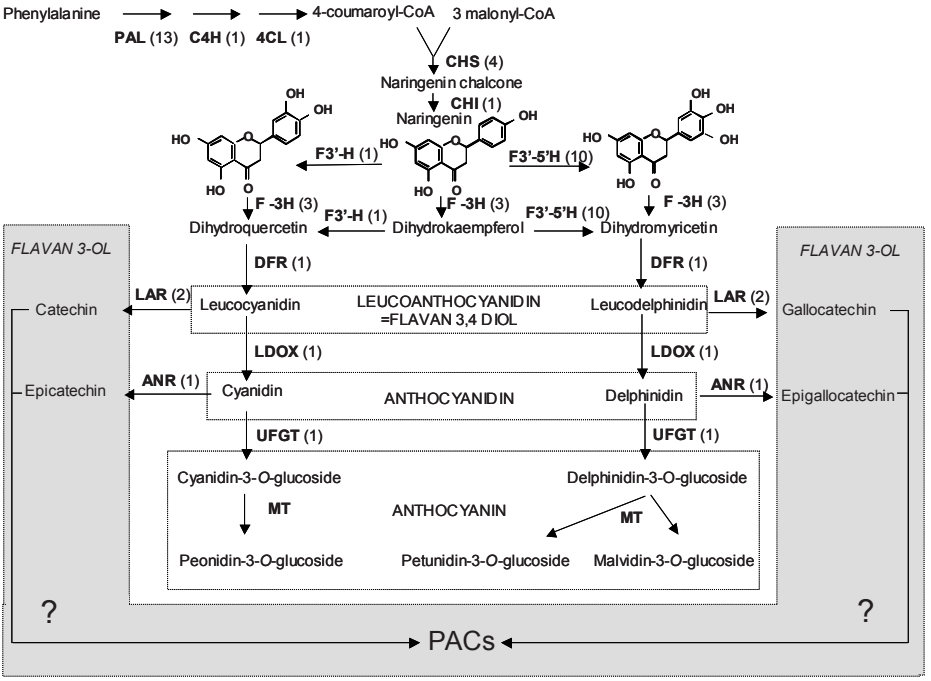


Fig. 4. The anthocyanin-PAC biosynthetic pathway. The enzyme names are in bold, numbers between brackets are the numbers of members in the gene family in grape according to Velasco et al. (2007). PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3´H, flavonoid 3´-hydroxylase; F3´5´H, flavonoid 3´,5´-hydroxylase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; UFGT, UDP-glucose flavonoid 3-O-glucosyl transferase; MT, methyltransferase.

3.1.2.1. Roles of ANR and LAR in epicatechin and catechin formation

The seeds of an *Arabidopsis* mutant that did not contain any PAC exhibited a red color due to the presence of anthocyanins. This mutant was therefore called BAN, named after a French red wine called Banyuls (Albert et al. 1997). The causal mutation was identified in a structural gene encoding a putative NADPH-reductase and was primarily thought to act as a LAR (Devic et al. 1999). However, Winkel-Shirley (2001) observed that leucoanthocyanidin dioxygenase (LDOX) mutants also resulted in a tt phenotype, suggesting that anthocyanidin formation is a compulsory step for PAC biosynthesis. Xie et al. (2003) confirmed that *BAN* encoded in fact an anthocyanidin reductase acting downstream the LDOX. Recombinant enzymes from *Medicago* and *Arabidopsis* were shown to catalyse the production of 2,3-*cis*-flavan-3-ols, i.e. (-)-

epicatechin, (-)-epiafzelecatechin and (-)-epigallocatechin from the corresponding anthocyanidins, respectively cyanidin, pelargonidin and delphinidin (Xie et al. 2003). The enzymatic reaction is dependent on the presence of a cofactor, NADPH and eventually NADH, depending on the sequence of the protein (Xie et al. 2004). In grape, one gene encoding an ANR was identified and its functional validation was obtained through heterologous expression in tobacco plants (Bogs et al. 2005). An increase in flavan-3-ol content in petals was observed, in parallel with a decrease in anthocyanin concentration.

The LAR activity was detected in extracts of cell suspension cultures from Douglas fir several years ago (Stafford and Lester 1984). The identification of the protein was performed by Tanner et al. (2003) from the legume *Desmodium uncinatum*, after purification, microsequencing of the protein and gene heterologous expression in a microorganism. The heterologous protein was able to produce (+)-catechin from 2R,3S-leucocyanidin as substrate (Tanner et al. 2003). Similar results were obtained with the *Medicago truncalata* LAR (Pang et al. 2007). Transgenic tobacco and white clover over-expressing *Desmodium uncinatum LAR* exhibited an increased *in vitro* LAR activity but no phenotypic characterization of the plant was presented (Tanner et al. 2003). Two *LAR* genes are present in the grape genome (Jaillon et al. 2007). The function of LAR1 was demonstrated after heterologous expression of the protein in *E. coli,* using leucocyanidin as substrate (Bogs et al. 2005). LAR1 and LAR2 yeast recombinant proteins accept the three leucoanthocyanidins as susbstrates, when generated *in situ* through the activity of recombinant DFR in a coupled assay from dihydroquercetin, dihydrokampferol or dihydromyricetin (Pfeiffer et al. 2006). However, for both studies, identification of the formed product including determination of stereochemistry is still lacking.

GFP fusion proteins of *Medicago trunculata* LAR and ANR were detected in the cytoplasm (Pang et al. 2007). However, these localizations were performed in *Nicotiana tabacum*, which raises the problem of mislocalization in an heterologous system due to non recognition of peptide signal or impairment in protein-protein interactions, especially in the case of the flavonoid pathway, where enzymes are assumed to be associated in complexes.

3.1.2.2. Questionable roles of LAR and ANR

Nevertheless, the respective roles of the LAR and ANR proteins are not so clear. In the case of ANR, (-)-catechin was also detected as a minor product of the *in vitro* reaction with cyanidin as substrate and even equal amounts of (-)-gallocatechin and (-)-epigallocatechin were formed from delphinidin (Xie et al. 2003). However the authors suggested that it might result from chemical epimerization rather than enzymatic reaction (Xie et al. 2003, Xie et al. 2004). In transgenic tobacco petals over-expressing grape *ANR*, epigallocatechin but also gallocatechin were both detected as monomers and eventually dimers (Bogs

et al. 2005) but their absolute configurations have not been determined.

Arabidopsis PACs only contain (-)-epicatechin (Routaboul et al. 2006), as expected since no *LAR* gene could be detected in its genome (Abrahams et al. 2003, Tanner et al. 2003). *Medicago trunculata* contains one *LAR* gene, but no catechin could be detected and the *LAR* transcript expression did not correlate with PAC accumulation (Pang et al. 2007). Moreover, a recent report failed to detect any activity for recombinant Lotus LAR when assayed as a single protein with leucocyanidin as substrate; weak activity was detected by thin-layer chromatography assay only in a coupled assay with DFR (Paolocci et al. 2007). Surprinsingly, the *in vivo* function of the LAR has never been assessed. Transgenic tobacco flowers over-expressing *MtLAR*, despite containing anthocyanins (and therefore leucoanthocyanidins, the putative substrates of LAR) do not contain any catechin, and their soluble PAC content was even reduced when compared to wild type plants (Pang et al. 2007).

3.1.3. Expression of biosynthetic genes in grape

As many biosynthetic proteins are encoded by multigenic families, the corollary question is whether this apparent redundancy is linked to a specialization towards one of the end-products of the pathway, i.e. flavonol, anthocyanin or PACs. However, none of the published works have presented an exhaustive expression analysis with an adequate detection of isogenes, because it was impossible before the completion of the genome sequence. Recently, Jeong et al. (2008) performed a multiple correlation between the expression of the isogenes of *CHS*, *CHI* and *F3H* and the flavonoid profiles in different parts of the plant at several stages. Some of the isogenes were specifically associated with one type of molecule such as *Chi2* with flavan-3-ols. It has been suggested that the PAC pathway may exist as a metabolic channel with a string of enzymes associated with cellular membranes (Winkel 2004). It remains to be demonstrated whether the specific expression of one isogene with one end-product could be either linked to special catalytic properties of enzyme or to particular association of the protein to other members of the pathway.

Some genes are involved in both PAC and anthocyanin pathways. This is compulsory for proteins which are encoded by a single copy in the grapevine genome, like *DFR* or *LDOX*, but also for isogenes belonging to multigenic families such as *F3´5´H*. Their expression profiles in berry skins therefore exhibit a ‘U’ shape, with a high level during the early stages of berry development, when PAC accumulation occurs, decreasing to very low levels at véraison when no flavonoid biosynthesis takes place and increasing again during ripening in parallel with anthocyanin accumulation (Boss et al. 1996, Bogs et al. 2006). In contrast, the expression of both *ANR* and *LAR2* genes is high in young berry and then constantly decreases all along fruit development, in ac-

cordance with their specific involvement only in PAC synthesis.

The PAC composition in the different organs of grapevine has to be correlated to the differential expression of the genes of the pathway. Grape PACs do not contain any monohydroxylated compound, i.e. (epi)afzelechin units. This absence is not due to substrate specificity of the different enzymes, ANR, LAR, and DFR, towards substrates with different B-ring hydroxylation patterns (Pfeiffer et al. 2006, Petit et al. 2007), suggesting a high expression and activity of the hydroxylases, F3´H and F3´5´H. By the way, almost no expression of *F3´5´H* was detected in the seeds, in accordance with the absence of (epi)gallocatechin unit (Bogs et al. 2006). *ANR* transcripts and, to a lesser extent, *LAR2* transcripts were also detected in the pulp of green berries (Verriès et al. 2008). In leaves, *ANR* was expressed at high levels all along development, *LAR2* expression was only detected in mature leaves and *LAR1* was expressed at very low levels in leaves at all developmental stage (Bogs et al. 2005).

LAR1 expression was almost restricted to seeds whereas *LAR2* was expressed in both skin and seeds (Bogs et al. 2005). Since no particular enzymatic difference was detected between the two proteins (Pfeiffer et al. 2006), it remains difficult to relate it with PAC composition.

3.2. Polymerization

The polymerization process is still unknown. Whether it happens chemically or is catalyzed by the so-called polymerizing enzymes remains a matter of debate. Several reactions have been postulated on the basis of chemical or biosynthetic evidences. All of them are based on the same mechanism, involving addition of a flavan-3-ol, through one of its C6 or C8 free positions (nucleophilic), onto an activated (electrophilic) species that serves as the precursor for the upper unit (substituted in C4).

The most commonly accepted pathway considers the flavan-3,4 diol formed by reduction of a dihydroflavonol (DFR activity), as the precursor of the upper unit (Fig. 5). In this process, a flavan-3-ol, either undergoes condensation (i.e. addition with loss of a water molecule) with the flavan-3,4 diol (Geissman and Yoshimura 1966), or adds to the quinone methide (or its protonated carbocation equivalent) formed by dehydration of the flavan-3,4 diol (Jacques and Haslam 1974). Spontaneous reaction of 3´,4´,5,7 tetramethyl flavan-3,4 diol with (+)-catechin in acidic conditions yielded high rates of methylated PACs (Geissman and Yoshimura 1966). A protocol involving chemical reduction of (+)-taxifolin (i.e. 2R,3S dihydroquercetin) followed by reaction of (+)-catechin or (-)-epicatechin onto the resulting flavan-3,4 diol has been developed to synthesize procyanidin dimers B3 ((+)-catechin-(4α→8)-(+)-catechin) and B4 ((+)-catechin-(4α→8)-(-)-epicatechin), respectively (Delcour

et al. 1983), meaning that these reactions can take place spontaneously when the right precursors are present. Optimum yields for the dimers are obtained with stochiometric amounts of taxifolin (or flavan-3,4 diol) and flavan-3-ol. When taxifolin is in excess, higher oligomers are formed as by products, suggesting that the degree of polymerization can be regulated by the flux of upper and lower unit precursors. In fact, flavan-3-ol monomers, oligomers, and polymers can add in the same way to the activated upper unit, so that polymerisation can be iterative, a DPn+1 species being formed at each step from a DPn species. Addition of flavan-3-ols onto the carbocation released by acid-catalysed cleavage of interflavanic bonds has also been demonstrated (Haslam 1980, Vidal et al. 2002). Mostly PAC dimers are formed when flavan-3-ol monomers are present in excess compared to all upper and intermediate units in the reaction medium. When flavan-3-ols monomers are present in insufficient amount, additions occur at random, yielding PACs of increasing molecular weight diversity.

Nevertheless, these mechanisms cannot explain the prevalence of 2R,3R stereochemistry in PAC upper and extension units observed within the plant kingdom, as (+)-taxifolin (2R,3S) is the only stereoisomer described in the biosynthetic pathway and the stereochemistry of all units is retained in these reactions.

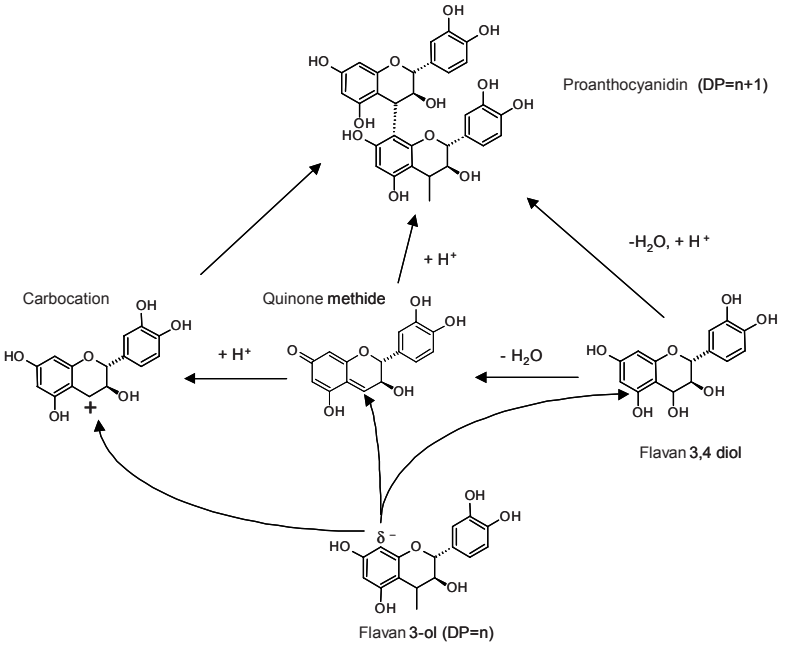


Fig. 5. Proposed pathway for polymerization with flavan-3,4 diol as precursor for upper unit.

Other precursors and mechanisms are required to obtain the 2R,3R isomers. A mechanism based on tautomeric rearrangement of the 2R, 3S quinone methide to the 2R, 3R quinone methide has been postulated (Fig. 6; Hemingway and Laks 1985) and the role of enzymes catalyzing these inter-conversions has been proposed. However, this still requires confirmation. Besides, other intermediates can be envisaged. As recently established for biosynthesis of (-)-epicatechin from cyanidin (ANR activity), formation of the 2R,3R extension units may involve an anthocyanidin intermediate in which the stereochemistry is lost. The anhydrobase base of the anthocyanidin, that is formed in neutral medium by deprotonation of the red flavylium cation, has been proposed as a precursor for the extension units (Jacques and Haslam 1974). Addition of flavan-3-ols onto anthocyanins (Fig. 7) has been demonstrated in model solutions. Starting from malvidin 3,5 diglucoside (Bishop and Nagel 1984) and from malvidin 3-glucoside (Remy-Tanneau et al 2003), anthocyanin-flavanol adducts were obtained. The reaction with (+)-catechin or (-)-epicatechin first formed the flavene adduct which then rearranged to an A-type flavanol dimer. Similar reaction with a procyanidin dimer yielded the corresponding flavylium forms (Salas et al. 2004). Conversion of the flavene to the B-type flavan-3-ol has not been observed.

The other possibility is that (-)-epicatechin itself serves as a precursor for the upper units (Fig. 8). Oxidative dimerization of two flavan-3-ol molecules has been proposed as an alternative polymerization mechanism (Weinges et al 1969a). However, the same authors have shown that oxidative coupling of catechin yields other derivatives called dihydrodicatechins B and A, which are isomers of B-type and A-type procyanidins, respectively (Weinges et al 1969b). In the formation of dihydrodicatechin B, one flavan-3-ol adds to the electrophilic *o*-quinone formed from the catechin *o*-diphenolic B-ring. The resulting linkage involving the B-ring of one unit is thus different from the C4-C6 and C4-C8 bonds of PACs. More recent studies have confirmed that oxidation involves prima- rily the o-diphenolic B-ring of flavan-3-ols and yields dimers in which the flavan-3-ol units are linked by biphenylether or biphenyl linkages, respectively, between the B-ring of one unit and the A-ring of the other (Young et al. 1987, Guyot et al 1996). The latter further oxidizes intramolecularly to yellow dihy-

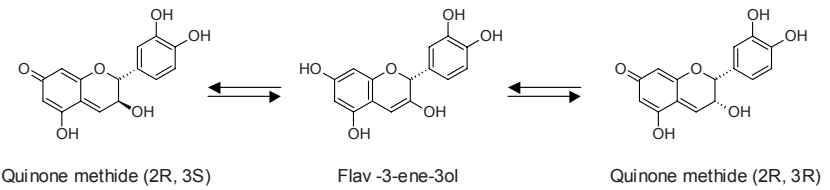


Fig. 6. Tautomeric rearrangement of the 2R, 3S quinone methide to produce the flav-3-ene-3-ol.

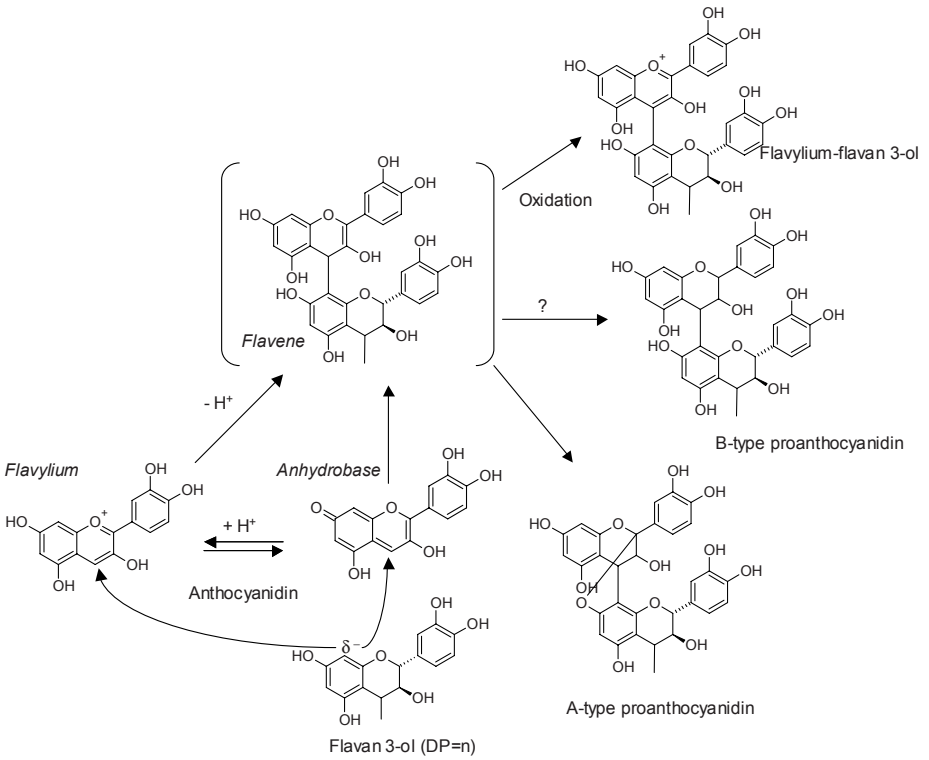


Fig. 7. Reactions involving anthocyanidin as precursor for upper unit.

drodicatechins A. The loss of brown coloration and larger amounts of procyanidins in the *Arabidopsis tt10* mutant, suggest that the laccase coded by the *TT10* gene oxidizes flavan-3-ols to brown unknown pigments, presumably related to dihydro-dicatechins (Pourcel et al. 2007).

The discovery of epicatechin 3′-glucoside as a potential intermediate in procyanidin biosynthesis (Pang et al. 2008) shed new light on an eventual role of oxidation in the polymerisation process. In fact, oxidation of this derivative, which is not an o-diphenol, could yield a quinone methine activated in the 4 position which could serve as the precursor for extension units. It is worth emphasizing that, in this case, the extension units may also be oligomers or polymers, so that the coupling would be random rather than sequential. However, the involvement of epicatechin 3′-glucoside in the polymerization process requires further validation.

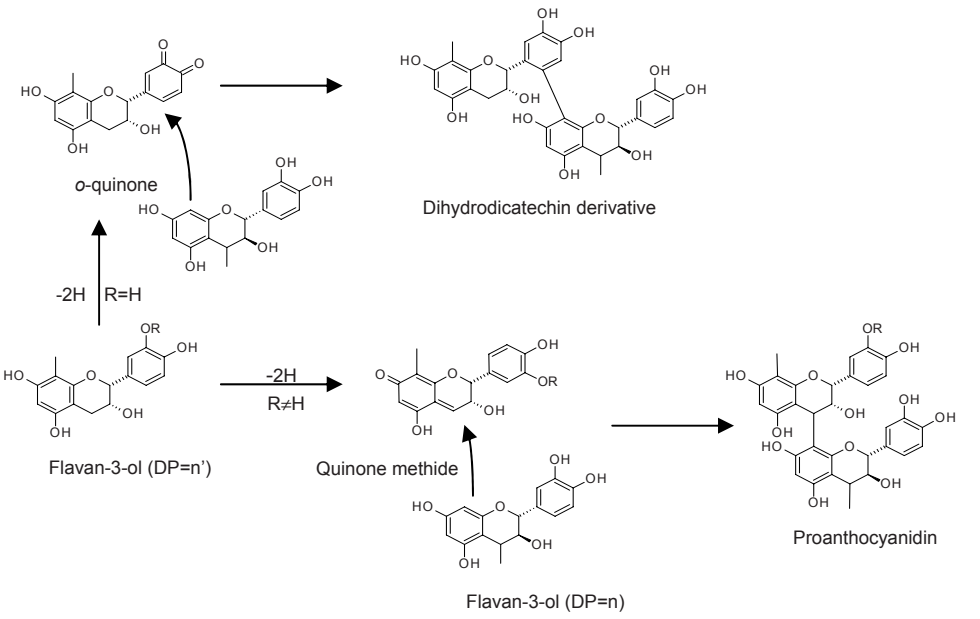


Fig. 8. Proposed mechanism for oxidative polymerization of flavan-3-ols

3.3. Transport and sequestration of PACs

In plants, PACs are often stored inside the vacuole and in grape skin cells (Cadot et al. 2006). In plants, synthetic enzymes of the upper part of the flavonoid pathway are localized to the cytosolic side of the endoplasmic reticulum (ER, Winkel 2004), while LAR and ANR may be localized to the cytosol (Pang et al. 2007). PACs or intermediate metabolites have therefore to cross the vacuolar membrane. Two non-exclusive different mechanisms have been described to explain the transfer of the molecules from ER to vacuole (Grotewold 2008). The first hypothesis implies that PACs or precursors reach the vacuole in vesicles derived from the ER (Baur and Walkinshaw 1974, Ibrahim 1992). The second one suggests an escort of the flavonoid from the synthesis site to the vacuole. This second hypothesis is supported by the fact that several mutants exhibiting abnormalities in their flavonoid sequestration are mutated for a Glutathione S-Transferase (*GST*) gene: *Bz2* and *AN9* for anthocyanins in maize and petunia, respectively (Marrs et al. 1995, Alfenito et al. 1998) and *tt19*, for both PACs and anthocyanins in *Arabidopsis* (Kitamura et al. 2004). Several authors have reported that those GSTs were also able to transfer glutathione tags, but those experiments were mainly performed with glutathionation of the non-

natural substrate 1-chloro 2,4-dinitrobenzene (CDNB, Alfenito et al. 1998). When assayed in parallel, activity with anthocyanin was 10-fold lower than with CDNB (Lo Piero et al. 2006). However, the GST enzymatic activity does not seem to be required for appropriate targeting of the compound in the vacuole, since no glutathionated anthocyanin could be detected through *in vivo* or *in vitro* labelling experiments (Mueller et al. 2000), suggesting that GST would rather act as a 'ligand' allowing the flavonoid molecule to cruise through the cytosol from the ER to the tonoplast.

In grape, high throughput expression studies all along berry development or between cultivars differing in skin color revealed that the expression of a *GST* was systematically associated with anthocyanin accumulation (Terrier et al. 2005, Ageorges et al. 2006). However, this *GST* is very unlikely to be involved in PA sequestration, since its expression was restricted to ripening stages of berry development.

Two kinds of transporters have been described transporting the flavonoid molecule from the cytosol into the vesicular or vacuolar lumen: the ATP binding casettes (ABC) primary transporters and the multidrug and toxic extrusion (MATE) secondary transporters. An ABC–type directly energized is involved in barley flavones vacuolar transport (Klein et al. 1996). ZmMRP3, a tonoplastic ABC transporter, is required for anthocyanin vacuolar accumulation in maize (Goodman et al. 2004). A multidrug resistance-associated protein ABC transporter would also facilitate flavonoid transport from cell to cell (Buer et al. 2007). To date, no ABC-type transporter has been described for PAC vacuolar sequestration.

On the other hand, a T-DNA insertion in a secondary MATE-type transporter resulted in a tt phenotype (Debeaujon et al. 2001). This *tt12* mutant is affected for both PAC and flavonol accumulation (Marinova et al. 2007). The protein encoded by the *TT12* gene was found to be localized to the tonoplast (Marinova et al. 2007). However, the localization of the GFP fusion protein was not performed in PAC producing cells. In *Arabidopsis* tapetum cells, flavonoids were detected in ER derived vesicles, but were present in the cytosol in *tt12* mutant, suggesting that this gene is also involved in transport of the flavonoids inside vesicles (Hsieh and Huang 2007).

In vitro transport experiments with vesicles purified from yeasts overexpressing *TT12* demonstrated that this MATE protein acts as a flavonoid/H^+ secondary antiporter and mediates cyanidin 3-glucoside (but not catechin) transport (Marinova et al. 2007). Potential role of decorations as recognition tags was suggested by diverse studies, glycosylation would be specific for secondary transporters and glutathione tag for primary ABC transporters (Yasaki 2005). Recently, a glucosyl tranferase was shown to be induced in parallel with PAC accumulation in *Medicago truncalata* hairy roots and to catalyze quite ex-

clusively the formation of epicatechin 3′-glucoside (Pang et al. 2008). Whether this molecule is the substrate of TT12 remains to be demonstrated.

A ΔpH across the tonoplast is necessary for H^+/metabolite antiporter to function. Both kinds of vacuolar proton primary pumps, V-PPase and V-ATPase are present in grape cell tonoplast all along berry development and are able to generate a H^+ gradient (Terrier et al. 2001). The *aha10 Arabidospis* mutant suggests that in addition, a P-type ATPase proton pump is required for correct vacuole biogenesis and PA accumulation (Baxter et al. 2005). However, the intracellular localization of AHA10 awaits validation.

In plants and in particular in grapevine, transport and sequestration of PACs remain partially understood.

3.4. Regulation of the PAC pathway

Since the PAC biosynthesis in grape exhibits a precise spatio-temporal differentiation, fine regulation mechanisms should operate to control the expression of the structural genes.

In *Arabidopsis thaliana*, 6 different mutations of transcription factors (TF) affected PAC composition. They belong to several TF families Myb, bHLH, WD40, WRKY, zinc finger and MADS box proteins (Lepiniec et al. 2006). Some of them, like *TT1* and *TT16* encoding respectively a zing finger protein and a MADS box, are rather involved in the cellular differentiation allowing PAC accumulation (Sagasser et al. 2002, Nesi et al. 2002). A complex formed by the association between R2R3Myb, basic helix-loop-helix (bHLH) and WDR (WD40 repeats) proteins encoded by *TT2*, *TT8* and *TTG1*, respectively/drives the expression of PAC biosynthesis structural genes (Baudry et al. 2004). In this activating complex, the Myb proteins would ensure the specificity of the induction between the different branches of the flavonoid and phenylpropanoid pathways (Zhang et al. 2003). Negative regulators of the pathway were also identified in *Arabidopsis thaliana*, like Myb4, a R2R3Myb repressor of the cinnamate-4-hydroxylase expression and therefore of the whole phenylpropanoid pathway (Jin et al. 2000) or small Mybs (R3Myb) like *MybL2*, whose over-expression reduces PAC content (Dubos et al. 2008).

In grapes, in addition to VvMybAs controlling the anthocyanin pathway (Kobayashi et al. 2002, Walker et al. 2007), several Myb factors were identified as involved in the phenylpropanoid/PAC pathway, VvMyb5a (Deluc et al. 2006), VvMybPA1 (Bogs et al. 2007) and VvMyb5b (Deluc et al. 2008).

Their expression profile in berries and ability to activate the promoters of some genes of the PAC pathway is summarized in Table 2 and 3, respectively. The expression of *VvMyb5a* decreases in both skin and seeds all along berry development (Deluc et al. 2006). Maximum expression for *VvMyb5b* was de-

Table 2. Expression level of three VvMyb factors involved in the regulation of the phenylpropanoid/PAC pathway all along berry development (from Deluc et al. 2006, 2008, Bogs et al. 2007).

	Skin			Seeds		
	Pre-véraison	Véraison	Ripening	Pre-véraison	Véraison	Ripening
VvMyb5a	+++	++	+	+++	++	+
VvMyb5b	+	-	++	++	-	+
VvMybPA1	-	+	++	-	++++	-

tected in ripe berries (Table 2, Deluc et al. 2008). Expression of *VvMybPA1* was the highest in seeds harvested at véraison, i.e. at maximal PAC accumulation rate in this tissue (Bogs et al. 2007).

VvMybPA1 is able to restore a wild type phenotype for PAC accumulation when overexpressed in *tt2* mutants (Bogs et al. 2007). Tobacco plants over-expressing *VvMYB5a* exhibit some modifications of their content in anthocyanins, flavonols, tannins, and lignins and those over-expressing *VvMyb5b* in anthocyanins and tannins, but other compounds were not quantified (Deluc et al. 2006, Deluc et al. 2007). Anyway, conclusions regarding their functions after ectopic expression in heterelogous plants are quite suspicious and should be validated in an homologous system. Actually, VvMyb5a and VvMyb5b induce anthocyanin biosynthesis in tobacco, whereas in grapevine they are expressed at green stages, when no anthocyanin is present yet.

None of these Myb factors activate *VvUFGT*, specific of the anthocyanin pathway, but they activate several genes of the general flavonoid pathway and some specific of the PAC pathway (Table 3). Surprisingly, VvMyb5a does not activate the *VvANR* promoter (Deluc et al. 2008), although transgenic tobacco petals over-expressing *VvMyb5a* contain epicatechin in significant amount (Deluc et al. 2006). Similarly, the activation of *VvF3´5´H* by VvMybPA1 is questionable since *VvMybPA1* is expressed almost exclusively in seeds (Bogs et al. 2007) whereas seeds do not contain any trihydroxylated units (*cf* 2.1).

To date, VvMybPA1 appears as the Myb factor, the most specific of the PAC pathway, while VvMyb5a and b rather appear as general regulators of the phenylpropanoid/flavonoid pathway. Nevertheless, the exhaustive identification of the transcription factors involved in the control of the PAC pathway is far from being achieved yet. Thanks to the complete genome, Matus et al. (2008) identified and classified the 108 R2R3-Myb proteins. According to their structure and similarity to TT2, eight genes were designated as putative candidates for PAC pathway regulators, but neither *VvMybPA1* nor *VvMyb5a* and *b* belong to this group.

Moreover, nothing is known on grape regarding the other members of the complex, i.e. *bHLH* and *WDR*, or whether the same genes are involved in both

anthocyanin and PAC pathways or if several members of a multigenic family are successively recruited.

Probably the main challenge concerning PAC biosynthesis in the next years will be to decipher the control points in the structure of the PAC, like degrees of galloylation and most importantly polymerization, from the mechanistic and regulation point of view. Grapevine appears as an important model plant to answer these questions because of its distinctive PAC composition when compared to other model plants like *Arabidopsis thaliana*, and because of the genomic tools developed for this plant during the last years.

Table 3. Promoter activation of several genes of the flavonoid pathway by Myb transcription factors using a transient expression assay (from Deluc et al. 2005, 2008, Bogs et al. 2007).

	Promoter activation					
	VvCHI	VvF3'5'H	VvLDOX	VvANR	VvLAR1	VvUFGT
VvMyb5a	+++	+	+++	-	++	-
VvMyb5b	++	+	+++	+	++	-
VvMybPA1	++++	++++	++++	++++	++++	-

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14

BIOLOGICAL ACTIVITY OF GRAPEVINE PHENOLIC COMPOUNDS

R. Amarowicz[1] & S. Weidner[2]

[1]Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences Tuwima Street 10, 10-747 Olsztyn, POLAND

[2]Chair of Biochemistry, University of Warmia and Mazury in Olsztyn, Oczapowskiego 1A10-957 Olsztyn, POLAND

1. INTRODUCTION

Phenolic compounds present in nearly all parts of grape berries are increasingly believed to exhibit antioxidant and antimicrobial activities and to play a significant role in the prevention of diseases including cancer and cardiovascular diseases (Bagchi et al. 2000, Ariga 2004). The majority of studies on grape phenolics properties has been conducted using proanthocyanidin-rich seeds extracts (GSEs).

Grape seed extract is rich in phenolic acids and different flavonoids but most important are the monomeric flavan-3-ols [(+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-*O*-gallate], oligomeric (2-5 units) and polymeric (6-20 units) procyanidins formed by monomer unit linked by C4-C8 and C4-C6 interflavanic bonds (B-type procyanidins) (Prieur et al. 1994, Monagas et al. 2005). In Fig. 1 the chemical structure of proanthocyanidins in grape berries is shown.

The phenolic content and composition of GSE depends on the solvent used for extraction. Jayaprakasha et al. (2001) obtained the highest yield of extraction using ethyl acetate-water (17:3, v/v). Extraction with methanol gave maximum yield of the extract but with a lower content in total flavanols, whereas methanol was the best solvent for the qualitative extractions of catechins from the grape seeds (Kalhithraka et al. 1995). Saucier et al. (2001) reported that a mean polymerization degree of grape seed proanthocyanidins was 3.6 ± 0.1. The extract contained only procyanidin polymers with some galloylation on epicatechin units (13.4% of galloyllation). The fractions obtained from the crude extract using liquid/liquid extraction were characterized by a degree of polymerization determined by thiolysis ranging from 3.1 to 12.2. The content of (-)-epicatechin-gallate, (+)-catechin, and (-)-epicatechin in the fractions were

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10.9-19.2%, 16.0-28.7%, and 52.4-64.8%, respectively. In the study of Sun et al. (1998) the mean degree of polymerization for oligomeric and polymeric proanthocyanidins in the seed extract were 9.8 and 31.5, respectively.

From a practical point of view, very important are the results of Yamakoshi et al. (2002). No evidence of acute oral toxicity of GSE at dosage of 2 and 4 g/Kg, and no evidence of mutagenicity was found in the reverse mutation test using *Salmonella typhimurum*, the chromosomal aberration test using CHL cells, and the micronucleus test using ddY mice. Administration of GSE at 0.02, 0.2, and 2% (w/w) to the rats for 90 days did not induce noticeable symptoms of toxicity. In conclusion, the results indicated lack of toxicity and supported the use of GSE in various food products.

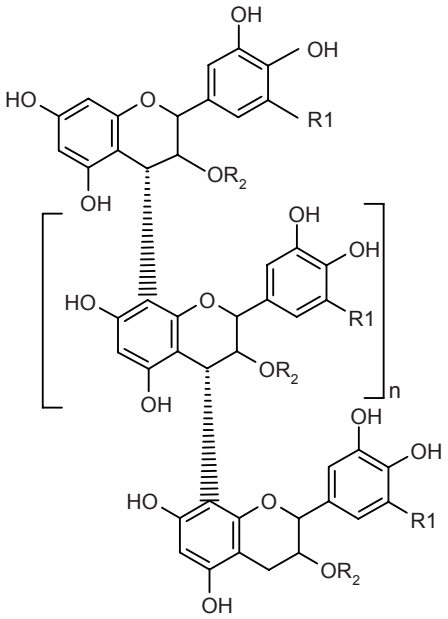

Fig. 1. Chemical structure of grape berry proanthocyanidin: R1 = H or OH; R2 = H or galloyl residue.

2. ANTIOXIDANT ACTIVITY

Antioxidant and antiradical activity of grape extracts were investigated using several methods, such as the liposome systems (Yi et al. 1997, Pazos et al. 2006), a β-carotene-linoleate model system (Jayaprakasha et al. (2001), lipid peroxidation (Gámez-Meza et al. 1999, Fujime et al. 2007), human LDL oxidation (Meyer et al. 1997), serum total antioxidant activity (Nuttal et al. 1998), scavenging of DPPH radical (Cruz et al. 2004, Faria et al. 2006, Weidner

et al. 2007) and ABTS cation radical (Fuji et al. 2007), the FRAP assay (Faria et al. 2006), and the ORAC assay (Monagas et al. 2005, Yilmaz and Toledo 2006). Some of the results are presented in Table 1.

Alonso et al. (2002) described the antioxidant activity of grape byproducts marcs, stalks, and dregs of both white and red varieties. Marcs and stalks presented a higher antioxidant activity than the dregs. Also the red varieties exhibited higher values than the white ones. Significant correlation was found between the content in total phenolics and the antioxidant activity (correlation coefficients for marcs, stalks, and dregs were 0.9069, 0.8438, and 0.7915, respectively).

The inhibition of LDL oxidation by GSEs was described by Meyer et al. (1997). Samples prepared by the longer extraction time showed significantly higher antioxidant activity than samples prepared by the short extraction time. The relative LDL antioxidant activity of phenolic extracts from 14 different types of fresh grapes was highly correlated with the level of flavan-3-ols ($r=0.86$, $p< 0.01$), total phenolics ($r=0.89$, $p< 0.05$), and hydroxybenzoates ($r= 0.77$; $p< 0.05$). Cruz et al. (2004) used winemaking waste solids from red grapes after fermentation for isolation of fractions with antioxidant activity. The best antioxidant properties were observed for the process of washing stage leading to liquors and washed solids, which were dried and subjected to hydrolytic processing with water as a reactive in an autocatalyzed reaction or with sulfuric acid solution to give an ethyl acetate fraction.

The grape seed extracts prepared by different solvents exhibited different antioxidant activity by using the bleaching of β-carotene assaying method. The solvent ethyl acetate-water (17:3; v/v) resulted to maximum antioxidant activity, as well as the highest reducing power of the extract (Jayaprakasha et al. 2001).

Furthermore, the posttreatments of extracts could improve their antioxidant properties (Pinelo et al. 2005). Chemical oxidation at 22 ^{o}C in water of an extract from *Vitis vinifera* was found to increase its antioxidant activity by 30%. The reason of this change was the process of polymerization of catechins to oligomers which possess higher antioxidant properties than monomers.

Grape extracts were also tested in food models. Horse mackerel fillets were supplemented with phenolic antioxidants by either spraying with an aqueous solution of grape procyanidins or glazing with the same solution. These treatments were effective and delayed lipid oxidation in the fish fillets as assessed by means peroxide and thiobarbituric acid related substances (TBARS) values and prolonged the shelf life time of fish about tree-fold when compared to controls. Preparation of grape procyanidins exhibited strong chelating capacity for the ferrous iron and reducing capacity measured by the FRAP method (Pazos et al. 2006).

Also, GSE phenolic compounds exhibited antioxidant properties in *in*

Table 1. Antioxidant activity/capacity of grapes and their extracts.

Material	Antioxidant activity/capacity	Unit	Method	Reference
Seed powder of:				
Muscadine	310.8 ± 3.7			
Merlot	344.8 ± 99.9	µmol Trolox/g d.w	ORAC	Yilmaz and Toledo (2006)
Chardonnay	637.8 ± 7.4			
Merlot	69.8 ± 33.2			
Chardonnay	102.8 ± 7.0			
Fraction of phenolic compounds from wine-making waste	0.16 – 1.06	EC50 (g/L)	DPPH$^{\bullet}$	Cruz et al. (2004)
Whole grapes (*Vitis vinifera*)	22-60 (10 µM GAE) 62-91 (20 µM GAE)	Relative inhibition (%)	Human LDL oxidation	Meyer et al. (1997)
Grape seeds	65 – 90	AA (%)	β-Carotene bleaching	Jayaprakasha et al. (2001)
Sixteen different commercial dietary product derivatived from grape seeds	2.71 – 26.4	µmol Trolox/ mg	ORAC	Monagas et al. (2005)
Five procyanidin fractions obtained from grape seed extract	10.803 – 13.348 7.332 – 11.810	µmol Trolox/ 2.5 µg/ml	DPPH• FRAP	Faria et al. (2006)
Grape seed extract	3.3 34.8 ± 0.5	mol of donated electrons/mol of phenolics % of chelated ferrus ion by 10 µmol of phenolics	Reducing power in fish microsomes Chelating activity in fish microsomes	Pazos et al. (2006)
Control	1.00			
Grape seed extract	1.04	mM	TEAC	
Cys-OLG	1.21			Fujime et al. (2007)
Control	8.80			
Grape seed extract	8.64	nmol/l	Lipid peroxidation	
Cys-OLG	7.91			
Grape procyanidin extract				
Day 0	408.1 ± 22.9	µmol Trolox/ l serum	Serum total antioxidant activity	Nuttal et al. (1998)
Day 5	453.3 ± 453.3			
Chardonnay seed flour	1076.4	Trolox equivalents µmol/g flour	ORAC	Parry et al. (2006)

Cys-OLG : oligomeric proanthocyanidin – L – cysteine complex; FRAP: ferric reducing antioxidant power; ORAC: oxygen radical absorbance capacity; DPPH$^{\bullet}$: 2,2′-diphenyl-1-picrylhydrazyl radical; TEAC: Trolox equivalent antioxidant capacity; Trolox (6-hydroxy-2,5,7,8 tetramethylchroman-caboxylic acid): water soluble vitamin E analogue.

vivo models. Treatment of mice with GSPE (100 mg/kg) decreased hepatic and brain lipid peroxidation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as well as production of reactive oxygen species by peritonel macrophages (Bagchi et al. 1998).

Grape seed proanthocyanidin extract had a protective effect on DNA in rat leukocytes when subjected for 47 days (Morin et al. 2008). Pretreatment of rats with GSE (0.04 and 0.4 g/g diet) decreased 8-oxodG level in leucocytes by 65% and 63% compared to the control group. However, the oxidative DNA damage as measured by urine excretion of 8-oxodG was not significantly affected by a GSPE supplemented diet. The results of Giovanelli et al. (2000) demonstrated that grape complex polyphenols and tannins can modulate *in vivo* oxidative damage in the gastrointestinal tract of rodents. In isolated colonocytes prepared from the colon mucosa of rats treated for ten days by oral gavage with wine complex polyphenols (57.2 mg/kg/day), the basal DNA oxidative damage was significantly decreased: −62% for pyrimidine and −57% for purine oxidation. Colonocyte oxidative DNA damage was analysed at the single cell level using a modification of the comet assay technique.

The antioxidant activity of catechin, epicatechin, peinidin 3-*O*-β-glucoside and malvidin 3-*O*-β-glucoside isolated from *Vinis vinifera* cultures were investigated using rat liver microsomes and human low density lipoproteins and DPPH. Catechin was twice as active as epicatechin, anthocyanins were less active than flavan-3-ols. No differences were observed between two anthocyanins tested. Catechins showed also twice higher antiradical activity against DPPH radical when compared with that of anthocyanins (Fauconneau et al. 1997).

New epicatechin conjugates obtained by depolymerization of grape polymeric flavonols in the presence of cysteamine or cysteine and with or without gallate were more efficient as antioxidant than epicatechin (Mitjans et al. 2004). The hemolysis of red blood cells mediated by an azo free radical initiator (AAPH) was used in this study.

Dietary procyanidins from *Vitis vinifera* seeds at a daily dose of 110 mg administrated to 10 healthy volunteers for 30 days exerted their antioxidant protection by sparing vitamin E and reducing DNA oxidative damage (Simonetti et al. 2002). The levels of α-tocopherol in red blood cell membranes increased significantly from 1.8 to 2.8 mg/g. The level of oxidized DNA in lymphocytes was reduced and the level of poly-unsaturated fatty acids in the red blood cells membrane was increased. In study of Natella et al. (2002) with 8 healthy volunteers the supplementation of a meal with GSE (300 mg per day) increased the plasma antioxidant capacity and enhanced the resistance to oxidative modification of LDL. The content of chylomicron lipid hydroperoxides was 1.5 fold higher after the control meal than after the GSE-supplemented meal.

3. PROTECTION FROM CARDIOVASCULAR DISEASES

Free radicals and oxidative stress play a crucial role in the pathophysiology of a broad spectrum of cardiovascular diseases including congestive heart failure, valvular heart disease, cardiomyopathy, hypertrophy, atherosclerosis and ischemic heart disease (Bagchi et al. 2003). Therefore, many authors believe that the antioxidant properties of grape extracts are the basis of the prevention of cardiovascular diseases. Some of the anti-artherosclerosic effects of grape phenolics are reported in Table 2.

The results of the experiments with cholesterol-fed rabbits described by Yamakoshi et al. (2000) suggested that proanthocyanidins from grape seeds

Table 2. Anti-atherosclerosic effects of grape phenolics.

Material	Effects/results	Reference
Grape seed extract	Transient reduction hypercholesterolemic response to semisynthetic diet in hyperlipidemic rabbits.	Frederiksen et al. (2007)
Grape seed extract	Prevention of atherosclerosis in postmenopausal women.	Nikitina et al. (2006)
Concord grape juice	Significant increases platelet aggregation and serum cholesterol in rabbits.	Shanmunganayagam et al. (2007)
Grape powder polyphenols	Reduction of atherosclerotic lesion area by 41% compared to the control in placebo mice following consumption of grape powder.	Fuhrman et al. (2006)
Grape seed extract	Prevention of the development of hamsters aortic atherosclerosis.	Auger et al. (2004)
Lyophilized grape preparation	Alteration of hepatic cholesterol metabolism, which may affect VLDL secretion rates and result in less accumulation of cholesterol in the aorta of ovariectomized guinea pigs.	Zern et al. (2003)
Phenolic fractions of grape seed extracts	Protection of LDL against oxidation.	Shafiee et al. (2003)
Grape seed extract	Promotion of cardiovascular health *via* a number of novel mechanisms.	Bagchi et al. (2003)
Grape seed extract	Reduction in the thickness of mice aortic valves consisting of foam cell and endothelium hyperplasia.	Yu et al. (2002)
Grape seed extract	Reduction of the postprandial oxidative stress by decreasing the oxidants and increasing the antioxidant levels in plasma, and, as a consequence, enhancing the resistance to oxidative modification of LDL in humans.	Natella et al. (2002)
Grape juice	Protection against the development of coronary artery disease and acute occlusive thrombosis.	Osman et al. (1998)

might trap reactive oxygen species in plasma and intestinal fluid of the arterial wall, thereby inhibiting oxidation of LDL and showing anti-atherosclerotic activity. The level of the plasma cholesteryl ester hydroperoxides induced by AAPH (2,2′-azobis(2-amidinopropane)-dichydrochloride) and the number of oxidized LDL-positive macrophage-derived foam cells in atherosclerotic lesions in the aorta was lower in the rabbits fed with GSE (1% in their diet). In this experimental group aortic malonaldehyde content was lower than in the control. Rats fed with diets containing 1% cholesterol showed an increase in total and low-density lipoprotein plasma cholesterol and a decrease in plasma high-density lipoproteins. The 2% addition of GSE to diets containing 1% cholesterol reduced plasma total cholesterol and LDL-cholesterol level (Tebib et al. 1994). Polymeric grape seed tannins also significantly increased the faecal excretion of lipids and cholesterol.

In Watanabe heritable hyperlipidemic rabbits, hypercholesterolemia was developing slower in GSE group compared to the controls as recorded by significantly lower plasma cholesterol in dosage week 7 and 11 (Fredriksen et al. 2007). Phenolic compounds present in grapes prevented cholesterol accumulation in human blood monocytes incubated with blood serum from postmenopausal women, and can be regarded as prospective components for the development of natural preparations for the prevention of atherosclerosis in postmenopausal women (Nikitina et al. 2006).

In addition, rabbits treated with Concord grape juice showed significantly lower total serum cholesterol and blood pressure, lower platelet aggregation, and development of atheroma than control group (Shanmunganayagam et al. 2007). The antiatherosclerotic effect of a standardized freeze-dried powder made from fresh grapes, rich in grape-specific polyphenols and free of alcohol, studied with apolipoprotein E deficient mice, was at least partly due to a significant 8% reduction in serum oxidative stress, an up to 22% increase in serum antioxidant capacity, a significant 33% reduction in macrophage uptake of oxidized LDL, and a 25% decrease in macrophage-mediated oxidation of LDL relative to controls (Fuhrman et al. 2006). Grape powder directly protected both plasma LDL and macrophages from oxidative stress *in vitro*.

On the other hand, the results of Auger et al. (2004) suggested that phenolic extracts from grape seeds and marc were beneficial in inhibiting atherosclerosis by indirect mechanism(s). These extracts fed at a moderate dose mimicking two glasses of red wine per meal reduced plasma cholesterol (-11% on average) but did not affect plasma antioxidant capacity of hamsters. The extracts prevented the development of aortic atherosclerosis. In an *ex vivo* experiment using rat aortic rings, induction of endothelium-dependent relaxation by the extracts was observed.

In female ovariectomized guinea pigs (a model for menopausal women),

plasma triglycerides and VLDL cholesterol were 39 and 50% lower, respectively in group fed with diet containing 10 g/100 g of a lyophilized grape preparation compared to controls (Zern et al. 2003). Also, significant modifications in LDL particles were found, including 58 and 30% lower triacylglycerols and phospholipids, respectively. Hepatic acyl CoA:cholesteryl acyltransferase activity was 27% lower ($P < 0.05$) in the grape diet-fed group compared with controls. In addition, concentrations of cholesterol in the aorta were 33% lower in guinea pigs fed the grape diet.

Polypenol fractions from grape seed rich in procyanidins achieved the best compromise between the direct and indirect (i.e. cell-mediated) types of action in protecting LDL against oxidation (Shafiee et al. 2003). They were efficient towards Cu^{2+} system, whereas they were rather poorly efficient towards AAPH, peroxinitrite-generating *in vitro* systems, and at impairing superoxide production in promonocyte cells. Furthermore, the results of Yu et al. (2002) indicated that the GSE has inhibitory effect on atherosclerosis in C57BL/6J mice, and the possible mechanism may be related to inhibition of the increase of plasma oxidized low density lipoprotein, and intercellular adhesion molecule-1 (ICAM-1), reduction of the damage of vascular endothelium.

Natella et al. (2002) investigated the effect of supplementing a meal with grape seed proanthocyanidins (GSPs) on plasma postprandial oxidative stress; eight healthy volunteers consumed the same test meal rich in oxidized and oxidizable lipids without (control) or with 300 mg of a proanthocyanidin-rich GSE. The content of lipid hydroperoxides in chylomicrons was 1.5-fold higher after the control meal than after the GSE-supplemented meal. Plasma lipid hydroperoxides increased only after consumption of the control meal. The plasma antioxidant capacity increased in the postprandial phase only following the GSE supplemented meal.

Platelet aggregation contributes to both the development of atherosclerosis and acute platelet thrombus formation followed by embolization producing cyclic flow reductions in stenosed and damaged human coronary arteries. According to Osman et al. (1998) the consumption of grape juice, containing phenolic inhibitors of palet agregation, may protect against the development of coronary artery disease and acute occlusive thrombosis. Also, Du et al. (2007) indicated that grape seed polyphenols could protect against cardiac cell apoptosis *via* the induction of endogenous antioxidant enzymes. This may be an important mechanism underlying the protective effects of GSE observed with various forms of cardiovascular disorders. Incubation of cardiac H9C2 cells with micromolar concentrations of catechin or proanthocyanidin B4 resulted in a significant induction of cellular antioxidant enzymes in a concentration-dependent manner. Furthermore, catechine or proanthocyanidin B4 pretreatment led to a marked reduction in xanthine oxidase/xanthine-induced intracellular reactive oxygen species accumulation and cardiac cell apoptosis.

Finally, Fritzpatrick et al. (1993) showed that grape skin extract caused endothelium-dependent relaxation (EDR) of rat aortic rings, and this activity was accompanied by elevated levels of cyclic GMP. The compounds present in grape seeds exhibiting the most EDR activity were proanthocyanidin trimers, tetramers, pentamers, and polymers and their gallates, as well a dimmer gallate (Frizpatrick et al. 2000).

4. ANTICARCINOGENIC ACTIVITY

Many authors have reported on the cytotoxic effects of grape seed polyphenols (GSPs) in tumor cells. Supplementation of the diet with GSPs decreased experimental carcinogenesis or delayed tumor onset. The modulation of various molecular targets by grape proanthocyanidins *in vitro* and *in vivo* tumor models suggests their importance, contribution and mechanism of action to the prevention of cancers of different organs (Katiyar 2006, 2007, 2008, Nandakumar et al. 2008, Meeran and Katiyar 2008). Some confirmation of the anticarcinogenic activity of grape phenolics are reported in Table 3.

In experiments of Mantena et al. (2006) with breast cancer cells, GSPs significantly inhibited cellular proliferation and viability and induced apoptosis in 4T1 cells in a time- and dose-dependent manner. GSPs increased the expression of *BAX*, induced the release of cytochrome c and expression of *APAF-1* while decreased the expression of *BCL-2* in 4Ta cells. Dietary GSPs inhibited *in vivo* growth of 4T1 breast tumor cells. In Balb/c mice they inhibited metastasis of the tumor cells from the primary tumor site to the lungs. The increase in the release of cytochrome c, induction of the expression of *APAF-1* and activation of caspase 3 in 4T1 tumors in Balb/c mice was caused by dietary GSPs.

Aromatase is the enzyme that converts androgen to estrogen. It is expressed at higher levels in breast cancer tissues than in normal breast tissues. Kijima et al. (2006) showed GSE to be potentially useful in the prevention/treatment of hormone-dependent breast cancer through the inhibition of aromatase activity as well as its expression.

The *in vitro* and *in vivo* studies of Eng et al. (2003) demonstrated that procyanidin B dimers from grape seeds could be used as chemopreventive agents against breast cancer by suppressing *in situ* estrogen biosynthesis. In experiment with rats, administration of GSE in a dry food diet resulted in a 50% reduction in mammary cancer (Kim et al. 2004).

The matrix metalloproteinases (MMPs) play a central role in the processes of tumor invasion, angiogenesis and metastasis. The regulation of the expression of MMPs in tumor cells is regulated by signalling cascade of mitogen-activated protein kinases (MAPK). Vayalil et al. (2004) showed that GSPs inhi-

Table 3. Anticarcinogenic activity of grape phenolics.

Material	Effects/results	Reference
Grape seed extract	Treatment of prostate cancer	Huang et al. (2008)
Grape seed extract	Treatment of skin cancers	Meeran and Katiyar (2008)
Grape seed extract	Increased apoptosis of colon cancer cells	Engelbrecht et al. (2007)
Fractions of grape seed extract	Inhibition HT29 human colon cancer cells cell proliferation	Lizarraga et al. (2007)
Grape seed extract	Inhibition of UVB-induced photocarcinogenesis in mice	Sharma et al. (2007)
Grape seed extract	Promotion of apoptosis in human epidermoid carcinoma A431 cells	Meeran and Katiyar (2007)
Grape seed extract	Chemopreventive agent against colorectal cancer	Kaur et al. (2006)
Fractions of grape seed extract and catechin	Decrease human breast cancer cells viability and proliferation	Faria et al. (2006)
Grape seed proanthocyanidins	Induction of apoptosis and inhibition of metastasis of highly metastatic breast carcinoma cells	Mantena et al. (2006)
Grape seed proanthocyanidins	Induction of mitochondria-associated apoptosis in human acute myeloid leukaemia 14.3D10 cells	Hu and Qin (2006)
Grape seed extract	Breast cancer chemopreventive potential due in part to its capacity to block carcinogen-DNA adduct formation, modulate activities of carcinogen-metabolizing enzymes, and suppress ROS in these noncancerous human breast cells	Singletary et al. (2007)
Grape seed extract	Prevention of photocarcinogenesis in mice	Mittal et al. (2003)

inhibited expression of matrix MMPs in human prostate carcinoma cells which is associated with the inhibition of activation of MAPK. GSP also inhibited nuclear factor κB (NFκB). The results of this investigations provided the molecular basis for the development of GSPs as a novel chemopreventive agent for prostate cancer therapy. Furthermore, the results of Tyagi et al. (2003) suggested that anticancer effects of GSE in prostate cancer is mediated *via* the impairment of mitogenic signalling and activation of c-Jun N-terminal kinase (JNK), causing growth inhibition and apoptosis, respectively. GSE significantly inhibited the growth of prostate cancer PC-3 cells in a concentration- and time-dependent manner, but had only a mild inhibitory effect on the kidney cells (Huang et al. 2008). Kaur et al. (2006) used LNCaP human prostate cancer cells as a model to assess GSE efficacy and associated mechanisms. GSE caused both caspase-dependent and caspase-independent apoptosis as evidenced by cytochrome c and apoptosis-inducing factor release into cytosol. Procyanidin B2-3,3'-di-*O*-gallate was found as a novel biologically active agent in GSE that should be studied in greater detail to determine its effects against prostate carci-

noma cells (Agarwal et al. 2007). Also, Meeran and Katiyar (2007) suggested that GSPs possess chemotherapeutic potential against human epidermoid carcinoma cells *in vitro*. The treatment of A431 cells with GSPs resulted in a dose-dependent increase in apoptotic cell death (26-58%), which was associated with an increased protein expression of proapoptotic BAX, decreased expression of antiapoptotic *BCL-2* and *BCL-XL*, loss of mitochondrial membrane potential, and cleavage of caspase-9, caspase-3 and PARP.

The results of Sharma et al. (2007) showed that GSP have the ability to protect the skin from the adverse effects of UVB radiation *via* modulation of the MAPK and NF-kappaB signalling pathways and provide a molecular basis for the photoprotective effects of GSPs in an *in vivo* animal model. Dietary GSPs resulted in inhibition of the expression of proliferating cell nuclear antigen, cyclin D1, inducible nitric oxide synthase, and cyclooxygenase-2 in the skin.

In the study of Engelbrecht et al. (2007) GSE (10-100 μg/ml) significantly inhibited cell viability and increased apoptosis in CaCo2 cells, but did not alter viability in the normal colon cell line (NCM460). The increased apoptosis observed in GSE-treated CaCo2 cells correlated with an attenuation of PI3-kinase (p110 and p85 subunits) and decreased PKB Ser(473) phosphorylation. GSE might thus exert its beneficial effects by means of increased apoptosis and suppression of the important PI3-kinase survival-related pathway. Lizarraga et al. (2007) observed that the most efficient fractions of GSE in inhibiting cell proliferation, arresting the cell cycle in G(2) phase and inducing apoptosis were the ones with the highest percentage of galloylation and mean degree of polymerization. Additionally, the antiproliferative effects of grape fractions were consistent with their oxygen radical-scavenging capacity and their ability to trigger DNA condensation-fragmentation.

The human keratinocyte cell line HaCaT and the mouse fibroblast cell line 3T3 were used by Ugartondo et al. (2007) to study the cytotoxicity of the different fractions of grape pomace extract after 24, 48, and 72 h of exposure. Antioxidant activity of the fractions was evaluated by measuring the inhibition of hemolysis mediated by AAPH [2,2´-azobis (2-amidinopropane) dichloride]. The results demonstrated that the polyphenolic fractions studied showed high antioxidant capacity at a concentration range that is not harmful to normal human cells.

GSE may be an effective chemopreventive agent against colorectal cancer. In experiments of Kaur et al. (2006a) with human colorectal cancer HT29 and LoVo cells in culture for proliferation, cell cycle progression, and apoptosis GSE (25-100 mg/mL) caused a significant dose- and time-dependent inhibition of cell growth with concomitant increase in cell death. GSE-induced cell death was apoptotic and accompanied by caspase-3 activation. GSE feeding to mice at 200 mg/kg resulted to time-dependent inhibition of tumor growth without any

toxicity and accounted for 44% decrease in tumor volume per mouse after 8 weeks of treatment. Finally, when polyphenolic red wine extract was mixed in diet at a dose of 50 mg/kg body weight and administrated for 16 weeks protected rats against azoxymethane-induced colon carcinogenesis by a mechanism probably involving increased apoptosis in tumors (Caderi et al. 2000). The tumors (adenomas and cancers)/rat in the group treated with wine polyphenolics was 1.63±1.6 whereas in the control group 2.54 ± 1.6.

5. ANTIMICROBIAL ACTIVITY

Results from several studies have confirmed the antimicrobial activity of grape seed extracts. Shoko et al. (1999) reported the antimicrobial activity of methanol extract from grape seeds. Gallic acid was identified as an active compound for the inhibition of *Escherichia coli* and *Salmonella enteritis*. In the sudy of Jayaprakasha et al. (2003) it was found that acetone and methanolic extracts of *Vitis vinifera* were the most effective antibacterial fraction against Gram-positive bacteria when compared to Gram-negative ones. The minimum inhibitory concentration (MIC) of the methanolic extract was found to be 900 ppm for *Bacillus subtilis*, *Bacillus cereus* and *Bacillus coagulans* and 1000 ppm for *Staphylococcus aureus*, 1250 ppm for *Escherichia coli* and 1500 ppm for *Pseudomonas aeruginosa*. The MIC of acetone extract was 850 ppm for *B. cereus*, *B. subtilis* and *B. coagulans* and the same for other organism as the methanolic extract. Condensed tannins (particularily tetramers and pentamers) were found to affect cell viability of wine lactic acid bacteria *Oenococcus oeni* and *Lactobacillus hilgardi* (Figueiredo et al. 2008).

Also, Gram-positive cocci were killed after 30-90 min of incubation in GSE (0.5-1.5 mg/ml), and after 8 hours in evodia extract (0.5-1 mg/ml), respectively. *C. albicans* was only susceptible to evodia (MIC 0.5 mg/ml; Thuile et al. 2003). The MIC of red grape skin, epicatechin polymer fraction, and commercial available GSE on *Streptococcus* mutans were 0.5, 1.0, and 2.0 mg/ml, respectively (Smullen et al. 2007). Epicatechin polymer fraction from red grape seed were bacteriostatic and prevented acid production when added at the MIC to cultures of *Streptococcus* mutans grown in a chemically defined medium supplemented with either glucose or sucrose. The extracts also reduced adherence of *Streptococcus* mutans to glass.

Furthermore, Sivarooban et al. (2007) evaluated the inhibitory activities of 1% GSE and of nisin (6,400 IU/ ml) against *Listeria monocytogenes* and the combination of nisin and GSE both in tryptic soy broth with 0.6% yeast extract (TSBYE) and on the surface of full-fat turkey frankfurters. The combination of nisin and GSE gave the greatest inhibitory activity in both TSBYE and on turkey frankfurters with reductions of *L. monocytogenes* populations to undetect-

able levels after 15 h and 21 days, respectively. This combination of two natural antimicrobials had the potential to control the growth and recontamination of *L. monocytogenes* on RTE meat products.

Finally, grape juice and skin and seed extracts of *Vitis vinifera* cv Ribier black table grapes were found to be highly inhibitory towards *Listeria monocytogenes*. This grape juice was also active against all other *Listeria* species tested but not against *Bacillus cereus*, *Salmonella menston*, *Escherichia coli*, *Staphylococcus aureus* or *Yersinia enterocolitica*. Fractionation of the extracts showed that the antilisterial activity was strongest in the polymeric phenolic fractions (Rhodes et al. 2006). Also, grape phenolic extracts obtained from *Vitis vinifera* cvs Cabernet Franc and Pinot noir and *Vitis* interspecific hybrids Baco noir and Noiret, especially from pomace, despite major differences in their phenolic content were highly effective against specific virulence traits of *Streptococcus mutans* (Thimothe et al. 2007).

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15

MOLECULAR ASPECTS OF GRAPEVINE-PATHOGENIC FUNGI INTERACTIONS

E. Gomès[1] & P. Coutos-Thévenot[2]

[1] Institut des Sciences de la Vigne et du Vin,
UMR INRA 1287 Ecophysiologie et Génomique Fonctionnelle de la Vigne,
Domaine de la Grande Ferrade, 33883 Villenave d'Ornon, FRANCE

[2] FRE CNRS 3091 Physiologie Moléculaire des Transports de sucres
Université de Poitiers, Bâtiment de Botanique,
40 Av. du Recteur Pineau, 86022 Poitiers Cedex, FRANCE

1. INTRODUCTION

Grapevine is a major and highly valuable fruit crop with roughly 2.25 mil ha grown worldwide in 2007 (source: U.S. Food and Agriculture Organization). Unfortunately, most of the premium cultivars used for winemaking, including the widely used European *Vitis vinifera* cultivars, are highly susceptible to several pathogenic microorganisms including fungi, oomycetes, bacteria, phytoplasma and viruses. In the past 15 years, the understanding of grapevine-pathogen interactions has entered the molecular era and will most certainly constitute a basis for future improvement of grapevine disease tolerance. After a brief presentation of the main fungal- or oomycete-induced diseases, this chapter aims to give an overview of some aspects of grapevine-pathogenic fungi or oomycete interactions, at the molecular level. It includes an overview of resistance gene analogs, elicitors that induce defense reactions in grapevine, signalling pathways and gene activation.

2. MAIN GRAPEVINE FUNGAL OR OOMYCETE-INDUCED DISEASES

2.1. Foliage and berry diseases

2.1.1. Powdery mildew

Powdery mildew, caused by the ascomycete *Uncinula necator* (syn.

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Erysiphe necator), an obligate biotrophic parasite of grapevine, is considered to be one of the most important fungal diseases in viticulture worldwide. Symptoms appear as grayish powdery or dusty patches of fungus growth on the upper side of the leaves and on other green parts of the vines, leading to a decrease in photosynthetic activity. In infected clusters, berries turn hard, brown, are smaller than uninfected ones, and may split open. Besides direct loss of yield, infected berries fail to properly mature and significantly alter wine quality (Calonnec et al. 2004). Almost no *V. vinifera* cultivar is immune to *U. necator*, but other grapevine species such as *Vitis labrusca*, *Vitis aestivalis* or *Vitis berlandieri* as well as *Muscadinia rotundifolia* possess various levels of resistance (Mullins et al. 1992).

2.1.2. Downy mildew

Downy mildew is caused by the oomycete *Plasmopara viticola*, also an obligate biotrophic parasite of grapevine. It still is one of the most destructive grapevine diseases in Europe and in the eastern half of the United States. Downy mildew affects the leaves, fruit, and shoots of grapevines. First symptoms occur as yellowish oily lesions on the leave upper surfaces; they rapidly give rise to white, felt-like "downy" fungal mass on the corresponding lower sides of the leaves. Infected berries first appear grayish then turn "downy" during pathogen sporulation. Yield losses occur through death of leaf tissue, low-quality fruit, and weakened young shoots.

2.1.3. Grey mould

Grey mould is the third major fungal disease affecting grapevine foliage and berries, particularly severe in areas where wet weather occurs between véraison and harvest. It is due to the broad host-range necrotrophic ascomycete *Botrytis cinerea*, which causes necrotic spots on leaves, total or partial destruction of the bunches before flowering and later on, rotting of berry clusters. Besides losses of fruit yield, infection of berries by *B. cinerea* also deteriorates wine quality by inducing the appearance of mushroom earthy off-odors (La Guerche et al. 2006).

Grey mould, powdery and downy mildews are controlled at the vineyard mostly by chemical spraying, sterol demethylation inhibitors or quinone outside inhibiting fungicides. However, besides negative environmental impacts, pathogens develop resistances towards these pesticides (Délye et al. 1997, Leroux et al. 1998, Chen et al. 2007).

2.2. Wood decay diseases

2.2.1. *Eutypa* dieback

Eutypa dieback is a wood decay disease caused by the ascomycete *Eutypa lata*. Symptoms do not usually appear until vines are at least six years old. Shoot symptoms are most evident during the beginning of the spring, with shoot arising from infected trunks being stunted with small chlorotic leaves (Moller and Kasimatis 1978). Berries fail to develop or develop very poorly, inducing yield losses ranging from 30 to 60% on highly susceptible cultivars (Munkvold and Marois 1994). *Eutypa* dieback shoot symptoms are always accompanied by a canker, which often appears V-shaped in a cross-section of the perennial wood. Cankers progress toward the trunk, killing the distal portions of the vine, and eventually, the entire vine may die in an average period of 10 years after the initial infection (Pascoe 1999). Currently, there is no cure for *Eutypa* dieback.

2.2.2. Esca

Esca, a.k.a. 'apoplexy' or 'lack measles' is a complex trunk disease involving at least five fungi, *Fomitiporia punctata*, *Stereum hirsutum*, *Phaeoacremonium aleophilum*, *Phaeomoniella chlamydospora*, and *E. lata*, that obstruct the vascular system (Larignon and Dubos 1997). It affects both young and older vines. Cross section in infected trunks shows a central soft, white necrosis (touchwood), surrounded by a brownish hard zone. Esca develops slowly in the grapevine until the plant exhibits a sudden apoplectic decline, eventually killing the vine within a few days. No chemical is currently available to control esca.

3. GRAPEVINE RESISTANCE GENES ANALOGS

The European grape *V. vinifera*, which accounts for about 90% of worldwide grape production for wine making, shows very low disease resistance. On the other hand, wild American species, such as *Muscadinia rotundifolia*, are resistant to various pathogens, including *U. necator* (powdery mildew), *P. viticola* (downy mildew) or *Xylella fastidiosa* (Pierce's disease; Olmo 1986). Enhancing the resistance of cultivated grapevine to diseases therefore constitutes a major goal for breeders (Bisson et al. 2002).

3.1. R-genes and the plant immune system

In the past decade, our understanding of the molecular basis of plant disease resistance has increased steadily. Plants lack the adaptive immune system, which is the privilege of the vertebrates. To detect and successfully ward off pathogens, plants rely solely on innate immunity of each cell and on systemic signals emanating from infected sites (Dangl and Jones 2001). It is now widely admitted that the plant immune system uses two distinct defense systems (Chisholm et al. 2006). A first line of defense uses transmembrane pattern recognition receptors to recognize slowly evolving pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin, lipopolysaccharide or fungal/oomycete cellulose binding elicitor proteins to activate basal defenses (Ausubel 2005). Pathogens, however, have evolved to acquire mechanisms that help them to by-pass PAMP-triggered immunity. Plants struck back by setting up a second line of defense that relies on proteins which recognize pathogen effectors or modifications of their cellular targets, a mechanism called effector-triggered immunity. Both PAMP- and effector-triggered immunity rely on the so-called resistance genes (*R*-genes).

R-genes can be grouped into 5 classes (Ellis et al. 2000, Dangl and Jones 2001) encoding for: (i) cytoplasmic serine/threonine kinases such as the *Pto* gene in tomato (Fig. 1A); (ii) extracellular leucine-rich re-peats (LRRs) proteins anchored to a transmembrane domain, as exemplified by the tomato *Cf-9* gene

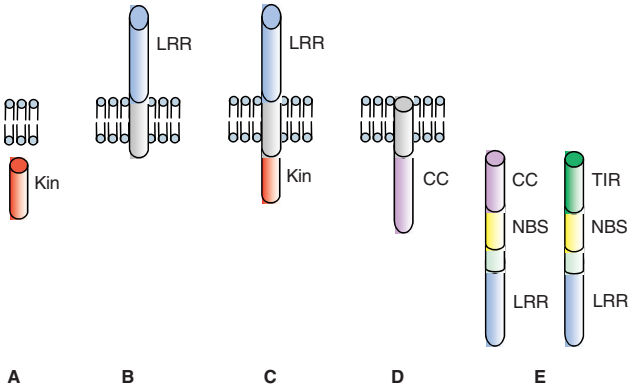

Fig. 1. Schematic representation of the *R*-genes products. (A) Intracellular Pto-like serine/threonine kinases (Kin). (B) Cf-9-like trans-membrane-anchored Leucine-Rich repeats (LRR). (C) Xa21-like proteins with an intracellular serine/threonine kinase domain and an extracellular LRR domain. (D) RPW8 protein with a transmembrane-anchored coiled-coil (CC) domain. (E) Intracellular NBS-LRR proteins with an LRR domain in their C-terminus, a nucleotide binding site (NBS) and either a toll/interleucine-1 receptor (TIR) or a CC domain in their N-terminus. Adapted from Dangl and Jones (2001), with permission.

(Fig. 1B); (iii) receptor-like kinases (RLKs) with an extracellular LRR and an intracellular serine/threonine kinase (i.e. the rice gene *Xa21*, Fig. 1C), (iv) proteins with an N-terminal transmembrane anchor and a cytoplasmic coiled-coil (CC) domain encoded by the *Arabidopsis RPW8* (Resistance to Powdery Mildew 8, Fig. 1D) genes; and (v) proteins with a nucleotide binding site (NBS), and a LRR domain in their C-terminus (NBS-LRR proteins, Fig. 1E). That later class of *R*-genes can be sub-divided in two sub-classes based on their N-terminal domain (Bai et al. 2002), which can either be a toll/interleukine-1 receptor (TIR-NBS-LRR, specific to dicotyledonous species) or a coiled-coil domain (CC-NBS-LRR, present in all angiosperms).

3.2. R-genes in grapevine

NBS-LRR genes are the most largely represented *R*-genes in plant genomes, as exemplified by the 149 genes found in the *Arabidopsis* genome (Meyers et al. 2003), or the 480 in the rice genome (Zhou et al. 2004). Grapevine is no exception and in a survey of the grape cv Pinot Noir draft genome Velasco et al. (2007) detected 233 genes encoding for proteins containing both NBS and LRR domains (InterPro IPR001611 and IPR002182, respectively). Among them, 84 genes belong to the CC-NBS-LRR subfamily while the TIR-NBS-LRR subfamily includes 37 genes. Additionally, 112 truncated NBS-LRR encoding genes are also present in the grape genome.

A complete inventory of defense-related RLKs genes is not easy to make, because these proteins are also implicated in a wide range of developmentally-related signalling pathways (Shiu and Bleecker 2001). Nevertheless, 53 genes encoding putative RLKs have been identified in grapevine (Di Gaspero and Cipriani 2003), eight of them being closely related to the Pto cytosolic protein from tomato and 3 to the products of the *R*-genes *Xa21* from rice and its *Arabidopsis* homolog *FLS2*.

To date, no true homolog of the *Arabidopsis RPW8* resistance genes have been identified in grapevine. Two genes, however, *VRP1-1* and *VRP1-2* (for *Vitis* Resistance to *Plasmopara* 1-1 and 1-2) encode CC-NBS-LRR with a RPW8 domain in their N-termini (Kortekamp et al. 2008). Such chimeric resistance proteins could link the pathogen effector-triggered (gene-for-gene) responses attributed to NBS-LRR proteins with the basal general resistance responses credited to RPW8 proteins (Xiao et al. 2005, Wang et al. 2007). Interestingly, *VRP1-1* and *VRP1-2* sequences show nucleotide polymorphism that led to amino acid substitutions at several positions when compared in the downy mildew resistant *Vitis* accession Regent and the susceptible Pinot noir (Kortekamp et al. 2008), making them potentially interesting to breed resistance.

3.3. Cluster of R-genes map to chromosomal region of grapevine genetic disease resistance

R-genes, particularly the NBS-LRR class, are arranged in clusters in plant genomes, a physical disposition that generates sequence variation and gene family expansion at a high rate, a point that is crucial to generate new resistance specificities (Bergelson et al. 2001, Meyers et al. 2003, Zhou et al. 2004). In grapevine, *TIR-NBS-LRR* gene clusters are preferentially located on linkage group (LG) 18, *CC-NBS-LRR* gene clusters on LG 9 and 14 and truncated *NBS-LRR* on LG 12 and 13 (Velasco et al. 2007, Moroldo et al. 2008). *RLK*s are more evenly dispersed in the grape genome, with LG 14 scoring the highest number of RLK coding genes (Moroldo et al. 2008).

In agreement with the role of *R*-genes in plant innate immunity, several clusters of *NBS* genes map to chromosomal regions where genetic resistance to bacterial, fungal or oomycete-induced diseases were previously assigned (Di Gaspero et al. 2007, Velasco et al. 2007). The *Run1* locus (Resistant to *Uncinula necator* 1), originating from *M. rotundifolia* (Pauquet et al. 2001), which confers resistance to powdery mildew, has a counterpart in the *Vitis* genome physically located on LG 12, in a region which contains several copies of *R*-genes (Barker et al. 2005). Additional loci for powdery mildew resistance have been reported on LG 15 and 14 in *Vitis* hybrids (Dalbo et al. 2001, Fischer et al. 2004). In the same region of LG 14, a primary locus for resistance to Pierce's disease causal agent, *Xylella fastidiosa*, was identified in the wild grape *Vitis arizonicai* (Krivanek et al. 2006). Quantitative trait loci for downy mildew resistance have been mapped with SSR markers to the distal part of LG 18 (Fischer et al. 2004), and in the middle of LG 7 (Grando et al. 2003) in *Vitis* resistant accessions, nearby regions where *NBS-LRR* genes are clustered. Another major determinant responsible for resistance to *P. viticola* has been identified on LG 12 (Merdinoglu et al. 2003). In conjunction with the knowledge of the grape genome sequence, the availability of linkage maps based on transferable molecular markers (reviewed by Doligez et al. 2006) will constitute valuable tools for pathogen resistance breeding in premium *Vitis* cultivars.

4. ELICITORS ACTIVE ON GRAPEVINE

Several molecules coming from microorganisms, plants or algae have been characterized as elicitors. These molecules, which encompass lipids, oligosaccharides and proteins, trigger defense responses in plants, such as the hypersensitive response (HR), the localized acquired resistance (LAR) or the systemic acquired resistance (SAR). Besides, some molecules coming from non-

pathogenic microorganisms potentiate ISR (Induced Systemic Resistance) in plants, leading to tolerance against many pathogens. These signal molecules, often recognized by a receptor (see *R*-genes, § 3), offer several possible applications as natural inducers of defense and tolerance in plants.

4.1. Oligosaccharide elicitors

Several elicitors such as β-1,3-glucans or α-1,4-oligogalacturonides are known to be active in many plant species. In grapevine some oligosaccharides appear to be efficient, like β-1,4-cellodextrins (Aziz et al. 2007), cyclodextrins (Morales et al. 1998, Bru et al. 2006), laminarin extracted from algae (Aziz et al. 2003) and induce tolerance against *B. cinerea* or *P. viticola*. Sulfated glucans like β-1,3-glucan sulfate enhance tolerance to *P. viticola* (Trouvelot et al. 2008). In addition, two novel oligosaccharidic elicitors were purified from *B. cinerea*. These molecules, obtained from crude mycelium cell wall extracts and culture filtrate preparations, were named Botrycin and Cinerein, respectively (Repka et al. 2001a, Repka 2002, 2006). In all cases, treatment with these elicitors triggered the classical PR (Pathogenesis Related) proteins accumulation, reactive oxygen species (ROS) production, as well as Ca^{2+}, jasmonic acid (JA) and salicylic acid (SA) signalling pathways.

4.2. Lipid elicitors

If several lipid molecules are known to act as elicitors in plants, in grapevine the main lipidic elicitor described up to now is the ergosterol molecule. This sterol, which is typical of fungi, was described as an inducer of a specific set of defense-related genes in tobacco and associated signal transduction pathways (Kasparovsky et al. 2004, Lochman and Mikes 2006, Rossard et al. 2006). Some PR-proteins (PR-14) and enzymes of the stilbene biosynthesis pathway are highly induced in grapevine by ergosterol treatment, most probably through the activation of WRKY trans-activator factors (Gomès et al. 2003, Laquitaine et al. 2006, Marchive et al. 2007). The putative specific receptors of ergosterol remain to be identified.

4.3. Proteinaceous elicitors

In terms of proteinaceous elicitors, two major lines of work have emerged these last years. Poinssot et al. (2003) described an endo-polygalacturonase secreted in the culture medium of *B. cinerea*, which is able to trigger full-scale early defense reactions in grapevine cell suspension cultures. Apparently, elici-

tor effect is not due to the enzyme activity. More recently, it was demonstrated that oligandrin, an elicitin of *Pithium oligandrum*, enhances *Vitis* tolerance towards *B. cinerea* (Mohamed et al. 2006). These results are of interest because elicitins were previously described to be active on tobacco but not on other plants (Ponchet et al. 1999). The fact that an elicitin could induce protection against fungal pathogens without HR response, but with modifications of the redox status of the cells, is indeed a very innovative concept.

5. EARLY CELLULAR EVENTS IN DEFENSE REACTIONS

Numerous studies of early plant defense reactions have been made in the last ten years. In Fig. 2 the principal steps, keys of the knowledge about this signal transduction pathway, are summarized. In model plants such as tobacco or *Arabidopsis* all these steps including perception, calcium flux activation, ROS synthesis, MAPKs (mitogen-activated protein kinases) or phosphatases activation are well characterized. In grapevine, the amount of information available, regarding early events of defense reactions, was less developed until recently (Busam et al. 1997, Jacobs et al. 1999). In the past few years, several publications aimed to decipher defense-related early signalling events in grape-

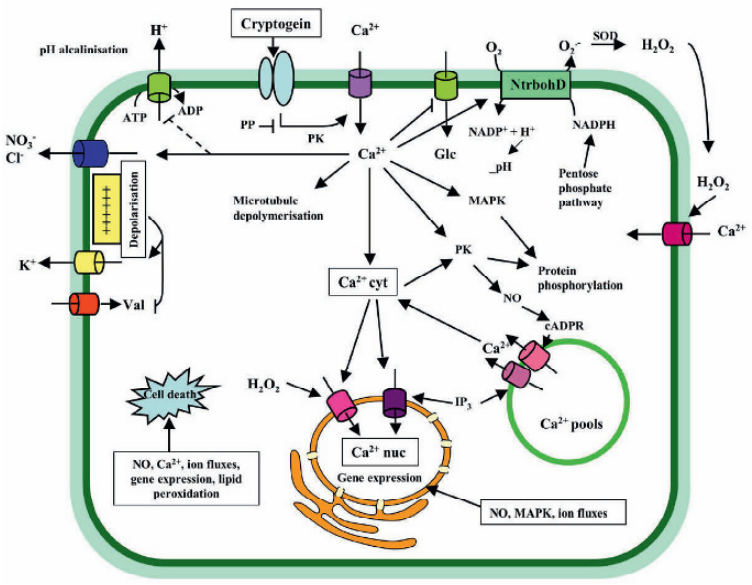

Fig. 2. Early cellular events triggered by pathogen recognition, as exemplified by the case of the signalling cascades induced on tobacco cells by cryptogein, an elicitin from *Phytophthora cryptogea*. Reprinted from Garcia-Brugger et al. (2006), with permission.

vine using model cell suspension cultures or entire plants (Repka 2006, Vandelle et al. 2006).

As described in paragraph 4, many elicitors have been characterized and some putative receptors identified in the Pinot Noir genome sequence. After the classical step of pathogen (or elicitor) perception, most of the crucial signalling events have been identified in grapevine. It seems that Ca^{2+} influx is the first event occurring after elicitation, by modulation of plasmamembrane Ca^{2+}channels. Later on, $NO^{\bullet}$ is synthesized and mobilizes internal stores of Ca^{2+}. Then come ROS ($O_2^{\bullet-}$, H_2O_2) production, MAPK and phosphatase activities that have also been evidenced in elicited plants.

A second class of signalling compounds (JA, SA and ethylene) are produced as endogenous signalling molecules and elicit pathogen protection process. Finally, a pool of defense genes gets activated, including PR proteins encoding genes, as well as phenylpropanoid and stilbene biosynthesis genes (Repka 2006, Vandelle et al. 2006). A particular class of PR-proteins is induced, the lipid transfer proteins (LTP, PR-14). These secreted proteins are able to bind JA, at least *in vitro* and trigger protection against *B. cinerea* (Girault et al. 2008). LTP-JA complexes could be one element of SAR signalling, leading to global protection of the plant against pathogens (Grant and Lamb 2006).

In conclusion, it seems that grapevine species possess all the signalling elements to respond to a pathogen attack. Nevertheless, it is clear that there are some differences between wild grapevine species and the European *V. vinifera*. For example, some *R* genes are absent from *V. vinifera* (*Run1*) and could explain the sensitivity of premium cultivars to pathogenic fungi (Barker et al. 2005).

6. GRAPEVINE PR-PROTEIN GENES

As already mentioned, one of the major steps in plant defense reactions is the synthesis of a particular class of proteins classically termed as ‘PR’ proteins. These proteins were distributed in 1999 in 11 classes. Recently, Van Loon et al. (2006) published a broader classification with 17 classes. The PR-proteins are defined as proteins that are not expressed in plants without pathogen interaction or largely induced during infection. For a protein to be classified as a PR-protein, it is necessary that its induction is described for two different plant/pathogen systems in two different laboratories. Not all the PR-proteins classes have been described in grapevine so far.

A putative sequence of a *V. vinifera* PR-1 protein was identified and cloned by Bertsch et al. (2003). The expression kinetics of a *PR-1* defense-related gene is strongly dependent on the nature of elicitor used (Repka 2001b). Comparison of *PR-1* expression in grapevine cell cultures after inoculation with

a host and a non-host pathogen revealed a high *PR-1* expression rate 3 weeks post-inoculation in *V. vinifera* cv Riesling and *Vitis riparia* cv Gloire de Montpellier, even if pathogens development was not blocked. Thus, the role of *PR-1* expression in impeding the downy mildew pathogen remains equivocal. It seems that expression of *PR-1* genes is a general stress response in some grapevine culture systems (Wielgoss and Kortekamp 2006). Three PR-1-like proteins were found to accumulate in grapevine leaves after infection by *U. necator*. Expression of these proteins was also induced by elicitor treatments in grapevine cell suspension culture (Repka et al. 2000).

The PR-2, -3, -4 and -5 classes are better documented in grapevine. In a susceptible *V. vinifera* cv, such as Riesling, inoculated with *Pseudoperonospora cubensis* (downy mildew of cucumber), a non-host pathogen in grapevine, ß-1,3-glucanases (PR-2) and chitinases (PR-3 and -4) are largely accumulated in comparison with a host situation (*P. viticola*). Following treatment with *P. cubensis*, sporulation intensity was significantly reduced in Riesling after subsequent inoculation with *P. viticol*a (Kortekamp 2006). Several PR-proteins are expressed in berries at maturity and liquid chromatography-mass spectrometry analysis of grape juice revealed the presence of several PR-3 and PR-5 (thaumatin-like) isoforms with different molecular masses, as a function of the varieties (Hayasaka et al. 2001).

U. necator, the causal agent of grapevine powdery mildew, induces expression of chitinases and ß-1,3-glucanases in leaves and berries in various grapevine cultivars, including susceptible ones. Indeed, Jacobs et al. (1999) showed that the hydrolytic activity was directly related to the severity of infection at the pathogen location. PR-2, -3 and -5 were also observed in infected berries at pre-véraison stage and were highly induced by ethephon treatment. These results demonstrate a paradox: even if these classes of PR-proteins are expressed during pathogen invasion this does not offer complete protection against *U. necator*. Probably, some other more specific proteins are necessary. Alternatively, the key of the protection might be more determined by the presence or the absence of *R*-genes (resistance genes).

Furthermore, the diversity of PR proteins expressed decreases during grape maturation (Monteiro et al. 2007) and could explain the enhanced susceptibility of the berries during the last stages of ripening. Accordingly, it was demonstrated that constitutive expression in transgenic *V. vinifera* of thaumatin-like protein protects grapevine plants against anthracnose (Jaysankar et al. 2003). These plants, however, were not protected against other fungi. In addition, induction of chitinase genes in *V. vinifera* depends on the infecting pathogen, but also the type of chitinase is different in a compatible or incompatible interaction (Robert et al. 2002). The selective expression of specific chitinases might be a reliable indicator of the SAR response in *V. vinifera* (Busam et al. 1997).

A grapevine class 10 PR-protein was cloned from *V. vinifera* leaves infiltrated with the incompatible bacterial pathogen *Pseudomonas syringae* pv Pisi (Robert et al. 2001). To our knowledge, it is the only example of *PR-10* gene characterized in grapevine. The accumulation of the corresponding mRNA was observed from 3 to 96 h post-inoculation and was followed by the accumulation, between 24 and, at least, 96 h after inoculation, of the encoded polypeptide, detected by immunoblotting.

The story of the PR-14 family is more complicated. These proteins (Lipid Transfer Proteins) were shown to be involved in several physiological processes. It is now quite clear that some isoforms are clearly involved in defense reaction signalling process (Maldonado et al. 2002, Blein et al. 2002, Grant and Lamb 2006). Several isoforms of LTP have been described in grapevine (Coutos-Thévenot et al. 1993). Some of them were induced by fungal elicitor treatments (Gomès et al. 2003). Ergosterol-induced protection of grape against *B. cinerea* relies on the expression of a type I lipid transfer protein, which is mediated by a WRKY trans-activating protein (Laquitaine et al. 2006). In addition, Girault et al. (2008) demonstrated that some grapevine LTP were able to bind JA, and that exogenous application of a LTP-JA complex induces protection of grapevine towards infection by *B. cinerea*. All LTPs, however, are not defense-related. Other isoforms seem to be involved in other physiological process, like somatic embryo development and epidermal layer formation. Accordingly, over-expression of the *VvLTP1* gene interferes with somatic embryo development in grapevine and abolishes the bilateral symmetry of embryos (François et al. 2008).

The last classes of PR-proteins that have been described in grapevine are the germin and germin-like proteins (PR-15 and -16). Recently, 7 members of the grapevine germin-like multigenic family were cloned in *V. vinifera* (Godfrey et al. 2007). Among them, one gene, *VvGLP3* (*V. vinifera* germin-like 3), have no basal expression level and is strongly induced by powdery mildew infection. Another member of the family, *VvGLP7*, responds to both *P. viticola* and *B. cinerea* infection. Some germin-like proteins exhibit oxalate oxidase or superoxide dismutase activities, but their exact role in plant defense reactions is far from being elucidated.

According to the literature, PR-6, -7, -8, -9, -11, -12, and -13, have not been described in grapevine yet. Nevertheless, using the sequences of the first member of each class to be published (Van Loon et al. 2006), a BLAST analysis indicate the presence of putative homolog genes in the Pinot noir genome (http://www.genoscope.cns.fr/externe/GenomeBrowser /Vitis/) for all these PR-protein classes, with the noticeable exception of PR-13 (thionins). Table 1 summarizes the BLAST results, by presenting for each PR-protein class the first

Table 1. Putative homolog genes for PR-6, 7, 8, 9, 11, 12 and 13 in the Pinot noir genome. For each PR-protein class, the grapevine genome database was probed with the sequence a typical member, as described by Van loon et al. (2006).

PR-protein class	Typical member	Properties	Putative grape homolog genes*	Linkage group
6	Tomato inhibitor I	Proteinase inhibitors	GSVIVT00020160001	5
			GSVIVT00020161001	5
			GSVIVT00029370001	13
7	Tomato P_{69}	Endoproteases	GSVIVT00001054001	2
			GSVIVT00001055001	2
			GSVIVT00001051001	2
			GSVIVT00001053001	2
			GSVIVT00001034001	2
			GSVIVT00001056001	2
8	Cucumber chitinase	Type III Chitinases	GSVIVT00006464001	Unknown
			GSVIVT00026961001	15
			GSVIVT00026949001	15
			GSVIVT00026950001	15
			GSVIVT00006463001	Unknown
			GSVIVT00020672001	14
9	Tobacco lignin-forming peroxidase	Peroxidase	GSVIVT00024722001	6
			GSVIVT00025396001	8
			GSVIVT00024724001	6
			GSVIVT00024717001	6
			GSVIVT00037460001	8
			GSVIVP00018771001	12
11	Parsley PR-1	Ribonuclease-like	GSVIVP00012304001	Unknown
			GSVIVP00012300001	Unknown
			GSVIVP00012296001	Unknown
			GSVIVP00005601001	Unknown
			GSVIVP00005604001	Unknown
			GSVIVP00005606001	Unknown
12	Radish Rs-AFP3	Defensins	GSVIVT00035146001	1
			GSVIVT00014577001	18
			GSVIVT00002075001	19
13	*Arabidopsis* THI2.1	Thionins	None detected	

* Putative grape PR-proteins transcripts are designated by their Genoscope annotation number (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/).

6 transcripts detected in the database (only 3 where detected for PR-12, defensins). Such an analysis is of course far from being exhaustive and is just intended to point the need of additional studies in the future to better characterize PR-protein families in grapevine.

7. GRAPEVINE PHYTOALEXINS BIOSYNTHESIS AND METABOLISM

7.1. Stilbene synthase genes

The knowledge of grapevine phytoalexin metabolism (Fig. 3) has increased vastly in the past decade. In woody plants, the classical phenylpropanoid pathway is split at the coumaroyl-CoA step by the stilbene synthase activity, which synthesizes resveratrol by condensation of 3-malonyl-CoA with one molecule of coumaroyl-CoA (Langcake and Pryce 1976, Langcake and Pryce 1977, Pont and Pezet 1990, Pezet and Pont 1995). This enzyme activity belongs to a multigenic family and has been already characterized by Wiese et al. (1994).

The first genes coding a *Vitis* stilbene synthase (*VST*) was cloned by Melchior and Kindl (1990), and afterwards several other genes were characterized (Richter et al. 2005). The recent publication of the grapevine Pinot Noir genome revealed the presence of 21 putative stilbene synthase genes, essentially on LG 10 and 16 (Velasco et al. 2007). This number fits nicely with a recent stilbene synthase gene expression analysis in infected grapes leaves (Richter et al. 2005). Stilbene synthases exhibit a high degree of homology with chalcone synthase and are known to be induced by several stimuli, including UV light, which is the major abiotic inducer (Adrian et al. 2000, Bonomelli et al. 2004).

Stilbene synthase genes are down-regulated during grape berry ripening, due to a competition between stilbene and chalcone synthase activities; that latter enzyme being activated after véraison for anthocyan and flavonoid accumulation in pellicula. A large group of biotic inducers are able to promote stilbene synthase expression and microorganisms were shown to have a direct effect on resveratrol accumulation. *B. cinerea* is the most common fungus able to act on phenylpropanoid pathway (Liswidowati et al. 1991, Bais et al. 2000, Keller et al. 2003). The level of the response, however, depends largely on the cultivar (Gabler et al. 2003), and it is possible to class the level of tolerance of cultivars in the field in regard to the capacity to accumulate resveratrol after UV light induction, as summarized in the Table 2 (Coutos-Thevenot et al. 2001).

Several other fungi were found to induce resveratrol production. *Aspergillus* induces expression of *VST* genes (Jean-Denis et al. 2006) and more problematic pathogens like three of the fungi associated with Esca, promote stilbene production (Bruno and Sparapano 2006a, 2006b). Bacteria-like *Pseudomonas*

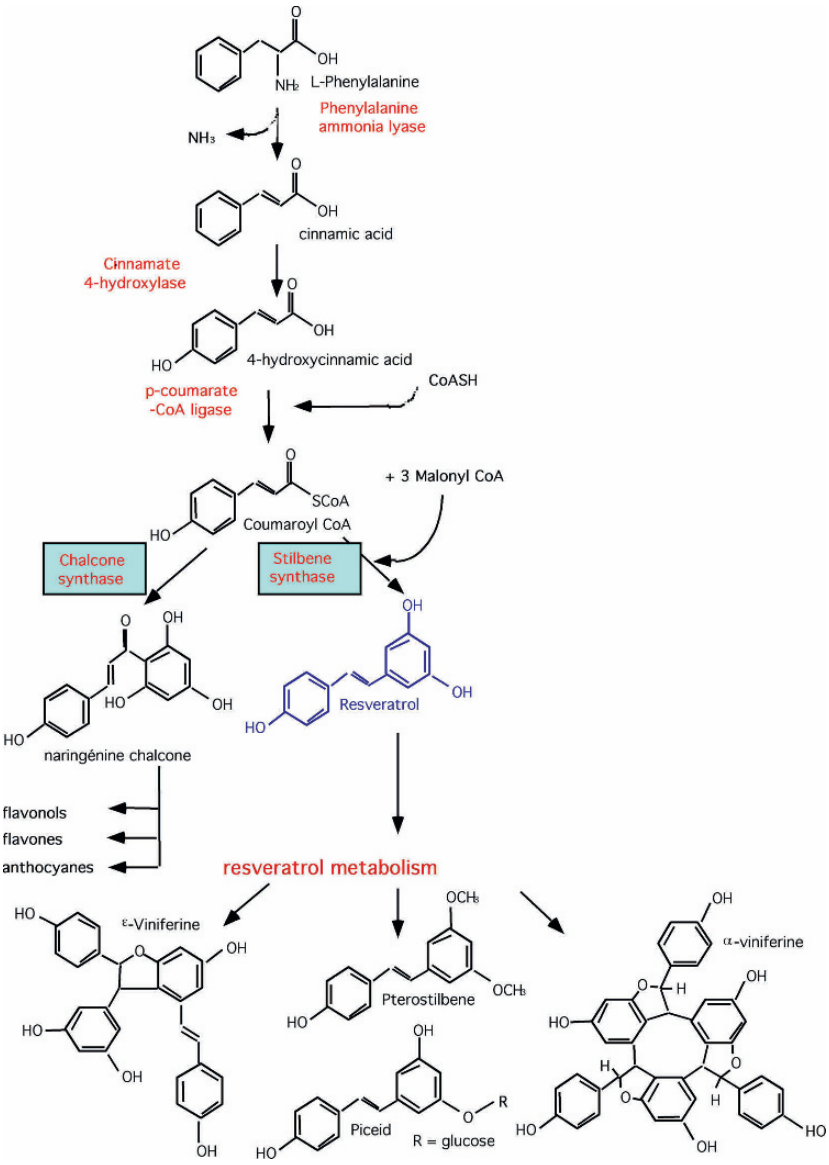

Fig 3. Biosynthesis pathway of grapevine stilbenes. Reprinted from Coutos-Thevenot et al. (2001) with permission.

syringae pv Pisi seem also to act as inducers during HR of grapevine (Robert et al. 2001). Various studies have demonstrated the ability of purified elicitors to induce *VST* gene activation or resveratrol synthesis. Ergosterol, a specific sterol of fungi and a non-specific elicitor, is highly efficient (Laquitaine et al.

2006). The endo-polygalacturonase BcPG1 produced by *B. cinerea* induces *VST1* mRNA accumulation 4 hours after treatment of grapevine cell suspension and is more active than oligogalacturonate elicitors (Poinssot et al. 2003). Several other molecules are also effective inducers of resveratrol production in grapevine (Borie et al. 2004, Laura et al. 2007), like oligogalacturonates (Aziz et al. 2004), β-1-3 glucane sulfate (Trouvelot et al. 2008), as well as chemicals like aluminium chloride (Adrian et al. 1996) or benzothiazole (Iriti et al. 2004, see also Chapter 12 in this book). Even if its mechanism of action is not well understood, resveratrol inhibits the growth of several fungi or oomycetes. Microbiologic tests revealed a good effect on *Botrytis* and *Eutypa lata* mycelium growth (Adrian et al. 1997, Coutos-Thevenot et al. 2001), *P. viticola* (Pezet et al. 2004) and *Venturia inaequalis* growth (Schulze et al. 2005). If resveratrol is active, its metabolites like pterostilbene (methylated form) and viniferins (oligomer metabolites) seem to be much more efficient (Pezet et al. 2004). On the other hand, the glycosylated form (piceid) seems to be less active and could be a soluble storage form (Pezet et al. 2004). It is suspected that enzymes, like methyl transferases and oxidases, could be involved in these mechanisms.

Table 2. Effects of *Botrytis* infection or UV treatment on resveratrol accumulation by various grapevine cultivars. Data are means of four independent experiments ± standard error. Nd: not detected. Reprinted from Coutos-Thévenot et al. (2001), with permission.

Variety / cultivars	Control (not induced)	*Botrytis* Resveratrol ($\mu g.g^{-1}$ DW)	UV light Resveratrol ($\mu g.g^{-1}$ DW)
Rupestris	nd	nd	350 (± 115)
41B Rootstock	nd	112 (± 30)	240 (± 120)
Ugni blanc 479	nd	86 (± 45)	210 (± 74)
Pinot Noir 386	nd	103 (± 31)	87 (± 49)
Folle blanche	nd	101 (± 16)	38 (± 11)

7.2. Hormones and signalling

Signalling pathways involved in stilbenes accumulation in grapevine are less clear-cut. It seems that stilbene synthase activation is probably due to a cross- talk between several pathways. The role of JA and methyl-JA are well documented with a very efficient induction (Zhang et al. 2002, Curtin et al. 2003, Larronde et al. 2003, Repka et al. 2004, Tassoni et al. 2005, Vezzulli et al. 2007), but the one of SA is less understood. It seems that SA acts on the phenylpropanoid pathway by inducing phenylalanine ammonia lyase and stilbene synthase genes expression (Chen et al. 2006, Wen et al. 2005, Wen et al.

2008). Ethylene also appears be involved (Grimmig et al. 2002).

7.3. Use in transgenic plants

After the fist evidence that tobacco plants, which do not naturally produce stilbenes, become tolerant to *B. cinerea* when transformed with *p35S-VST1* chimeric gene (Hain et al. 1993), the idea of generating transgenic grapevine that over-express stilbene synthase rapidly gained ground. The 41B rootstock was transformed with the *PR10* promoter, which is highly inducible by *B. cinerea* infection, fused to the *VST1* coding sequence. Some transgenic clones showed a high level of *in vitro* tolerance to *Botrytis* and could be also tolerant to *E. lata* (Coutos-Thevenot et al. 2001). More recently, these results were confirmed by several groups on various plant species, like grapevine (Fan et al. 2008), hop (Schwekendiek et al. 2007), oilseed rape (Husken et al. 2005), papaya (Zhu et al. 2004), poplar (Giorcelli et al. 2004) and apple (Szankowski et al. 2003). These examples prove the importance of stilbenes in plant defense mechanisms and open many future applications. Moreover, treatment of post-harvested fruits with resveratrol improves their resistance for conservation (Urena et al. 2003).

8. CONCLUSIONS

In the last 15 years, our understanding of molecular aspects of grapevine-fungi interactions has increased largely. The recent publication of the Pinot Noir genome will undoubtedly be a valuable tool for future studies. However, a fair deal of work remains to be done, to precisely decipher and finely characterize the different steps of the pathogen detection by the plant and the subsequent activation and establishment of defense reactions.

This applies, for example, to the characterization of the not yet described, but present in the grapevine genome, PR-protein families; or to the comparative studies of the genetic diversity of resistance genes and other defense-related genes, in the various cultivated and wild grapevines. The global outcome of the knowledge of grapevine defense reaction studies at the molecular level has already started to be integrated to breeding experiments, either through genetic transformation or through marker-assisted selection. It is reasonably safe to bet that this tendency will increase in the future.

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PROGRESS IN GRAPEVINE PROTOPLAST TECHNOLOGY

A.K. Papadakis[1], N. Fontes[2], H. Gerós[2] & K.A. Roubelakis-Angelakis[1]

[1]Department of Biology, University of Crete, P.O. 2209, 71209 Heraklion, GREECE

[2]Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, PORTUGAL

1. INTRODUCTION

During the past four decades, plant protoplasts –naked plant cells lacking cell walls- have been used for the development of new scientific knowledge involving cell membrane function, cell structure, synthesis of pharmaceutical products, and toxicological assessments. Furthermore, plant proptoplasts are valuable tools for biotechnological applications, such as somatic hybridization by protoplast fusion, and genetic transformation by gene transfer mediated through *Agrobacterium* and Biolistics. A prerequisite for the use of protoplasts in genetic engineering is the ability to regenerate plants from the transformed protoplasts. Plant cells possess the unique property of totipotency, that is the ability of somatic cells or protoplasts from fully differentiated, non-dividing cells, to dedifferentiate, re-enter the cell cycle, and proliferate, eventually regenerating the whole plant. Also, in the past decade, few laboratories have employed protoplast transient expression to dissect the function of *cis*-elements and *trans*-factors in many essential processes and signalling pathways (Sheen 2001).

Plant regeneration has been achieved in numerous plant species and genotypes (Davey et al. 2005). However, this has not been the case for most agronomically important plant species (perennial woody species, cereals and legumes), including the grapevine (Roubelakis-Angelakis 1993, Papadakis et al. 2001a, Papadakis and Roubelakis-Angelakis 2002). These protoplasts exhibit recalcitrance to plant regeneration. Plating efficiencies of grapevine protoplasts are low and plant development has been described only in two cases at very low frequencies (Reustle et al. 1995, Zhu et al. 1997). Nevertheless, following the

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first successful regeneration of grapevine protoplasts derived from embryogenic callus, efforts have been directed toward developing techniques for grapevine breeding using protoplast technology. Also, protoplasts are good experimental models to study important aspects of ultrastructure, genetics and physiology of plant cells.

In an effort to reveal the potential mechanism(s) affecting totipotency, several factors have been examined, such as the quality of the donor plant, the kind of donor plant organs/tissues, the isolation and culture conditions, the biochemical and ultrastuctural characteristics of newly synthesized cell walls, and the cell membrane integrity. Furthermore, oxidative stress has been considered as a potential crucial factor affecting recalcitrance, as supported by the profiles of generated reactive oxygen species (ROS) and ROS-scavenging enzymes, the modified cell redox state, as well as the altered endogenous titers of polyamine and phytoalexin levels.

Following our previous review (Papadakis et al. 2001), this revised chapter is focused mainly on the new knowledge which has been accumulated eversince.

2. ISOLATION, ASSESSMENT OF QUALITY AND CULTURE OF GRAPEVINE PROTOPLASTS

The isolation of sufficient amount of high quality protoplasts is a prerequisite for using protoplasts either as experimental model for basic research or for biotechnological applications. The yield and the quality of protoplasts are determined by several factors, such as the donor plant material (genotype, type of tissue, growth conditions of donor plants, physiological and ontogenic stage, sampling method, storage conditions, etc.) and the procedure of protoplast isolation (sterilization, type and concentration of cell wall degrading enzymes, duration of enzymic action, conditions of enzymic action, purification, etc.).

Thereafter, the fate of protoplasts is greatly dictated by the culture conditions, such as the composition of the culture medium and environmental parameters. In the last three decades, significant progress has been made in the optimization of the isolation and culture procedures; of special significance has been the use of axenic leaves from *in vitro* grown plants for the isolation of protoplasts. Also, significant progress has been made in the use of grapevine protoplasts for biotechnological applications, such as somaclonal variation, somatic hybridization and gene transfer. The regeneration of plants from embryogenic callus-derived protoplasts has been successful only in two cultivars, although mesophyll protoplasts represent much more genetically stable material. Therefore, more efforts are necessary to develop regeneration protocols for mesophyll

protoplasts and embryogenic callus-derived protoplasts from more grapevine cultivars. All the above aspects have been extensively presented in Papadakis et al. (2001a).

Grapevine protoplasts also provide a good experimental model for toxicological assessments (effect of pesticides and elicitors of natural disease resistance), especially those concerning the berries due to their uniqueness for both human consumption and wine production. Protoplasts from grape berry mesocarp tissue have been successfully isolated and purified (Fontes et al. 2007). Due to the low pH of berry tissue and its fragility, and to the wide range of secondary metabolites present, cell wall digestion and protoplast purification were performed in very special environment to maintain their integrity and viability (Fig. 1). Enzyme concentrations were extremely low (0.03 % cellulase Y-C and 0.003% pectolyase Y-23), and were combined with a relatively long digestion period. After separating the protoplasts from the enzymatic solution by sedimentation, they were purified in a discontinuous gradient of sucrose and sorbitol (500*g* for 10 min) and recovered in the interface. Besides toxicological assessments, this approach has opened good perspectives to study basic physiological mechanisms of grape development and ripening, such as sugar, organic acid and water transport, metabolism of polyphenols and host-stress interaction.

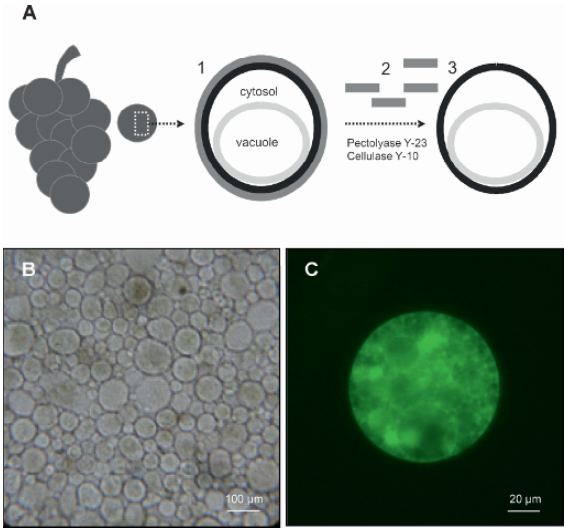

Fig. 1. (A) Isolation and purification of protoplasts from grapevine berry mesocarp. Berry discs are digested with pectolyase Y-23 and cellulase Y-10, under gentle agitation, followed by protoplast purification. 1, grapevine berry mesocarp cell; 2, cell wall debris; 3, protoplast. (B) Population of protoplasts isolated from grape mesocarp tissue observed under the light microscope. (C) Isolated protoplast from grape berry observed under UV light (epifluorescence) after staining with fluorescein diacetate to measure viability. Reconstructed from Fontes et al. (2007).

The quality of freshly isolated protoplasts is assessed as the percentage of viable protoplasts. The viability test is based on the use of various staining reagents, such as FDA (fluorescein diacetate), Evans blue (Theodoropoulos and Roubelakis-Angelakis 1990), and neutral red (Barbier and Bessis 1990). The intact plasma membrane is permeable to FDA, which is converted to a green fluorescent dye, fluorescein, by internal esterases displaying a green fluorescence in viable cells (Jones and Senft 1985). At any case, before any protoplast population is efficiently used, as either research model or for biotechnological applications, a high viability rate must be ensured.

Figure 2 depicts a protoplast population prepared from grapevine suspension cultured cells labelled with FDA observed under visible light (A) and UV light (epifluorescence, B). Comparison of the epifluorescence light with visible light images showed that most protoplasts remained viable immediately after isolation, displaying an intense green fluorescence (Fontes et al. 2008). Lee and Wetzstein (1988) and Mii et al. (1991) could regenerate callus from protoplasts with 90% viability at the beginning of culture, and Zhu et al. (1997) could regenerate plants starting with 90% of viable protoplasts. However, high viability rates of mesophyll protoplasts do not ensure high plating efficiency and sustained divisions, thus leading to the formation of regenerating calluses.

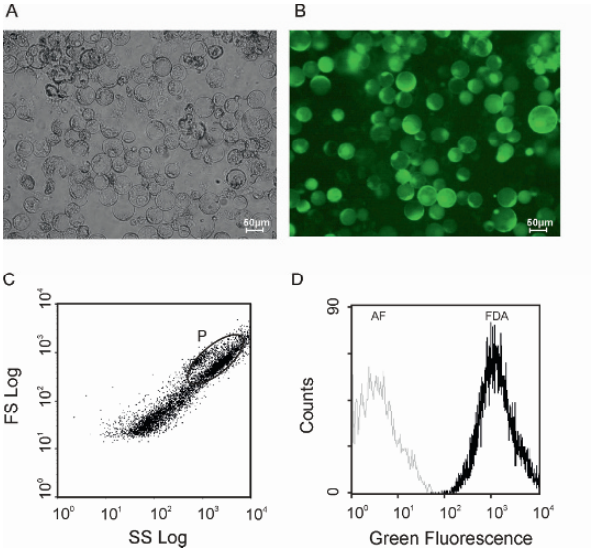

Fig. 2. Protoplast population isolated from grapevine suspension cultured cells (CSB, Cabernet Sauvignon berry) labelled with FDA (A) observed under visible light and (B) UV light (epifluorescence. (C) Flow cytometric analysis of a protoplast population labellled with FDA: scattergram for grapevine cells protoplasts after FDA staining and (D) overlay of green fluorescence and autofluorescence histograms of the same protoplast suspension of gated region. Reconstructed from Fontes et al. (2008).

Flow cytometry analysis requires that microscopical biological particles to be in suspension and allows the simultaneous quantification of multiple fluorescence emissions in the same cell or biological particle, and scattered light related to morphology (O'Connor et al. 2001). Therefore, individual cells or sub-cellular particles from heterogeneous subpopulations can be physically isolated on the basis of their fluorescence or light scatter properties (Herrera et al. 2006).

Fontes et al. (2008) exploited flow cytometry to characterize the protoplast samples purified from suspension cultured cells in order to assess the purity of the sub-populations. For each sample 15,000-20,000 protoplasts were analysed at a low flow rate. An acquisition protocol was defined to measure forward scatter (FS), side scatter (SS) and green fluorescence (FL1) on a four decades logarithmic scale. The analysis of the bi-parametric histograms, plotting log SS against log FS, revealed some heterogeneity, in both relative complexity and size (Fig. 2C). However, the sub-population of protoplasts can be easily identified since they easily stain with FDA (gated region P). Above and to the right of this region, there is also a sub-population that probably consists of protoplast aggregates. The sub-populations with the lowest scatter (below and to the left of region P) mainly correspond to sub-microscopical particles as some cell debris and cell wall residues - of relative low complexity and size, which copurified with the protoplasts. Figure 2D depicts the overlay of the green fluorescence and auto-fluorescence histograms of the gated region P.

3. RECALCITRANCE

The term *recalcitrance* has been used in plant tissue culture to denote lack of cell proliferation or lack of morphogenetic response *in vitro.* The regeneration process from protoplasts includes the stages of cell wall reconstitution, cell elongation, cell division, callus formation and plantlet formation, either by direct organogenesis or by somatic embryogenesis (Roubelakis-Angelakis 1993).

In protoplasts, recalcitrance can be expressed at three points (Roubelakis-Angelakis 1993):

- ✓ Protoplasts, shortly after isolation, exhibit low viability rates and are unable to complete reconstitution of cell wall.
- ✓ Protoplasts exhibit high viability rates, reconstitute cell walls but either fail to divide or exhibit low plating efficiencies.
- ✓ Protoplasts divide, form colonies and calluses, but either cease proliferating or do not show morphogenetic activity.

Grapevine mesophyll protoplasts seem to belong to the 2nd or/and the 3rd group. They complete cell wall reconstitution (Katsirdakis and Roubelakis-

Angelakis 1991, 1992, Fig. 3) but exhibit low plating efficiency. Several factors have been proposed to contribute to the recalcitrance of plant protoplasts including misfunction of cell membrane and oxidative stress (Roubelakis-Angelakis 1993, Papadakis and Roubelakis-Angelakis 2002, and refs therein).

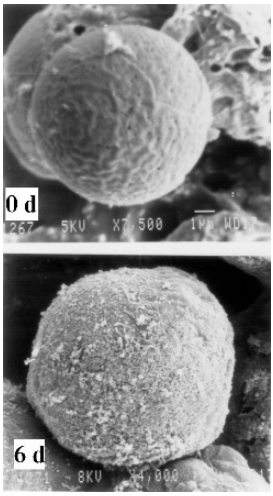

Fig. 3. Electron scanning microscope image of grapevine protoplasts after 0 and 6 days in culture. Reconstructed from Katsirdakis and Roubelakis-Angelakis (1992).

3.1. Recalcitrance and protoplast integrity

Several studies on the uptake characteristics of sugars, amino acids and polyamines by grapevine protoplasts (reviewed by Papadakis et al. 2001a) have shown that protoplast plasma membrane is intact and functional (Theodoropoulos and Roubelakis-Angelakis 1989, 1991, Christakis-Hampsas 1995, Christakis-Hampsas and Roubelakis-Angelakis unpublished). Thus, recalcitrance of grapevine protoplasts does not seem to be linked to the misfunction of plasma membrane.

Cultured grapevine protoplasts rapidly depleted glucose from the culture medium. Concentration dependent uptake of labelled glucose was linear for concentrations higher than 1.5 mM, at lower concentrations a saturating pattern was observed (Fig. 4A). The transport system was hexose specific and the stereospecificity was closely related to carbon-1 of the glucose molecule. Glucose structural analogues were not metabolized beyond the stage of phosphorylation. The sugars entered the plasma membrane by a carrier, which was driven by a proton motive force, probably in an uncharged form (Theodoropoulos and Roubelakis-Angelakis 1991).

The sugar uptake characteristics were remarkably similar to those exhib-

ited by intact (walled) suspension-cultured cells (CSB), which were recently used as a model system to study glucose transport and its regulation (Conde et al. 2006). Transport of D-[^{14}C]glucose in these cells obeyed simple first order Michaelis-Menten kinetics which was more evident for sugar concentrations above 1mM (Fig. 4B). The saturating component is a high-affinity, broad-specificity H^+-dependent transport system (K_m 0.05 mM). Glucose concentration in the medium tightly regulated the transcription of *VvHT1* (*Vitis vinifera* hexose transporter 1), a monosaccharide transporter previously characterized in grape berry, as well as VvHT1 protein level and monosaccharide transport activity. Recently, it was shown that an Hg^{++}-sensitive channel may mediate the diffusional component of glucose transport in plant cells (Conde et al. 2007a).

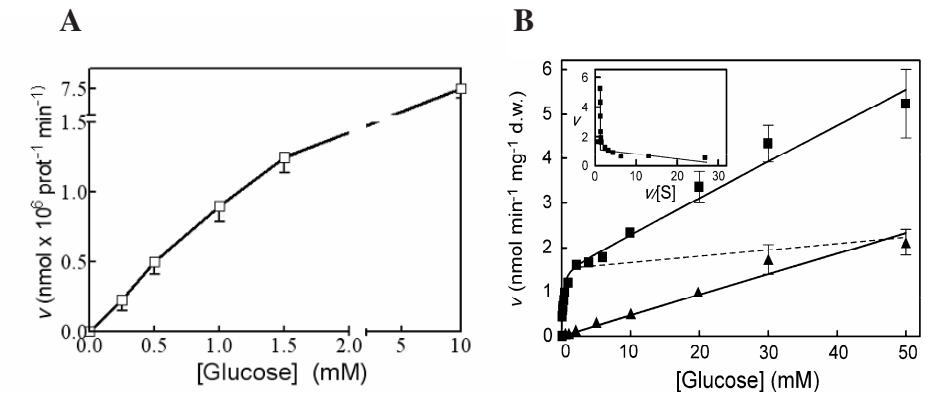

Fig. 4. (A) Uptake of labelled glucose by grapevine protoplasts, reconstructed from Papadakis et al. (2001a). (B) Uptake of of D-[^{14}C]glucose (squares) and L-[^{14}C]glucose (triangles) by intact (walled) suspension cells (CSB). Dotted line represents the difference between D-glucose and L-glucose uptake. Inset, Eadie-Hofstee plot of the initial D-glucose uptake rates. Reconstructed from Conde et al. (2006).

Endocytosis in plant cells has only been recently documented, and it can act as a parallel mechanism for sugar uptake directly into the vacuole (Etxeberria et al. 2005a, b, Baroja-Fernandez et al. 2006). The utilization of fluorescent 2-deoxy-glucose (2-NBDG), a fluorescent non-metabolizable glucose analogue, can be used to visualize the incorporation of sugar by fluorescent microscopy. Two-NBDG is a potent competitive inhibitor of D-glucose uptake (Oliveira et al. 2002, Conde et al. 2006) and the same behaviour was demonstrated for the corresponding fluorescent probe (Conde et al. 2007a). The results showed that 1 hour post-incubation both walled grapevine cells and protoplasts contained a brightly fluorescent cytosol and vacuole. Several fluorescent spots, most probably vesicles, were evident (Fig. 5A) and the uptake of the fluorescent glucose analogue was prevented by wortmannin, a well known endocytic inhibitor, indi-

cating that sugar may also permeate the plasma membrane by endocytosis. To confirm the involvement of endocytic vesicle formation, the styryl FM dye N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)-styryl)pyridinium dibromide (FM1–43) was used (Emans et al. 2002, Conde et al. 2007a). As shown in Fig. 5B and C, both intact walled-cells and protoplasts showed an intracellular endocytic vesicles traffic revealed by the fluorescent membrane marker (Fontes N, Delrot S and H Gerós, unpublished).

The above results strongly suggest that plasma membrane of grapevine protoplasts is functional and therefore can not be considered as contributing to their recalcitrance.

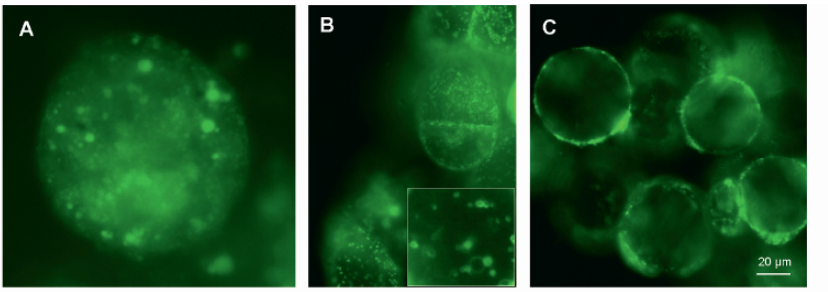

Fig. 5. Assessment of plasma membrane integrity of intact cells and protoplasts by using fluorescent markers. (A) Intact protoplast from grapevine mesocarp cells after incubation with the fluorescent glucose analog 2-NBDG and (B) walled suspension cultured cells and (C) derived protoplasts after incubation with FM1-43. *Inset*: close-up view of the cytosolic vesicle trafficking (Fontes N, Delrot S and H Gerós, unpublished).

3.2. The potential role of the oxidative stress

Oxidative stress results from imbalance between ROS generation and the antioxidant capacity of cells. ROS, such as superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) are by-products in plant cells during normal metabolism of photosynthesis and respiration (Alscher et al. 1997, Apel and Hirt 2004, Foyer and Noctor 2005, Asada 2006, Halliwell 2006). When the increase in ROS is relatively small, the housekeeping antioxidant capacity is sufficient to reset the original balance between ROS production and scavenging, thus re-establishing redox homeostasis. In addition to a direct radical-scavenging activity performed by chemical species, such as ascorbate, glutathione, aminoacids, polyamines, etc., a chain of enzymatic reactions cooperate for the removal of the ROS (Apel and Hirt 2004, and refs therein). Among them are peroxidases (EC 1.11.1.7, POX), catalases (EC 1.11.1.6, CAT), superoxide dismutases (EC 1.15.1.1, SOD), and also the enzymes that catalyze the reactions of the Halliwell-Asada cycle, ascorbate per-

oxidase (EC 1.11.1.11, APO), glutathione reductase (EC 1.6.4.2, GR), monodehydroascorbate reductase (EC 1.6.5.4, MHAR) and dehydroascorbate reductase (EC 1.8.5.1, DHAR, Asada 2006, Halliwell 2006). This cycle operates for the reduction of ascorbate and glutathione, which determine to a great extent the redox state of the cell.

The delicate redox balance is easily disturbed under growth limiting environmental conditions, leading to significant ROS accumulation and transient oxidative burst (Dat et al. 2000, Mittler et al. 2004). Under abiotic stress, the rate of carbon fixation is limited, causing an increase in photoinhibition and overproduction of ROS (Foyer and Noctor 2005); also, the incompatible plant-pathogen interactions are characterized by a biphasic oxidative burst (Doke 1997). ROS at high concentrations can certainly behave as extremely reactive molecules, able to react with almost all cellular components provoking destructive protein modifications, mutagenic DNA strand breaks, purine oxidations, and protein-DNA cross links, finally leading to membrane leakage, cell lysis and necrosis (Van Breusegem and Dat 2006). To avoid such collateral damage, a tight regulation of ROS homeostasis is necessary, which is provided by a complex gene network (Mittler et al. 2004). Additionally, ROS can signal in different cellular processes (Foyer and Noctor 2005).

3.2.1. ROS generation and accumulation during protoplast isolation and culture

Several enzymatic mechanism(s) have been implicated in the generation of ROS (Apel and Hirt 2004, and refs therein). The NADPH-dependent oxidase system, similar to that present in mammalian neutrophils, has received major attention. In animals, NADPH-oxidase found in phagocytes and B lymphocytes, catalyzes the production of $O_2^{-\cdot}$ by the one-electron reduction of oxygen using NADPH as the electron donor. The core of the phagocyte oxidase comprises five components: p40PHOX, p47PHOX, p67PHOX, p22PHOX, and gp91PHOX. In the resting cell, three of these five compnents, p40PHOX, p47PHOX, and p67PHOX, are present in the cytosol as a complex and the other two, p22PHOX and gp91PHOX, are localized in membranes of secretory vesicles, ensuring that the oxidase remains inactive.

When the resting cells are stimulated, the cytosolic component p47PHOX becomes heavily phosphorylated and the entire cytosolic complex is recruited to the membrane, where it associates with the two membrane-bound components to assemble the active oxidase. Activation requires also the participation of two low molecular weight guanine nucleotide-binding proteins (Segal 2008). Antibodies raised against human p22PHOX, p47PHOX, and p67PHOX cross-reacted with plant proteins of similar sizes (Tenhaken et al. 1995, Desikan et al. 2000), and in several plant species *rboh* genes (respiratory burst oxidase

homologues) of p91PHOX have been found (Keller et al. 1998, Torres et al. 1998), but homologues of the p47PHOX and p67PHOX regulators of the mammalian NADPH-oxidase were not found in the *Arabidopsis* genome (Dangl and Jones 2001).

In addition to a plant-specific NADPH-oxidase, alternative mechanisms of ROS production have been proposed. Many peroxidases are localized in the apoplastic space and are ionically or covalently bound to cell wall polymers. Peroxidases can act in two different catalytic modes. In the presence of H_2O_2 and phenolic substrates they operate in the peroxidatic cycle and are engaged in the synthesis of lignin and other phenolic polymers. However, if the phenolic substrates are replaced by NADPH or related reduced compounds, a chain reaction starts that provides the basis for the H_2O_2-producing NADH-oxidase activity of peroxidases; furthermore, *in vitro* studies of horseradish peroxidase suggested that the enzyme can reduce hydrogen peroxide to hydroxyl radicals (Chen and Schopfer 1999).

ROS were generated during the enzymatic maceration of cell walls during cereal protoplast isolation (Ishii 1987). Endoxylanase, an enzymatic component of some commercial cellulase and pectinase preparations, induced alterations in membrane integrity in tobacco (Bailey et al. 1992) and generation of O_2^-(Ishii 1988). In an effort to identify the mechanisms of ROS generation during protoplast isolation, two plant species were compared; tobacco, an easily regenerating species and grapevine, a recalcitrant one. Extensive wounding and/or addition of purified cellulase Worthington did not affect ROS accumulation, whereas non-purified cellulase Onozuka resulted to a significant accumulation of ROS in tobacco protoplasts, which was dose-dependent (Papadakis and Roubelakis-Angelakis 1999). In grapevine, the response was much less and affected mainly the H_2O_2 level.

The induced $O_2^{-\cdot}$ generation by tobacco protoplasts was sensitive to four inhibitors of the mammalian neutrophil NADPH oxidase (DPI, quinacrine, imidazole, pyridine), whereas none of them inhibited $O_2^{-\cdot}$ production in cellulase Onozuka-treated grapevine protoplasts; in contrast, KCN and NaN_3, inhibitors of plant peroxidases, both affected $O_2^{-\cdot}$ production and completely inhibited H_2O_2 generation in both plant species (Papadakis and Roubelakis-Angelakis 1999). Extracellular SOD activity was not implicated in ROS levels.The above data indicate that different systems are responsible for cellulase Onozuka-elicited ROS generation in each plant species; in tobacco, a mammalian-like oxidase seems to be responsible for superoxide production, since its inhibitors were able to reduce the superoxide accumulation, but this enzyme had no effect on H_2O_2 generation. A peroxidase-like activity is possibly contributing to H_2O_2 generation with an additional role in superoxide production. These two enzymes seem to operate in tandem or have different kinetics. In grapevine protoplasts,

only peroxidase activity was identified with the use of inhibitors, which more likely is responsible for the generation of both ROS (Papadakis and Roubelakis-Angelakis 1999). Two distinct sources of elicited ROS were also described in tobacco epidermal cells; a flavin-containing oxidase system and a cell wall peroxidase-like activity (Allan and Fluhr 1997).

In preparations of tobacco plasma membrane, the $O_2^{\cdot-}$-generating activity was higher than in grapevine, independently of the substrate used. Superoxide production from tobacco plasma membrane preparations showed differences in substrate (NADPH or NADH) affinity, although both obeyed Michaelis-Menten kinetics. The K_m was 7.9±2.7 μM for NADPH and 102.5±6.1 μM for NADH. In grapevine, the respective values for the K_m were 32.6±7.8 and 43.1±8.3 μM (Papadakis and Roubelakis-Angelakis 1999). Treatment with inhibitors suggested that in tobacco plasma membrane there were two different $O_2^{\cdot-}$-synthase activities; one that exhibited specificity for NADPH and sensitivity to DPI and imidazole and a second that can uses either NADPH or NADH and was sensitive to KCN and NaN_3. The first activity fits to a mammalian-like oxidase and the second to a NAD(P)H oxidase-peroxidase. In grapevine only the second activity was detected (Papadakis and Roubelakis-Angelakis 1999).

Freshly isolated protoplasts using purified cellulase accumulated $O_2^{\cdot-}$ and H_2O_2 intracellularly and extracellularly. Use of purified cellulase for 4 h resulted in protoplasts with the highest viability and plating efficiency during subsequent culture. Isolation of protoplasts with non-purified cellulase resulted in increased levels of intracellular and extracellular ROS in both, tobacco and grapevine. The increases in ROS levels were accompanied by a decrease in viability and plating efficiency of the resultant tobacco protoplasts, but they still could regenerate quite readily. However, when the maceration procedure was extended to 16h, the resultant tobacco protoplasts accumulated high ROS levels and lost their totipotency. ROS accumulation was also increased in grapevine protoplasts, but they did not regenerate regardless of the quality of maceration enzymes or the duration of the maceration period; by 8d, they exhibited a negligible number of divisions in all treatments (Papadakis et al. 2001b).

In regenerating tobacco protoplasts, total ROS levels decreased during the 8d culture period. In grapevine protoplasts, the $O_2^{\cdot-}$ level decreased during culture, while H_2O_2 decreased by day 4 and increased again later, mostly in the extracellular medium. The correlation between ROS levels and expression of totipotency was further demonstrated when alkaline DMSO, which elicits generation of $O_2^{\cdot-}$, was added to the regenerating tobacco protoplasts. This treatment increased ROS content and decreased viability and plating efficiency; in contrast, addition of ascorbate further increased the viability and plating efficiency of tobacco protoplasts (Papadakis et al. 2001b).

3.2.2. Scavenging of ROS during protoplast culture

Induction of superoxide dismutase (SOD) in stress conditions was an immediate response of cells, in order to scavenge excess $O_2^{\cdot-}$; the various SOD forms were induced with different kinetics during sustained stress conditions (Alscher et al 2002, and refs therein). In freshly isolated tobacco protoplasts, SOD activity was slightly higher than in leaf tissue and increased during the 8d culture period. This increase was associated with an increase in immunoreactive protein and in the steady-state levels of cytoplasmic Cu/ZnSOD isoenzyme mRNA. In grapevine protoplasts, no such response of SOD was detected (Papadakis et al. 2001b).

Catalase (CAT), the enzyme that removes H_2O_2 with high efficiency, was studied in regenerating tobacco and recalcitrant grapevine protoplasts (Siminis et al. 1994). The multiple CAT isoforms from both species were immunologically related. Immunoblots of leaf proteins, resolved by native IEF and SDS-PAGE, showed that tobacco and grapevine CAT isoenzymes consisted of two subunits, α- and β. The molecular mass of α-subunit was 57 kD in both species, and that of the β-subunit was 56 and 55 kD, in tobacco and grapevine, respectively. CAT increased in tobacco leaf following incubation with cell wall degrading enzymes, concommittally with increase of its immunoreactive protein; in contrast, no such increases were found in grapevine leaf. The cathodic and anodic CAT isoenzymes consisted exclusively of α- and β- subunits, respectively in tobacco and of β- and α- subunits, respectively in grapevine. The CAT specific activity increased only in grapevine protoplasts during culture, but not in tobacco protoplasts. In tobacco protoplasts, *de novo* accumulation of the CAT β-subunit gave rise to the less catalatic, acidic isoenzymes, whereas in grapevine protoplasts, after 8d in culture, only the catalatic, basic isoenzymes remained due to *de novo* accumulation of α-subunit (Siminis et al. 1994).

Halliwell-Asada pathway is also operating for H_2O_2 scavenging and includes many enzymes and antioxidant molecules; ascorbate oxidized by ascorbate peroxidase (APO) is reduced by monodehydro-ascorbate reductase (DHAR) and dehydroascorbate reductase (DHAR) in reactions that exploit NADPH and glutathione; glutathione reductase (GR) completes the cycle maintaining a high cellular ratio between the reduced (GSH) and the oxidized form (GSSG) of glutathione (Alscher et al. 1997, Asada 2006). APO specific activity steadily increased in tobacco protoplasts during culture. In grapevine protoplasts, it also increased up to 4d in culture and remained constant thereafter, with significant lower levels compared to tobacco. The importance of APO activity as H_2O_2 scavenger for expression of regenerating potential in cultured tobacco protoplasts has been shown by using specific inhibitors; protoplasts died soon after the specific inhibitor of APO, *p*-chloromercuribenzoate (pCMB) was added to the culture medium (de Marco and Roubelakis-Angelakis 1996a). The

activity of DHAR, which regenerates the reduced ascorbate pool, was reduced in both protoplasts during culture. However, the specific activity of DHAR was always several fold higher in tobacco than in grapevine protoplasts. GR specific activity significantly increased in tobacco protoplasts during culture, while in recalcitrant grapevine protoplasts it did not change compared to the leaf tissue (Papadakis et al. 2001b).

Two new isoforms of APO have been found in tobacco protoplasts, one cytoplasmic and another chloroplastic, the second extremely labile in the absence of its substrate (de Marco and Roubelakis-Angelakis 1999). The isoenzyme 2 significantly increased in tobacco protoplasts and the steady-state levels of cytoplasmic *APO* mRNA were relatively high. Isoenzymatic analysis of GR revealed two activity bands in tobacco protoplasts, which significantly increased during culture. In grapevine protoplasts, two isoenzymes of APO and GR were detected in leaf tissue and their activity declined during culture, while Northern blot analysis did not show any induction of cytoplasmic *APO* mRNA (Papadakis et al. 2001b).

Ascorbate and glutathione have central and interrelated roles in oxidative stress, acting both chemically and as substrates in enzyme-catalyzed detoxification reactions (Foyer and Noctor 2005). The ratio of the reduced to oxidized form of ascorbate or glutathione, termed as redox state, describes the redox state of the cell. Reduced endogenous ascorbate (ASA) slightly decreased and oxidized ascorbate (DHA) was fairly constant in tobacco protoplasts during culture, whereas they both significantly increased in recalcitrant grapevine protoplasts. Furthermore, in the recalcitrant grapevine protoplasts, the GSSG level was greater than GSH during the entire culture period. The very low GSH/GSH+GSSG and ASA/ASA+DHA ratios in grapevine protoplasts suggested an altered redox state (Papadakis et al. 2001b).

3.2.3. Potential role(s) of ROS in the fate of protoplasts

The results presented so far suggested that a collapse of defense mechanism against oxidative stress occurred in grapevine protoplasts; thus, that suppressed expression of totipotency could be attributed to the reduced antioxidant machinery (Fig. 6). The expression of totipotency in cultured protoplasts involves at least two developmental pathways; suppression of events leading to cell death, such as ROS accumulation and induction of cell division. In grapevine protoplasts, the titers of ROS, produced during isolation, were lower than in regenerated tobacco protoplasts, but due to the collapse of the defense mechanism, they were probably adequate to induce cell death. Van Breusegem and Dat (2006) have summarized the role of ROS on cell death; when ROS production exceeds the cellular scavenging capacity, ROS levels may rise above two different threshold levels both leading to cell death. Under conditions favor-

ing leakage from electron transport chains together with reduced antioxidant potential, unleached ROS accumulation will build up to phytotoxic levels attacking and damaging proteins, lipids, and DNA and finally leading to a necrotic phenotype. When the accumulation of ROS is insufficient to kill the cell directly, changes in cellular redox homeostasis appear to switch on a signalling cascade, leading to PCD, as typically encountered during the hypersensitive response.

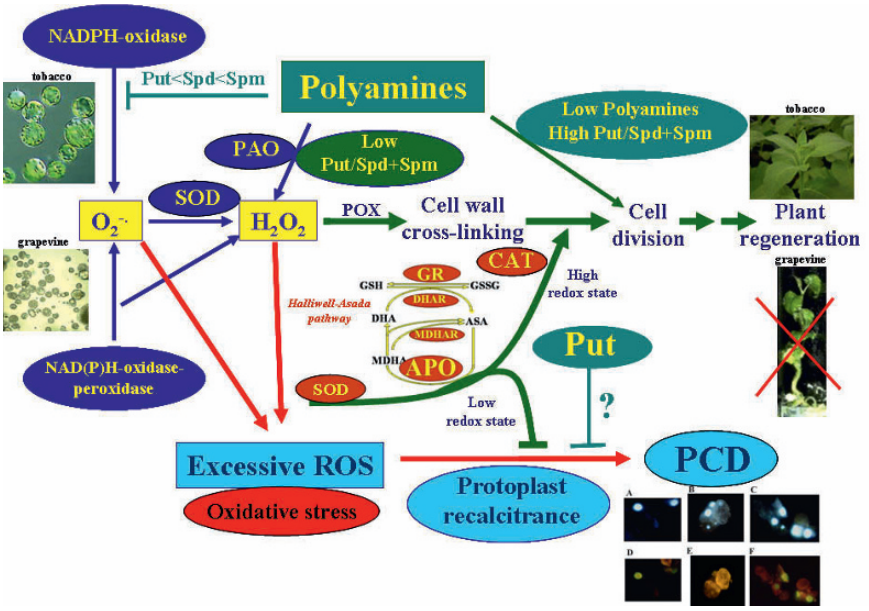

Fig. 6. Reactive oxygen species are generated during isolation and culture of plant protoplasts, by either NADPH-oxidase and/or NAD(P)H-oxidase-peroxidase, depending on plant species. The generated $O_2^{\cdot-}$ are scavenged by SOD to produce H_2O_2, which plays a bivalent role in the regenerating process. On one hand, it ensures the POX-mediated cell wall reconstitution and also it signals the expression of antioxidant genes (*APO*, *MDHAR*, *DHAR*, *GR*, *CAT*) in the totipotent tobacco protoplasts. As a result, ROS are detoxificated and the increased redox state promotes cell division and regenerating developmental processes. In contrary, in recalcitrant grapevine protoplasts the antioxidant machinery is not induced, the cell redox state is lowered due to mostly oxidized forms of glutathione and ascorbate, and instead of cell divisions, a programmed cell death syndrome is initiated. Put prevents this event, but in contrary, cell death is enhanced by the H_2O_2 generated *via* the polyamine oxidase-mediated catabolism of higher polyamines, spermidine and spermine. Reconstructed from Papadakis and Roubelakis-Angelakis (2005).

ROS are not just toxic products that need to be removed; they can be beneficial as well, as spatial and temporal fluctuations of ROS levels are inter-

preted as signals required for plant growth and development (Apel and Hirt 2004) and as second messengers in several plant hormone responses (Kwak et al. 2006). Recent data have suggested that ROS may control development through their role in regulating cell growth, which increases the number of cells in an organ and their expansion (Gapper and Dolan 2006). Protoplast regeneration relies on the control of cell growth, which is ensured by both the modulation of the extensin extrusion and polymerization (Cooper et al. 1994) and probably by regulating the indole-3-acetic acid (IAA) concentration, known to stimulate cell elongation (Hoson et al. 1995). Peroxidases have an essential role in cell wall modifications; they are involved in lignin and suberin biosynthesis and intramolecular isodityrosine generation in hydroxyproline-rich glycoproteins (Fry 1986). POX specific activity in grapevine leaf extract was substantially higher than in tobacco leaf (Siminis et al. 1993). Freshly isolated protoplasts exhibited very low POX activity. During the subsequent culture, POX activity in tobacco protoplasts increased constantly, whereas in grapevine protoplasts the respective increase was much lower. In addition, significant differences were found in the profile of POX isoenzymes. In tobacco, 3 basic and 7 acidic peroxidases were induced during protoplast culture. In freshly isolated grapevine protoplasts, only one basic POX isoenzyme was present with decreasing intensity during culture; at the 6^{th} day two barely detectable acidic POXs appeared; no new peroxidases were induced during culture, and this was correlated with the inability of grapevine protoplasts to enter the cell cycle (Siminis et al. 1993).

When POX activity was inhibited by KCN, protoplasts still survived but lost the dividing potential further suggesting that division is dependent on a modification of plasticity of the primary cell wall. Apoplastic H_2O_2 seemed to be necessary in order to ensure cell division since addition of CAT to the culture medium prevented it; dividing potential was partially recovered when 3-amino-1,2,4-triazole (AMT) was added to block the activity of exogenously added CAT. Such results could indicate that oxidative stress may reduce the regeneration potential of protoplasts, but only protoplasts that are able to supply H_2O_2 can actually divide (de Marco and Roubelakis-Angelakis 1996b). It is well-documented that H_2O_2 plays a central role as signal molecule regulating a variety of stress responses and physiological adjustments, so called 'master hormone' as it serves as intrinsic signal in plant growth and development (Slesak et al. 2007). Somatic embryogenesis is stimulated by H_2O_2 in *Lycium barbarum* (Cui et al., 1999) and extacellular H_2O_2 seems to contol plant development in poplar trees (Srivastava et al. 2006).

Hydroxyl radicals generated by the plasma membrane NAD(P)H oxidase, play a central role in plant cell growth; in dividing plant cells, cell walls are kept in a loose state by triggering $O_2^{\cdot-}$ production and subsequently increasing $OH^{\cdot}$

levels, while H_2O_2 produced by $O_2^{-\cdot}$ dismutation is kept at low levels, thus promoting fast extension growth (Schopfer 2001). Additionally, $OH^{\cdot}$ generated by a peroxidase-catalyzed reaction from $O_2^{-\cdot}$ and H_2O_2 in the cell wall cleaves load-bearing bonds of cell wall polysaccharides and in this way acts as an unspecific, non-enzymatic wall loosening agent (Schopfer and Liszkay 2006). Recalcitrant grapevine protoplasts were able to complete cell wall reconstitution within 3-4 d in culture and their ultrastructural appearance resembled that of grapevine leaf callus cells. In addition, U-[^{14}C]glucose uptake by regenerating tobacco protoplasts was significantly higher than by recalcitrant grapevine ones, and was followed by quantitative differences in the distribution of ethanol-soluble, cellulosic and non-cellulosic fractions and qualitative differences in non-cellulosic monosaccharides of the newly synthesized cell walls (Katsirdakis and Roubelakis-Angelakis 1992).

3.3. The role of polyamines and phytoalexins

The diamine putrescine (Put), the triamine spermidine (Spd) and the tetramine spermine (Spm) are the main polyamines (PAs) found in all living cells. PAs may occur in the free molecular form (S, soluble), or/and associated with small molecules like phenolic acids (SH, soluble hydrolyzed, soluble conjugated forms), or/and with various macromolecules like proteins (PH, pellet hydrolyzed, insoluble conjugated forms) due to their polycationic nature at physiologically relevant ionic and pH conditions (Martin-Tanguy 1997). PAs have been implicated in a number of cellular and molecular processes in eukaryotic cells; in higher plants, PAs act as modulators of several developmental processes, e.g. root growth, flower and fruit development and somatic embryogenesis. They also have a significant impact in plant defense and adaptation to stress conditions (Bouchereau et al. 1999, Groppa and Benavides 2008, Moschou et al. 2008).

3.3.1. Polyamine biosynthesis during protoplast isolation and culture

Total PA levels in freshly isolated tobacco protoplasts were higher whereas in fresh grapevine protoplasts total PAs were at the same levels as in donor leaf tissue (Papadakis et al. 2005). During protoplast culture, total PAs increased and exhibited higher titers in regenerating tobacco protoplasts (Fig. 7). The SH-fraction of total PAs (Put+Spd+Spm) predominated and was followed by the PH-fraction; both SH- and PH-PAs increased during culture. In contrast, S-PAs fraction was low and rapidly decreased during culture.

In freshly isolated recalcitrant grapevine protoplasts, S-PAs predominatd but they gradually decreased during culture. Total (S+SH+PH) Put, Spd and Spm increased during culture in tobacco protoplasts and were significantly

higher compared to grapevine protoplast (Fig. 7). In tobacco protoplasts, total Put predominated, followed by total Spd and Spm; in grapevine protoplasts total Put predominated, followed by total Spm and Spd. Furthermore, during the entire culture period, the highest ratio of total Spm/total PAs was found in grapevine protoplasts (Papadakis et al. 2005). Changes in the response to abiotic stress during the isolation of leaf protoplasts were also compared between a

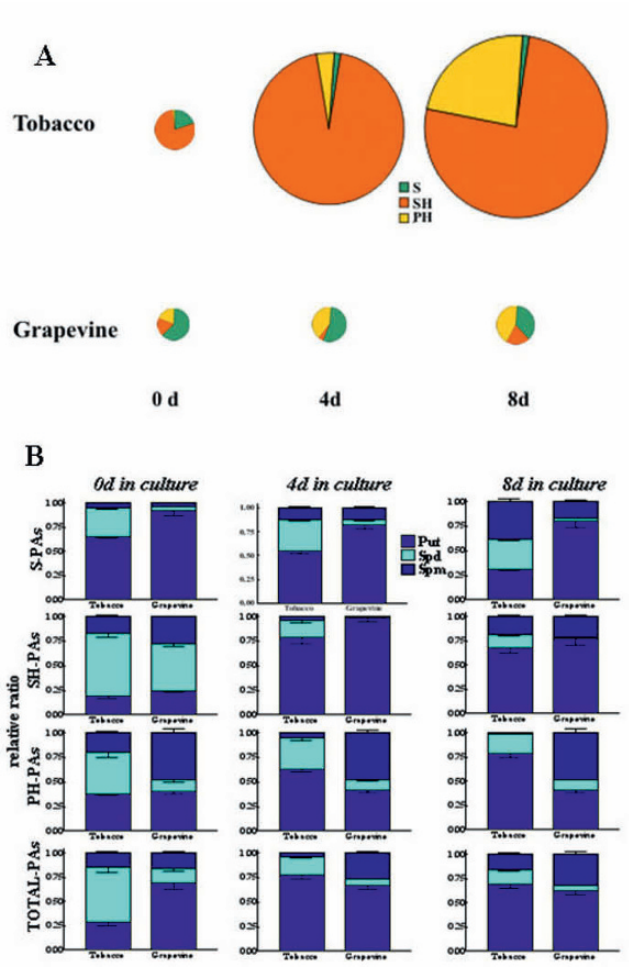

Fig. 7. (A) The relative size of soluble (S), soluble hydrolyzed (SH) and pellet hydrolyzed (PH) fractions of PAs to total PAs during tobacco and grapevine protoplast culture. (B) The relative size of putrescine (Put), spermidine (Spd) and spermine (Spm) in different PA fractions (S-, SH-, PH-PA) during culture. Reconstructed from Papadakis et al. (2001).

recalcitrant species of *Brassica napus* and regenerating species of *Petunia hybrida* (Watanabe et al. 2002). Results showed an increase in total PAs during protoplast isolation associated with the initiation of senescence, especially in *B. napus* where the ratio of soluble free Put/total PAs almost doubled, whereas that of Spd and Spm significantly declined.

Polyamine biosynthesis is mediated by ornithine decarboxylase (ODC; EC 4.1.1.17), arginine decarboxylase (ADC; EC 4.1.1.19), S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50), spermidine synthase (SPDS; EC 2.5.1.16) and spermine synthase (SPMS; EC 2.5.1.22) (Bagni and Tassoni 2001). ODC and ADC catalyze the synthesis of Put from L-ornithine and L-arginine, respectively. Spd and Spm are synthesized by the subsequent addition of an aminopropyl moiety onto Put and Spd, respectively by reactions catalyzed by the enzymes SPDS and SPMS. The aminopropyl moiety results from the decarboxylation S-adenosylmethionine by SAMDC. In freshly isolated tobacco protoplasts, ADC specific activity was similar to that in leaf tissue, remained at the same level during the first days in culture and declined at day 8 (Papadakis et al. 2005). In contrast, ODC specific activity was greater than in intact leaf just after isolation and increased thereafter. In grapevine protoplasts, ODC and ADC activity decreased during the entire culture period. Arginine is converted to ornithine in a reaction catalyzed by L-arginine amidino hydrolase (arginase; EC 3.5.3.1); arginase may then affect the availability of arginine and ornithine. In protoplast populations, arginase increased just after isolation compared to leaf tissue. During culture, it increased significantly only in tobacco protoplasts. SAMDC specific activity did not change during isolation of tobacco protoplast but decreased in freshly isolated grapevine protoplasts compared to the intact leaf. During culture, SAMDC activity increased in tobacco protoplasts and decreased in grapevine protoplasts (Papadakis et al. 2005).

Thus, ornithine is probably the major source for PA biosynthesis in the regenerating protoplasts and most of it is directly produced from arginine by arginase as part of the urea cycle and not from glutamate. When ODC activity was inhibited, tobacco protoplasts died soon after isolation, suggesting an important role of the enzyme or its product Put on protoplast fate. On the other hand, ADC mediates the enhanced levels of Put produced under stress conditions (Bouchereau et al. 1999, and refs therein). Additionally, SAMDC activity increased in regenerating tobacco protoplasts during the first days in culture further providing the substrate for Spd and Spm synthesis, and decreased in grapevine protoplasts, where Spd and Spm levels were lower (Papadakis et al. 2005).

3.3.2. Effect of exogenous polyamines on protoplast fate

Cellular PAs can act as endogenous antioxidant molecules. Spd completely inhibits the activity of microsomal NADPH-oxidase during chilling injury

of cucumber (Shen et al. 2000). In tobacco protoplasts, exogenous PAs decreased the accumulation of $O_2^{\cdot-}$, which was induced by the maceration treatment and this effect was positively correlated with the number of amino groups (Spm>Spd>Put; Papadakis and Roubelakis-Angelakis 2005). This antioxidant action of PAs could be due to the inhibition of the activity of NADPH- oxidase, which was concentration-dependent and positively correlated with the number of amino groups of PAs. Additionally, $O_2^{\cdot-}$ accumulation in grapevine, which is not generated through NADPH oxidase, was not affected by exogenous PAs (Papadakis and Roubelakis-Angelakis 2005).

The levels of ROS were estimated in fresh tobacco and grapevine protoplasts which were isolated from Put-, Spd- or Spm-treated plant material (Papadakis and Roubelakis-Angelakis 2005). Tobacco protoplasts exhibited significantly lower levels of $O_2^{\cdot-}$ but the level of H_2O_2 was not affected. In grapevine protoplasts, the levels of ROS were not altered just after isolation. In the subsequent culture, the intra- and extracellular accumulation of $O_2^{\cdot-}$ significantly decreased in PA-treated tobacco protoplasts; in fact, the greater the number of amino groups of PA used, the lower the $O_2^{\cdot-}$ level. In contrast, the intra- and extracellular accumulation of H_2O_2 increased in Spd- and Spd-treated protoplasts. In PA treated-grapevine protoplasts, the accumulation of H_2O_2 followed the same pattern as in tobacco protoplasts, whereas the level of $O_2^{\cdot-}$ was not affected. Put addition to the maceration medium resulted to a significant increase in the viability and plating efficiency of tobacco and grapevine protoplasts. However, treatments with Spd or Spm decreased both the viability and the plating efficiency of protoplasts, leading to the execution of a cell death program, which was prevented by Put (Papadakis and Roubelakis-Angelakis 2005).

PAs are catabolized by diamine and polyamine oxidases (DAO; EC 1.4.3.6 and PAO; EC 1.5.3.11). DAO activity did not significantly change during culture of tobacco protoplasts, whereas PAO activity increased in Spd- and Spm-treated tobacco protoplasts Furthermore, during protoplast culture, PAO activity increased in tobacco and grapevine protoplasts. In PA treated-grapevine protoplasts no significant change was detected in DAO activity, while PAO increased during culture (Papadakis and Roubelakis-Angelakis 2005).

Exogenously supplied Put during protoplast isolation improved the viability and plating efficiency and prevented the programmed cell death (PCD) syndrome, acting as an anti-PCD factor (Papadakis and Roubelakis-Angelakis 2005). The accumulation of Put in regenerating tobacco protoplasts probably exerts a growth stimulatory effect and promotes the expression of totipotency. In contrast, recalcitrant grapevine protoplasts exhibited the highest PH-Spm level, which corresponds to almost 50% of PH-PA fraction during the entire culture period, and total Spm was higher than that of tobacco protoplasts (Papadakis et al. 2005). Exogenous Spd and Spm exhibited cytotoxicity to proto-

plasts; they were catabolized by PAO, leading to H_2O_2 generation which contributes to oxidative stress and to induction of PCD (Fig. 6, Papadakis and Roubelakis-Angelakis 2005). Furthermore, when SPMS was blocked, the viability and plating efficiency of T-tobacco protoplasts was improved (Papadakis et al. 2005).

Exogenous PAs exerted an antioxidant activity by inhibiting NADPH-oxidase activity and $O_2^{\cdot-}$ generation (Papadakis and Roubelakis-Angelakis 2005). However, exogenous Spd and Spm exhibited cytotoxicity to protoplasts through their catabolism and subsequent H_2O_2 generation. As a result, oxidative stress induced the PCD syndrome which was documented by the membrane misfunction, DNase activity, DNA fragmentation and the positive TUNEL reaction. Put may prevent the induction of cellular damage linked to subsequent cell death, acting as anti-PCD agent with unknown yet mode of action (Fig. 6).

3.3.3. Polyamine homeostasis in protoplasts

Protoplast isolation leads to changes in PA homeostasis in tobacco and grapevine protoplasts meaning alteration of the cellular PA pool and PA biosynthetic activities (Papadakis et al. 2005). Regenerating tobacco protoplasts exhibited higher accumulation of total PAs during culture supporting that the higher the PA accumulation, the higher the regenerating potential of protoplasts, in agreement with data indicating that the actively dividing cells in tobacco leaf contained the highest titers of PAs (Paschalidis and Roubelakis-Angelakis 2005). Exogenous PAs caused a decrease in the maceration-induced accumulation of $O_2^{\cdot-}$ by inhibiting NADPH-oxidase, and this effect was positively correlated with the number of amino groups (Spm>Spd>Put; Papadakis and Roubelakis-Angelakis 2005); the high accumulation of PAs could account for the low ROS levels in regenerating tobacco protoplast (Papadakis et al. 2005). PAs exerted an antioxidative action during the recovery of browning tissues into normal callus cultures and thus improved plant regeneration in pine (Tang et al 2004).

Regeneratig tobacco protoplasts successfully deal with culture conditions; they cope with generated ROS by inducing the antioxidant machinery and further proceed to cell division and regeneration (Papadakis et al. 2001). These protoplasts also accumulated the highest SH-PAs and further correlated the increased antioxidant capacity and the regenerating potential with high SH-PAs, and especially with SH-Put (Papadakis et al. 2005). It has been proposed that the antioxidant effect of PAs is expressed by the conjugated forms, which have scavenging activities when compared to those of known efficient radical scavengers, e.g. ascorbate. Monocaffeoyl Put, an effective scavenger of oxyradicals, was detected in the apoplast of tobacco leaves exposed to ozone (Langebartels et al. 1991).

Put predominated to Spd and Spm in all protoplast populations and all PA fractions and the highest titers were found in regenerating tobacco protoplasts (Fig. 6, Papadakis et al. 2005). High cellular levels of Put were associated with resistance to salt tolerance (Urano et al. 2004, Moschou et al. 2008). Additionally, Put directly affected cell division and growth (Kakkar et al. 2000); when Put biosynthesis was inhibited, protoplasts did not survive (Papadakis et al. 2005). Increased Spd and Spm during the transition from G1 to the S phase of the cell cycle, preceding the onset of DNA synthesis, is a universal phenomenon in animal and plant cells (Kakkar et al. 2000). High Spd and Spm synthesis coincided with cell division in tobacco tissues and a low synthesis with cell expansion (Paschalidis and Roubelakis-Angelakis 2005). Regenerating tobacco protoplasts with high total Spd and Spm contents were able to re-enter cell cycle and regenerate, while grapevine protoplasts did regenerate (Papadakis et al. 2005, Fig. 6).

3.3.4. The role of phytoalexins

Commun et al. (2003) reported the production of stilbene phytoalexins by protoplasts of *Vitis* spp. and detected *trans*-resveratrol as early as 4 h after the beginning of enzyme digestion. When observed under long UV, over 30% of Chardonnay (CH) leaf protoplasts exhibited a bright deep blue fluorescence characteristic of stilbenes. When analyzed by HPLC, freshly isolated leaf protoplasts contained a compound with a retention time identical to that of authentic pure *trans*-resveratrol. Grapevine protoplasts accumulated other phytoalexin-type compounds as well, an inducible resveratrol dehydrodimer analogous to ε-viniferin being detected after 24 h of culture. Resveratrol production could be modulated by changes in the digestion medium. The substitution of the Cellulase Onozuka RS with the Cellulase Fluka, led to an increase in resveratrol production at the end of the digestion although both enzymes were extracted from *Trichoderma viride*.

On the contrary, when macerating enzyme (0.02% Macerozyme R10) was added to the digestion medium, only a slight increase in resveratrol production was observed. The production of stilbene phytoalexins occurred through activation of the *VST1* gene encoding stilbene synthase. Therefore, the results suggest that digestion conditions used for grapevine protoplasts induce defense-related genes, involved here in phytoalexin accumulation. The presence of resveratrol and its derived phytoalexins, epsilonviniferin and pterostilbene, may account for loss of protoplast viability, but the mechanisms responsible for phytoalexin induction were not clarified (Commun et al. 2003). The authors discussed that elicitors resulting from the activity of fungal degrading enzymes on host cell walls may indeed induce phytoalexin production, as well as the osmotic pressure and the nature of the osmoticum and plant regulators.

4. APPLICATIONS OF PROTOPLAST TECHNOLOGY

The 'recalcitrance problem' which characterizes the most agronomically important plant species, including the grapevine, has been the main barrier for the application of protoplast technology in breeding programs. Nevertheless, some efforts to develop techniques have been started 10 years ago, with the first success in regeneration of grapevine protoplasts derived from embryogenic callus (Reustle et al. 1995, Zhu et al. 1997). The efforts to use protoplasts as a selection system for agronomicaly interesting characters, the detection of somaclonal variants in grapevine regenerants from protoplasts and the production of somatic hybrids by protoplast fusion has been summarized in detail in Papadakis et al. (2001a).

4.1. Genetic transformation

Protoplasts are an excellent tool to introduce foreign genes to plant cells due to the removed cell wall. Polyethylene glycol (PEG)-mediated or electroporation are the mostly commonly used methods, whereas *Agrobacterium* mediated transformation of protoplasts has been used only in a few cases. However, very few groups have been working on genetic engineering of grapevine protoplasts. Valat et al. (2000) established a protocol for transformation of grapevine protoplasts isolated from transgenic 41B rootstock to transfer virus particles and viral RNA into protoplasts *via* electroporation. Protoplast electroporation was used as a rapid screening techinque of transgenic grapevine clones expressing viral particle genes to identify virus-resistant grapevine clones at cell- and plant-level (Valat et al. 2006). Other efforts have been described in detail in Papadakis et al. (2001a). Nowdays, embryogenic cell suspensions are routinely used for grapevine genetic transformation *via* both *Agrobacterium*- and biolistic-mediated DNA delivery systems.

4.2. Protoplasts as a test system

As single cell systems, protoplasts are good experimental models for research on ultrastructure, physiological aspects and genetics of plant cells. For instance, transport studies in protoplasts offer several advantages over the same studies in plant tissues, because in intact plant bulk diffusion, tissue penetration barriers and cell heterogeneity impair kinetic studies. In addition, in the protoplasts, the plasma membrane is readily amenable for challenging with exogenous substrates, analogues, transport inhibitors and drugs. Thus, they can be of great importance for identifying new and more efficient fungicides and elicitors

of natural disease resistance, measuring their accumulation, compartmentation and toxicity in the cells.

In particular, the effect of pesticides on grapevine is being studied using protoplasts from grape berry mesocarp as *in vitro* model. The incubation of freshly isolated protoplasts with tebuconazole, which is present in several fungicides, promotes a significant and dose-dependent loss in cell viability within 4 h (H. Gerós and A.C.P. Dias, unpublished). Fluorescein diacetate and propidium iodide double staining was used to estimate cell viability, as described earlier (Jones and Senft 1985), coupled to flow cytometry analysis.

Efforts to use grapevine protoplasts as a test system for toxicity of *Eutypa lata* and to grapevine fan leaf virus infection in transgenic grapevines have been described in detail in Papadakis et al. (2001a).

4.3. Use of protoplasts for subcellular fractionation

Controlled lysis of protoplasts permits the isolation of subcellular fractions, including membranes, organelles, and vacuoles, with the latter being used to study accumulation of compounds, such as sugars (reviewed by Davey et al. 2005). In addition, the increasing impact of proteomic studies in plant biology has generated an unpredicted interest in the purification of this extremely fragile organelle and led to two independent proteomic studies focused on intact vacuoles from *Arabidopsis* (Carter et al. 2004, Shimaoka et al. 2004). Vacuoles play central roles in plant growth, development, and stress responses. The central vacuole, which can occupy more than 80% of the total plant cell volume, is separated from the surrounding cytosol by the tonoplast membrane that controls the passage of inorganic and organic solutes to and from the cytoplasm through a wide range of pumps, carriers, ion channels and receptors (Neuhaus 2007), but these proteins are generally less well known than the corresponding plasma membrane proteins.

In the mesocarp of fleshy fruits, the large central vacuole plays a prominent role in cell expansion, fruit size and fruit quality. This is the case for the vacuoles of the grape berry cells, which accumulate high concentrations of sugars, organic acids and secondary metabolites. These compounds, which all play a key role for the taste and flavour of the fruit are imported and/or compartmentalized in various cells (mesocarp, skin), thanks to specific plasma membrane and tonoplast transporters (reviewed by Conde et al. 2007b). Despite the importance and uniqueness of fruit vacuoles, relatively little is known about vacuolar transporters. Proteomic methodologies can provide important insights into the potential functions of these proteins, based on subcellular localizations or changes in expression level in response to a stimulus. Additionally, molecular characterization and functional analysis of tonoplast transporters should help

our understanding of vacuole function and may promote the genetic engineering of fruits and vegetables (Maeshima 2001, Carter et al. 2004).

Within this context, approaches aiming at the isolation and purification of intact vacuoles from grape cells are a prerequisite to understand the physiology of this organelle during grape berry development and ripening. The first large scale isolation of mature vacuoles from higher plants was based on the osmotic shock of protoplasts in 200 mM K-phosphate (pH 8.0) and 3 mM $MgCl_2$ (Wagner and Siegelman 1975). Since then, several methods have been described using both, cultured-cells and mature plant tissue as starting material. Most of them include several steps of protoplast purification followed by protoplast lysis by application of both osmotic and thermal shocks of the isolated protoplasts.

Fontes and co-workers (2008) isolated vacuoles by a ficoll step gradient centrifugation after osmotic disruption of the protoplast plasma membrane at a relatively high temperature. The methodology was adapted from the protocol used to obtain vacuoles from *Arabidopsis* protoplasts (Carter et al. 2004). In a typical fractionation procedure, an average number of 4.0×10^6 vacuoles was obtained, corresponding to about 12 % of the total number of protoplasts subjected to lysis. Only 2% of the cytosolic marker enzyme glucose-6-phosphatase was recovered in the vacuolar fraction, indicating high degree of purity, which was further confirmed by microscopic observation (Fig. 8) and flow cytometry analysis (Fig. 9). Under fluorescent microscopy, the tonoplast membrane stained strongly with the styryl dye FM 1-43. This fluorescent probe exhibits weak fluorescence in aqueous medium, but shines brightly when inserted into membranes (Betz et al. 1996).

Impure vacuoles can also be easily visualized by incubation with the styryl dye FM 1-43. The vacuole sample was also labelled with Fluo4/AM, a fluorescent dye used for the quantification of cellular Ca^{2+} concentrations. The high fluorescence observed suggested a strong accumulation of Ca^{2+} in the vacuoles that may be dissipated by calcymicin. Most of the intact vacuoles maintained an internal acidic pH, exhibiting a red colour after being labellled with neutral red. Regarding flow cytometry analysis of vacuoles (Fig. 9), a well-defined sub-population in the centre of the histogram can be seen, corresponding to the vacuole population (gated region V). When the vacuole sample was stained with the calcium probe Fluo4/AM, the fluorescence of the gated region V increased indicating the vacuole ability to accumulate calcium.

The low pH of the vacuole of fruit cells is the result of two processes: 1) pumping of protons across the tonoplast, which directly results in a drop of vacuolar pH, and 2) synthesis and accumulation of organic acids in the vacuolar sap (Shiratake and Martinoia 2007). Two distinct primary proton pumps, the vacuolar ATPase and the vacuolar inorganic pyrophosphatase (V-PPase) gener-

ate a proton electromotive force, which, in turn, allows the secondary active transport of inorganic ions, sugars and organic acids. For proton-pumping measurements, tonoplast enriched-vesicles and intact vacuoles were incubated in ACMA (9-amino-6-chloro-2-methoxyacridine). This probe exhibited a significant fluorescence quench in the presence of membranes, as a response to

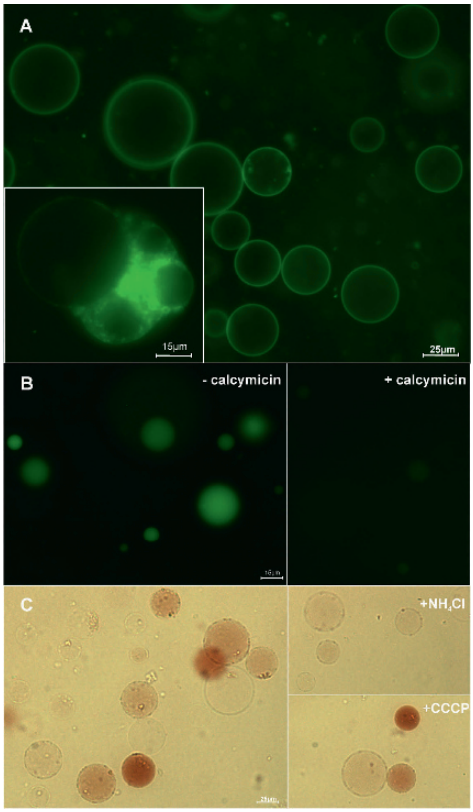

Fig. 8. Intact vacuoles, (A) purified after protoplast lysis, labellled with the fluorescent membrane marker FM1-43 (*inset*: membraneous bodies attached to the central vacuole forming an 'impure vacuole') and (B) the calcium fluorescent probe Fluo4/AM in the absence/presence of 300 μM calcymicin, observed under the fluorescence microscope. (C) Intact vacuoles labelled with neutral red in the absence/presence of 2.5 mM NH_4Cl and 25 μM CCCP. Reconstructed from Fontes et al. (2008).

change in their energization states (Getz and Klein 1995). Results showed that intact vacuoles are able to maintain and generate a pH gradient through the activity of V-ATPase and V-PPase. The later is the predominant pump in the tonoplast, with a very high affinity for pyrophosphate. Intact vacuoles also displayed proton-dependent calcium and phosphate transport activities. However,

in spite of the high concentrations of glucose and fructose measured in intact vacuoles, no evidence was obtained for sugar-induced proton movement. As a whole, intact vacuoles isolated from grape protoplasts appeared a reliable model to study the mechanisms of tonoplast energization and the activity of secondary active transport systems dependent on proton and electrical gradients generated by both the tonoplast PPase and ATPase (Fontes et al. 2008).

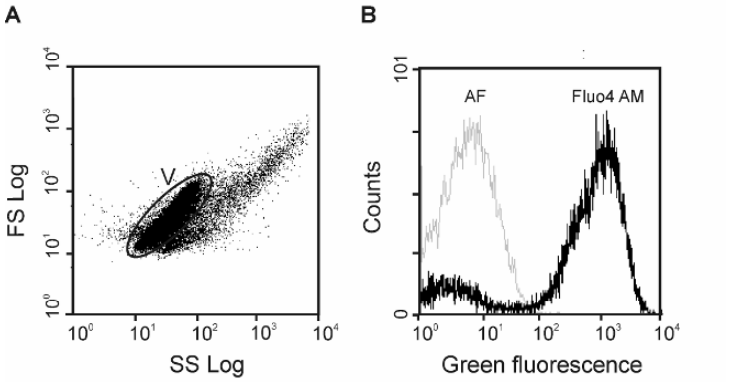

Fig. 9. Flow cytometric analysis of a vacuole population obtained from grapevine-cultured cells labelled with Fluo4/AM: (A) scattergram (dotplot) of a vacuole suspension after Fluo4/AM staining and (B) overlay of green fluorescence histograms of stained and autofluorescence of vacuole suspension for gated region V. Reconstructed from Fontes et al. (2008).

5. CONCLUSIONS AND PERSPECTIVES

The plant regeneration process from a protoplast can be divided into 4 main phases: reconstitution of new cell wall, cell elongation and first cell divisions, sustained cell divisions to micro- and macrocallus callus formation, and plant regeneration by direct organogenesis or somatic embryogenesis. According to the literature, grapevine protoplasts can reach different phases. A successful protoplast-to-plant system for grapevine protoplasts derived from embryogenic callus was developed by Reustle et al. (1995) for *V. vinifera* cv Seyval blanc, and by Zhu et al. (1997) for *V. vinifera* cv Koshusanjaku. The use of mesophyll protoplasts has several advantages, compared to protoplasts from embryogenic callus, and therefore research efforts must continue in order to reveal the potential factors contributing to their recalcitrance.

ROS generation during protoplast isolation and accumulation during culture could have a great impact on protoplast fate. ROS are used as substrates for cell wall reconstitution and loosening and as signals for the activation of the antioxidant defense. Both processes are elementary for the successive progression of cell cycle, cell proliferation and eventually plant regeneration. In recalcitrant

grapevine protoplasts, sufficient ROS are generated to ensure cell wall reconstitution. However, the antioxidant enzymatic and non-enzymatic machinery is not induced. As a result, the altered redox homeostasis of recalcitrant protoplast could result in suppressed expression of totipotency, cell cycle arrest and cell death. Attempts by using molecular approaches may further elucidate whether in fact oxidative stress impairs expression of totipotency or is the result of other mechanism(s), which block the developmental process.

Generally speaking, the use of protoplast technology for plant breeding in easily regenerating plant species has not been as extensive as it was expected. Also, somaclonal variation, spontaneous or induced, was found to be a scarce source of variability. Plant resistance to pathogen attack depends on a cascade of defense mechanism(s), limiting the use of protoplasts for selection of resistant lines. In fact, selection of each of these defense mechanism(s) at protoplast and cell level seems to be difficult. Also, since the hypersensitive response is the main mechanism mediating plant resistance to pathogens, the selection of protoplasts based on hypersensitivity is not possible, as the infected cells will die and thus can not be regenerated.

Due to lack of cell walls, the transfer of genes into the plant genome *via* protoplast transformation is not complicated. Furthermore, if appropriate regeneration systems are available, selection of transgenic plants is facilitated and the formation of chimeric regenerates can be avoided. However, the difficulties with protoplast regeneration, and the availability of more efficient alternative systems for gene transfer *via Agrobacterium* mediated transformation and particle bombardment, led to an arrest of using protoplasts for production of transgenic plants in many species. Hereby, the problem of regenerating chimeric plants is compensated by the much easier and more efficient regeneration systems from leaf discs or embryogenic tissue. In grapevine, transformation and regeneration starting from embryogenic tissue and using *Agrobacterium* transformation or particle bombardment are well established and selection systems for non-chimeric transgenic plants seems to be very efficient. Also, symmetric and asymmetric hybridization as well as cybridization of protoplasts, offer the possibility to develop an interesting gene pool of the *Muscadinia* species available for grapevine breeding programs. However, prerequisite to this is the existence of regeneration protocols, at least for one of the used fusion partners.

Meanwhile, protoplasts are mostly used as single cell systems for basic reseach. Plant protoplasts exhibit physiological perception and responses to hormones, metabolites, environmental cues, and pathogen-derived elicitors, similar to cell-autonomous responses of intact tissues and plants. The development of defined protoplast transient expression systems for high-throughput screening and systematic characterization of gene functions has greatly contributed to elu-

cidating plant signal transduction pathways, in combination with genetic, genomic, and transgenic approaches (Sheen, 2001).

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17

STRATEGIES FOR EFFECTIVE SOMATIC EMBRYOGENESIS IN GRAPEVINE: An appraisal

L. Martinelli[1] & I. Gribaudo[2]

[1] Fondazione Edmund Mach-IASMA Research and Innovation Centre, Genomics and Crop Biology Area, Via E. Mach 1, 38010 San Michele all'Adige (TN), ITALY

[2] Plant Virology Institute IVV-CNR, Grugliasco Unit, Via Leonardo da Vinci 44, 10095 Grugliasco (TO), ITALY

1. INTRODUCTION

Following our previous review on the progress in somatic embryogenesis in the *Vitis* genus until the beginning of this millennium (Martinelli and Gribaudo 2001), the present review reports the state-of the-art of this morphogenic process stressing the milestones, the most recent advances and the main points of interest. With this chapter, the authors aim to offer an experienced background to researchers who intend to study grape morphogenesis and to scientists who want to achieve successfully somatic embryogenesis in this fruitcrop.

As previously reviewed (Martinelli and Gribaudo 2001), somatic embryogenesis has been defined as the initiation of embryos from plant somatic tissues closely resembling their zygotic counterparts (Ammirato 1983). This morphological event has been documented as occurring spontaneously in some plant species (Yarbrough 1932, Taylor 1967); however, it is usually induced in tissue cultures, potentially from almost any part of the plant body, either directly from the explant or after the callus stage. In both cases, somatic embryos can be of unicellular or multicellular origin (Quiroz-Figueroa et al. 2006). Somatic embryogenesis has been described as following patterns similar to those of zygotic embryogenesis (Altamura et al. 1992, Dodeman et al. 1997); however, in studies conducted on model plants, it was shown to be more variable in several morphological and molecular aspects (Mordhorst et al. 1997, Chug and Khurana 2002, Quiroz-Figueroa et al. 2006). In any case,

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establi-shment of embryo polarity seems to be the crucial event (Mordhorst et al. 1997).

In vitro somatic embryogenesis was first reported in 1958 (Reinert 1958, Steward et al. 1958) in *Daucus carota* tissue cultures; since then, it has been described for many species and is widespread in various *taxa*. In model plants, somatic embryogenesis is the most used experimental tool for cell totipotency and developmental studies and is one of the most powerful techniques for genetic improvement of important agricultural species.

Somatic embryogenesis was first developed in grape in the 1970s and was initially pursued with the aim to recover dihaploid plants for genetic improvement programs (Martinelli and Gribaudo 2001). Since then, most attempts were unsuccessful and the production of haploid plants *via* anther cultures has rarely been documented (Zou and Li 1981, Cao 1990, Gray 1995). Nevertheless, in recent years somatic embryogenesis has been widely applied in different breeding programs, and today it is one of the most suitable tools for the application of *in vitro* manipulation in the *Vitis* genus. In particular, embryo tissues are the best sources for regeneration of genetically modified plants (Martinelli and Mandolino 2000, Kikkert et al. 2001). In addition, somatic embryogenesis has been proposed as a strategy aimed at inducing somaclonal variation (Torregrosa et al. 2001), virus elimination (Goussard et al. 1991, Gambino et al. 2006), germplasm storage by synthetic seed technology (Gray and Compton 1993, Jayasankar et al. 2005, Das et al. 2006) and chimerism detection (Franks et al. 2002, Bertsch et al. 2005).

The first successful somatic embryogenesis was induced from cultured anthers (Gresshoff and Doy 1974, Hirabayashi et al. 1976) and the first somatic embryos able to germinate were obtained from the hybrid Gloryvine (*V. vinifera* x *V. rupestris*; Rajasekaran and Mullins 1979). In spring 1977, the first commercial planting of vines (*Vitis* sp. cv Seyval) from somatic embryos was established in Maryland, USA (Krul and Mowbray 1984), and in 1985 a protocol for grape somatic embryogenesis was patented in the USA (Krul and Narragansett 1985). Eversince, successful protocols for somatic embryogenesis from numerous genotypes of *V. vinifera*, *V. labruscana* and *V. rotundifolia* have been published. However, the method still needs improvement.

2. PROTOCOLS FOR SOMATIC EMBRYOGENESIS AND RELATED ASPECTS

As already mentioned, several protocols for somatic embryogenesis from grape genotypes have been published. Some of them have been patented in the USA (Krul and Narragansett 1985, Gray et al. 2002). There are important

differences among the protocols mainly related to the genotype and to the kind of cultured explants. Several strategies have also been exploited for preserving morphogenetic competence in the embryogenic cultures. At any case, the main developmental phases include:

(i) Induction of callogenesis and embryogenic competence in cultured explants.

(ii) Culture of embryogenic calli and expression of the embryogenic program.

(iii) Long term culture of embryogenic callus and preservation of the embryogenic ability of the cultures or re-initiation of embryogenic calli from somatic embryos.

(iv) Development of somatic embryos into plantlets.

2.1. Induction and culture of embryogenic calli

The main successful protocols developed for achieving somatic embryogenesis are presented in Table 1. Genotype is commonly considered to be one of the most important factors, which determine success. Anthers have been the most widely used starting material for culture, and successful initiation of regenerable embryogenic calli has been obtained from anther filaments of a considerable number of grape genotypes (Nakajima and Matsuda 2003, Perrin et al. 2004). In addition, other plant parts have also been exploited, and in many cases some of them were successful.

Immature ovule cultures were also employed in one of the first papers on grape somatic embryogenesis (Mullins and Srinivasan 1976) as well as in subsequent studies. In our experience, ovaries have also proven to be suitable material for obtaining somatic embryogenesis in several cultivars of *V. vinifera* and *Vitis* rootstocks (Fig. 1; Martinelli et al. 2001b and 2003, Gribaudo et al. 2004, Gambino et al. 2007, Cadavid-Labrada et al. 2008) and in most cases they were more efficient than anthers. In accordance with our findings, similar results for various *Vitis vinifera* cultivars have been reported in recent publications, showing that ovaries have been re-evaluated as suitable explants (Kikkert et al. 2005a, Pinto-Sintra 2007). Stigmas and styles have also proven to be suitable alternatives to anthers, giving rise to highly efficient induction of embryogenesis (Morgana et al. 2004, Carimi et al. 2005).

Leaves were also successfully used in the past (Krul and Worley 1977, Martinelli et al. 1993a, Harst 1985, Nakano et al. 1997); recently good embryogenic potential of leaf disks was obtained with some Indian *Vitis vinifera* cultivars (Das et al. 2002). Petioles (Robacker 1993), tendrils (Salunkhe et al. 1997) and nodal sections (Maillot et al. 2006) are less widely used tissues. Protoplasts turned out to be suitable when isolated from leaf-derived embryoge-

Table 1. Successful somatic embryogenesis protocols in *Vitis*.

Explants	Induction of embryogenic callus (μM)	Culture of embryogenic callus (μM)	Genotypes	References
Anthers	2,4-D (5) + BA (1)	PGR-free	*V. vinifera* x *V. rupestris* Gloryvine and others cvs, *V. rupestris, V. longii, V. vinifera* Grenache	Rajasekaran and Mullins 1979, Mullins and Rajasekaran 1980
	2,4-D (4.5) + BA (1.1)	IAA (5.7) + BA (2.2)	*V. vinifera, V. rupestris, V. riparia* and *Vitis* hybrids	Bouquet et al. 1982
	2,4-D (4.5) + BA (1)	NOA (0.5) + BA (1)	*V. vinifera* Cabernet-Sauvignon	Mauro et al. 1986
	2,4-D (5) + BA (0.9)	PGR-free	*V. riparia*	Moszar and Sule 1994
	2,4-D (9) + BA (0.9)	NOA (10) + BA (0.9) + IASP (17)	*V. vinifera* (4 cvs)	Perl et al. 1995
	2,4-D (4.5) + BA (1.1)	PGR-free	*V. vinifera* Grenache noir	Faure et al. 1996a
	2,4-D (5) +BA (1)		*Vitis* spp. (10 cvs)	Torregrosa 1998, Cutanda et al. 2008
	2,4-D (4.5) + BA (9)	NOA (10) + IAA (20) + BA (1)	*V. vinifera* Sultana	Franks et al. 1998
	2,4-D (20) + BA (9)	NAA (10) + BA (9)	*V. latifolia*	Salunkhe et al. 1999
	2,4-D (9) + TDZ (11.35)		*V. vinifera* (9 cvs)	Bouamama et al. 2007
Anthers, ovaries	2,4-D (5) +BA (1)		*V. longii* Microsperma	Gray and Mortensen 1987
	2,4-D (9) + BA (4.4)	NOA (10) + IAA (20) + BA (1)	*Vitis vinifera* (2 cvs), *V. berlandieri* x *V. rupestris* 110R, *V. berlandieri* x *V. riparia* 5BB	Martinelli et al. 2001b
	2,4-D (2.5) + NOA (2.5) + 4-CPPU (5)	PGR-free	*V. vinifera* (6 cvs), *Vitis* hybrid Chancellor, *V. labruscana* Concord and Niagara	Kikkert et al. 2005
	2,4-D (4.5) + BA (8.9)	BA (13.3)	*V. vinifera* Touriga Nacional	Pinto-Sintra 2007

Ovules	2,4-D (5) + BA (1)	NOA (10) + BA (1)	*V. vinifera* Cabernet Sauvignon and Grenache, *Vitis* hybrid Gloryvine	Srinivasan and Mullins 1980
	2,4-D (1) + TDZ (0.2)	2,4-D (1)	*V. labruscana* Kyoho	Nakajima et al. 2000
Ovaries	2,4-D (9) + IASP (17) + BA (1), then 2,4-D (2) or 2,4-D (2) + IASP (4)	NOA (10) + IASP (17) + TDZ (1) + ABA (1)	*V. labruscana* Fredonia and Niagara	Motoike et al. 2001
Styles and stigmas	NOA (5) + BA (9)	PGR-free	*V. vinifera* Sugraone	Morgana et al. 2004
Whole flowers, anthers, ovaries	2,4-D (4.5) + BA (9)	NOA (10) + IAA (20) + BA (1)	*Vitis vinifera* (8 cvs) and *V. berlandieri* x *V. rupestris* 110R	Martinelli et al. 2001b, 2003, Gri-baudo et al. 2004, Gambino et al. 2007, Cadavid-Labrada et al. 2008
Leaves, petioles, internodes, young florets	2,4-D (4.5) + BA (0.4)	NAA (10.7) + BA (0.4)	*Vitis* hybrid Seyval	Krul and Worley 1977
Leaves (from *in vivo* and *in vitro* plants), anthers	NOA (5) + BA (0.9)		*V. vinifera* and *V. rupestris* (several cvs)	Stamp and Meredith 1988a
Leaves, ovaries, anthers	2,4,5-T (10) + TDZ or CPPU (10)	2,4-D (1)	*V. vinifera* (10 cvs) and *V. labruscana* Delaware	Nakano et al. 1997
Leaves	2,4-D (5-10) + TDZ or KT30 (5-10)	2,4-D (1)	*V. vinifera* Koshusanjaku	Matsuta and Hirabayashi 1989
	2,4-D (9) + BA (9)	NOA (5) + BA (0.9)	*V. rupestris* du Lot	Tsolova and Atanassov 1996
	2,4-D (0.45) + BA (4.5)		*V. vinifera* Pusa seedless, Beauty seedless, Perlette and Nashik	Das et al. 2002
Leaves, petioles	2,4-D (4.5) + BA (0.4-4.4)	IAA (5.7) or IBA (0.5)	*V. rupestris*	Martinelli et al. 1993a
	2,4-D (9)+BA (4.4), then NAA (10.7) +BA(0.9)	PGR-free	*V. rotundifolia* Regale and Fry	Robacker 1993

Leaves (from *in vitro* plants)	2,4-D (5) + BA (1.1)	IAA (5) + BA (1.1)	*V. vinifera* x *V. rotundifolia* (2 cvs), *Vitis* hybrid VMH1	Torregrosa et al. 1995
	NOA (20) + BA (40) or TDZ (4)		*Vitis* hybrids Seyval blanc and Chancellor, *V. thunbergii*	Harst 1995
	2,4-D (9)+BA (4.4) for 6 wks, then NAA (5.4) + BA (4.4)	IAA (0.5)	*V. vinifera* Podarok Magaracha	Kuksova et al. 1997
Leaves, internodes (from *in vitro* plants)	2,4-D (4.5) + BA (9-20)	NAA (10.7) + BA (2.2-4.4) if callus from internodes, NAA (2.7-16) + BA (22.2) if from leaves	*V. vinifera* Krymskaya Zhemchuzhina, *V. monticola, Vitis* hybrids (2 cvs)	Marchenko 1991
Nodal sections	2,4-D (9) + BA (4.5)	NOA (10) + IAA (20) + BA (1)	*V. vinifera* (6 cvs), *Vitis* hybrid Fercal	Maillot et al. 2006
Seed integuments (from young berries)	2,4-D (9) + BA (4)	PGR-free	*V. vinifera* Autumn Royal seedless	Xu and Lu 2009
Protoplasts (isolated from leaf-derived embryogenic callus)	NAA (10.7) + BA (2.2)		*V. vinifera* Koshusanjaku	Zhu et al. 1997
Protoplasts (isolated from leaf-derived embryos and embryoids)	NOA (20) + TDZ (4)	PGR-free	*Vitis* hybrid Seyval blanc	Reustle et al. 1995
Zygotic embryos	NOA (5) + BA (0.9-4.5)		*V. vinifera* (4 cvs), *V. longii*	Stamp and Meredith 1988b
Zygotic embryos (from in ovulo embryo culture)	NOA (5) + BA (0.9)	PGR-free	*V. rotundifolia* (5 cvs)	Gray 1992

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; 2,4,5-T: 2,4,5-trichlorophenoxyacetic acid; BA: 6-benzyladenine (6-benzylaminopurine); CPPU: N-(2-chloro-4-pyridyl)-N'-phenylurea; cvs: cultivars; IAA: indole-3-acetic acid; IASP: indole-3-aspartic acid; IBA: indole-3-butyric acid; KT30: N-(2-chloro-4-pyridyl)-N'-phenylurea; NAA: 2-naphtaleneacetic acid; NOA: 2-naphthoxyacetic acid; PGRs: plant growth regulators; TDZ: N-(1,2,3-thidiazol-5-yl)-N'-phenylurea (thidiazuron).

nic calli or embryos (Reustle et al. 1995, Zhu et al. 1997). Recently, attempts to enhance protoplast technology have been reported in the frame of somatic hybrid production (Xu et al. 2007).

In somatic embryogenesis, both the developmental stage of explants as well as their preconditioning may be of great importance. For example, it is common practice to collect anthers at an early stage (uninucleate microspores). In a trial aimed at defining the best anther developmental stage to initiate embryogenic cultures in relation to the microsporogenesis and the outward appearance of anthers and flowers, six stages were identified (Gribaudo et al. 2004). These ranged from the stage, when the pollen mother cells were in premeiotic phase, the inflorescence was compact and the anthers were translucent green, to the stage, when anthers were opaque yellow and uninucleate pollen was formed. Optimal somatic embryogenesis was obtained in *V. vinifera* Chardonnay and Barbera when explants were collected at early stages, while in the rootstock 110R somatic embryogenesis occurred more frequently at later stages.

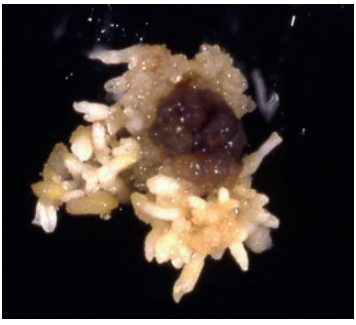

Fig. 1. Somatic embryogenesis from ovary cultures of *Vitis vinifera* cv Grignolino. Embryos develop asynchronously from the callus (from Bondaz et al. 2004).

The optimal developmental stage seems to be related to the genotype; for example, Nakajima et al. (2000) obtained best results in *V. labruscana* when anthers were collected 20 days before anthesis, while Bouhama et al. (2007) identified the tetrad stage as optimal in eight *V. vinifera* Tunisian cultivars. Chilling of floral explant at 4°C for a few days is usually suggested, even though some authors consider this step to be unnecessary (López-Pérez et al. 2005, Cutanda et al. 2008).

The basal compositions formulated by Murashige and Skoog (1962) and Nitsch and Nitsch (1969) have often been adopted in media used for somatic embryogenesis induction. Regarding growth regulator balance in the culture media, different combinations of a phenoxy-auxin, such as 2,4-dichloro-phenoxyacetic acid (2,4-D) or 2-naphthoxyacetic acid (NOA) with 6-benzyl-

adenine (BA), or - less frequently - other cytokinins, have been employed for inducing embryogenic calli. For further culture of the embryogenic calli, auxins are generally removed, decreased or substituted by other auxinic compounds. In some cases, however, these two morphological steps are obtained with the same growth regulator composition.

Refinements of the protocols have been frequently proposed and may contribute to increasing the final success rates. Composition of basal culture medium (Perrin et al. 2001), pH and light intensity (Das et al. 2002) have also been shown to influence successful cultures. Liquid media were used in the first papers on grape somatic embryogenesis (Mullins and Srinivasan 1976, Srinivasan and Mullins 1980); in subsequent research, liquid media were adopted in the initial culture step only for inducing formation of embryogenic calli (Bouquet et al. 1982, Faure et al. 1996a, Nakajima and Matsuda 2003). The type of solidifying agent used also affects significantly callus initiation and formation of embryogenic structures, with new gelling polymers being superior to agar (Perl et al. 1995, Torregrosa 1998). The addition of activated charcoal to the medium was essential for somatic embryo differentiation and maturation in several *Vitis* cultivars (Motoike et al. 2001, López-Pérez et al. 2005, Jittayasothorn et al. 2007).

2.2. Somatic embryogenesis from embryonic tissues

Somatic and zygotic embryos have been shown to be highly efficient in somatic embryogenesis protocols. Immature and mature whole zygotic embryos have been used for induction of somatic embryogenesis in both *Euvitis* (Stamp and Meredith 1988b, Emershad and Ramming 1994) and *Muscadinia* subgenera (Gray 1992). Zygotic embryos, however, are unsuitable for breeding programs where cultivar integrity must be preserved. In fact, their genetic constitution is unknown and they can be genotypically quite different from the parental cultivars due to the high heterozigosity of grapevine. On the other hand, they have been adopted as a model system for studies involving regeneration and exogene transfer (Stamp and Meredith 1988b).

Re-initiation of embryogenic calli from somatic embryos (secondary embryogenesis) has been used for preserving the embryogenic potential of the cultures (Martinelli et al. 1993a, 2001a, Kuksova et al. 1997). This procedure has been successfully applied in experiments on foreign gene transfer (Kikkert et al. 2001) where entire somatic embryos (Scorza et al. 1996, Martinelli and Mandolino 2000, Martinelli et al. 2002) or sections of somatic (Mullins et al. 1990) and zygotic (Scorza et al. 1995) embryos were used for secondary embryogenesis during co-culture with *Agrobacterium tumefaciens*.

Secondary embryogenesis from somatic embryos has been obtained in

various *Vitis vinifera* cultivars (Krul and Worley 1977, Matsuta and Hirabayashi 1989, Vilaplana and Mullins 1989, Perl et al. 1995, Kuksova et al. 1997, Morgana et al. 2004, Pinto-Sintra 2007), hybrids (Vilaplana and Mullins 1989) and different species, such as *Vitis rupestris* (Newton and Goussard 1990, Altamura et al. 1993, Martinelli et al. 1993a, 2001a) and *Vitis rotundifolia* (Robacker 1993). A genotype effect on the ability to regenerate secondary embryos was shown by Mozsar and Viczian (1996).

Both direct and indirect secondary embryogenesis have been described. As for direct embryogenesis, it has been generally observed that somatic embryogenesis occurs more frequently on the root/shoot transition zone and in the root region, confirming a gradient of embryogenic competence along the embryo tissues (Martinelli et al. 1993a; Fig. 2).

Secondary embryogenesis has been described to be superficial, i.e. originating from the epidermal layer of the embryo (Altamura et al. 1993, Margosan et al. 1994). In particular, striking alterations have been observed by scanning electron microscopy and light microscopy in *Vitis rupestris* somatic embryo tissues during secondary embryogenesis (Martinelli et al. 2001a). The most essential alteration concerned the external layer where, like meristematic cells, the cells exhibited an active proliferation and initiated clusters progressively, forming numerous huge protruding masses. Thus, callusing was occurring from the surface cells. This could be the reason why application of a co-culture of *Agrobacterium tumefaciens* with such embryogenic tissues resulted to an efficient strategy for gene transfer.

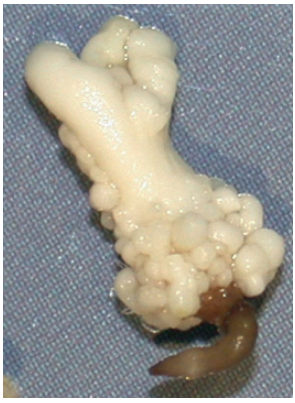

Fig. 2. Secondary embryogenesis on a *Vitis rupestris* somatic embryo: morphogenesis is mostly produced on the root region.

2.3. Long term embryogenic cultures

In view of their many applications, and the labor needed to obtain them, once induced and established, grape embryogenic cultures are precious materials. However, during the subsequent subcultures, embryo development and eventual conversion into plants may lead to considerable reduction in/or loss of the primary embryogenic callus. Secondary embryogenesis is a very important property of somatic embryos providing a long-term source of somatic embryos.

Various strategies have been adopted for maintaining the embryogenic competence of the cultures indefinitely. Subcultures have been carried out in the presence of auxin, with (Gray and Mortensen 1987, Stamp and Meredith 1988a, Perl et al. 1995) or without cytokinins (Matsuta and Hirabayashi 1989, Martinelli et al. 1993a), on a growth regulator-free medium (Krul and Worley 1977, Lebrun and Branchard 1986, Gray and Mortensen 1987), or with a reduced concentration of growth regulators (Motoike et al. 2001, Pinto-Sintra 2007). Our strategy, based on the protocol of Franks et al. (1998), is a two-step cycle that could be repeated indefinitely. In the first step, callus initiation and propagation is obtained within a two month-culture, while in the second step, development of somatic embryos is induced within an additional two-month culture (Franks et al. 1998, Martinelli et al. 2001b, Gambino et al. 2007).

Embryogenic cultures have been reported to be maintained for as long as several years (Gray 1989, Torregrosa 1998). In our hands, the oldest cultures of *Vitis vinifera* and *Vitis rupestris* produced in our laboratories still retain their embryogenic competence after 10 and 18 years, respectively.

Long-term culture preservation has also been obtained by establishing liquid embryogenic suspension cultures started from somatic embryos and embryogenic calli. The best suspensions should produce pro-embryogenic masses capable of proliferating without differentiation and retaining their totipotency until further induction produces highly synchronized somatic embryos (Jayasankar et al. 1999, Jittayasothorn et al. 2007). Success of the cultures has been attributed to both, the culture conditions and the status of the embryos.

The starting material is an important factor, and primary globular- and heart-stage embryos have been shown to be the most suitable source, while more advanced developmental stages failed to produce secondary embryogenesis (Bornhoff and Harst 2000). Removing these cited less suitable developmental stages from the cultures has been considered as necessary. The meticulous work of differential size fractionation is usually performed manually with a forceps or by sieving or through discontinuous density gradient centrifugation in Ficoll solutions (Colova-Tsolova et al. 2007);

alternatively, attempts have been made using ultrasonic standing waves, although only a modest success rate in grape has been achieved (Maitz et al. 2000). To prevent culture browning, the addition of antioxidants, such as dithioerythritol (DTE; Perl et al. 1996), the use of glucose and pH adjustment to 5.8 (Bornhoff and Harst 2000) have also been proven to be beneficial. Various growth regulators at various concentrations and combinations, as well as the omission of these compounds, have been tested by different researchers (Zlenko et al. 2005).

Culture density and in particular a high cell population has proven to be an important parameter in establishing suspension cultures and in blocking further embryo development, probably due to the accumulation of toxic compounds and extra-cellular macromolecules. For this reason, it has been suggested that the cultures be maintained at low density by daily subcultures in a fresh medium (Maës et al. 1997). Recently, the use of a conditioned medium, i.e. a culture medium added with the filter-sterilized supernatant collected from an established suspension culture, was shown to be suitable in establishing highly proliferating embryogenic cell suspensions of some grapevine cultivars and rootstocks. The presence of extracellular proteins secreted in the added medium, in particular arabinogalactan proteins, has been put forward as an explanation of this favorable effect on cell proliferation (Ben Amar et al. 2007).

The embryogenic suspension cultures proved to be appropriate for successful gene transfer, particularly when a biolistic approach is used (Kikkert et al. 2005b).

2.4. Morphogenic potential of somatic embryos

Besides secondary embryogenesis, somatic embryos were also found to be suitable for plantlet regeneration *via* direct organogenesis. This morphogenic potential opens up interesting perspectives for massive plantlet production from selected embryos, as for instance in the case of successful gene transfer.

Chardonnay cotyledonary leaves dissected from somatic embryos showed a highly efficient shoot differentiation capability along the central vein (Martinelli et al. 2001b). Each cotyledonary leaf produced many shoots that regenerated whole plantlets and this direct regeneration system was compatible with embryo germination. Adventitious bud induction was also obtained from hypocotyls and cotyledons of dormant somatic embryos by Vilaplana and Mullins (1989), although that particular strategy was described as an alternative to embryo germination.

Another regeneration system, differing from the direct organogenesis described above, was set up for *Vitis rupestris* (Martinelli and Mandolino 1994) and for several other genotypes (unpublished); somatic embryos with a non

canonical shape failed to germinate, but after the production of a green disorganized callusing tissue many individual shoots could be regenerated *via* organogenesis from each embryo.

2.5. Somatic embryo teratology

It has been generally observed that embryogenic cultures are never spontaneously synchronized and that heterogeneity in the developmental steps (globular, heart-shaped, torpedo and cotyledonary stages) and eventual morphological heterogeneity can be distinguished (Martinelli and Gribaudo 2001). Mature somatic embryos with canonical shape are characterized by an evident polarization i.e. the root and the shoot axes, and they present a hypocotyl and two cotyledons (Fig. 3). However, abnormal embryos can be frequently observed at the end of their development.

Fig. 3. A grape somatic embryo showing the canonic shape, i.e. evident polarization with the root and shoot axes and the presence of a hypocotyl and two cotyledons.

In a study conducted on the rootstock 41B, Goebel-Tourand et al. (1993) separated eight classes of somatic embryos, each characterized by a specific shape and morphology: mono-, di- and poli-cotyledonary embryos, trumpet-embryo (the most frequent class), embryos with long hypocotyls and vestigial thread-like cotyledons, zygotic-like embryos, cauliflower-like embryos, fused embryos with moderate fasciation. The various shapes were correlated with specific growth regulator compositions added to the media during maturation, with the abscisic acid/zeatin combination being the most suitable in inducing the canonical aspect. We also found analogous abnormal morphologies in somatic embryos of *Vitis rupestris* and *Vitis vinifera* cvs Chardonnay and Brachetto (Martinelli et al. 2001b).

In general, somatic embryos are bigger than their zygotic counterparts (Goebel-Tourand et al. 1993). Moreover, continuous growth after the torpedo stage has been described, giving origin to giant structures which rarely undergo germination (Faure et al. 1996b). Abnormal developments of the root pole - with either lack of root tip, bifurcation or more than one root formation - have been described (Altamura et al. 1992; Martinelli and Gribaudo 2001).

Normal embryos have a functional apical meristem between the two well-defined cotyledons; conversely, in aberrant embryos incomplete development or lack of apical meristem has been observed (Goebel-Tourand et al. 1993, Faure et al. 1996a, Martinelli and Gribaudo 2001). In these cases, embryo germination was strictly dependent on apex functionality.

Teratology has also been related to culture conditions, genotype and physiological state. The growth regulator composition of the medium has been considered as a principal cause of anomalous embryo cotyledon number (Ammirato 1977, Goebel-Tourand et al. 1993). However, a general explanation of events leading to abnormal morphologies of grape somatic embryos has not yet been put forward.

The occurrence of genetic mutations associated with *in vitro* cultures has also been considered. Ploidy level and nuclear DNA content in embryos of *Vitis vinifera* cv Grenache noir have been measured by flow cytometry and *in situ* DNA microspectrophotometry (Faure and Nougarède 1993); homogeneous nuclear populations and typical diploid levels were assessed and neither aneuploid nor polyploid nuclei were detected. Moreover, identical results were observed in a sample of zygotic embryos at the same stage of development and therefore a genetic component in embryo teratology has been excluded.

Analogous results have been reported in a regenerated population of *Vitis vinifera* cv Podarok Magaracha plants (Kuksova et al. 1997), where cytological analysis showed normal chromosome morphology, a negligible level of tetraploidy and neither chimeral nor aneuploid plant production. Accordingly, only a few examples of somaclonal variation have been documented in grape (Torregrosa et al. 2001).

3. CONVERSION OF SOMATIC EMBRYOS INTO PLANTLETS

As already pointed out (Martinelli and Gribaudo 2001), the efficiency of somatic embryo conversion into plantlets is a key factor in the success of the regeneration method. Embryo conversion is accompanied by development of the primary root, greening of hypocotyls and cotyledons and formation of the shoot apex with one or two foliar primordia (Redenbaugh et al. 1986; Fig. 4a).

Although somatic embryogenesis induction has been obtained in several grape species and cultivars, further germination and plant recovery seem to vary considerably. The behavior of somatic embryo cultures appears to be quite different depending on the various protocols and genotypes used. In addition, the morphological and physiological state of embryo development and the culture conditions are crucial aspects in promoting embryo conversion. Abnormal embryo morphogenesis has been found to be linked to low germination efficiency.

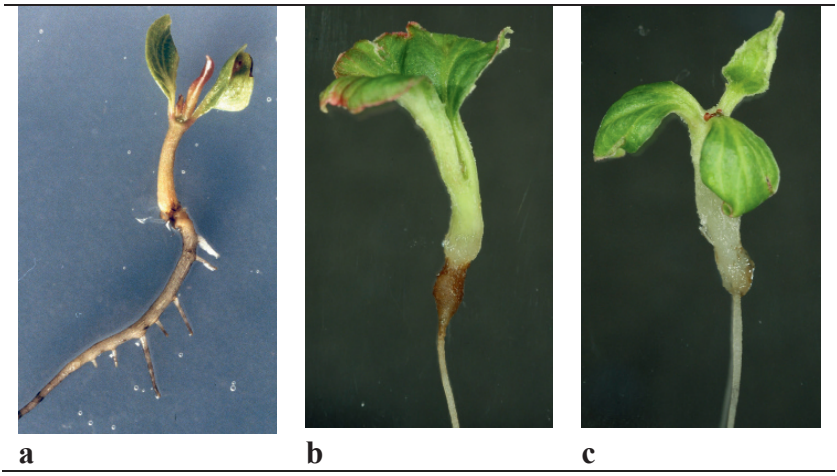

Fig. 4. Seedlings from grape somatic embryos respectively showing (a) the canonic shape leading to germination and teratologies regarding the cotyledon number, (b) mono- or fused-cotyledons and (c) three-cotyledons. From Martinelli and Gribaudo (2001).

3.1. Dormancy

When canonical, well-developed somatic embryos at an advanced stage of development fail to germinate, physiological rather than morphological anomalies appear to hinder germination efficiency and therefore dormancy may be considered as the most critical aspect of embryo conversion into plantlets.

Grape seeds (with zygotic embryos) exhibit dormancy that is overcome by cold treatment, usually stratification in sand at low temperature (2-4°C), which results in a decrease in endogenous abscisic acid (ABA) in the seed. Accordingly, chilling Gloryvine (*V. vinifera* x *V. rupestris*) embryogenic cultures was shown to induce a rapid reduction in endogenous ABA content in somatic embryos and to improve germination as well, while the addition of exogenous ABA to the medium resulted to inhibition of germination (Rajasekaran et al. 1982). Chilling was also essential for maturing somatic embryo

germination of another *V. vinifera* x *V. rupestris* hybrid (Takeno et al. 1983) and of four *V. vinifera* genotypes (Das et al. 2002). In *V. rupestris*, chilling was shown to increase the speed of embryo germination (Martinelli et al. 1993a). Changes in the metabolism of endogenous gibberellin-like substances may also be involved in chilling-induced release from dormancy (Takeno et al. 1983, Pearce et al. 1987).

Following a comparison between Chardonnay and Thompson seedless somatic embryos cultured in solid and liquid cultures, respectively, no dormancy and precocious germination was obtained in liquid-cultured embryos while the others presented dormancy, like their zygotic counterparts (Jayasankar et al. 2003). A detailed histological comparison revealed morphological differences when either solid or liquid cultures were adopted for embryo development. In the embryos cultured in liquid, a large, persistent suspensor, smaller cotyledons and a more anatomically defined shoot apical meristem were found. The persistent suspensor was assumed to be responsible for the different germination performances, this tissue being a site of gibberellin synthesis. The poor apical development observed in the solid-derived embryos was related to the lack of an adequate suspensor resulting in a deficient supply of growth factors and other nutrients. Dormancy-breaking treatments were found to be effective.

3.2. Promotion of germination

Various alternatives to the chilling strategy described above were proposed as dormancy-breaking treatments. Specific growth regulator compositions have been studied for germination of embryos. BA is often used for obtaining an efficient plant yield (Gray 1987 and 1989, Robacker 1993, Torregrosa 1995, Maillot et al. 2006, Pinto-Sintra 2007). In *V. rupestris*, BA was effective in promoting embryo germination if a low amount of IBA was also present (Martinelli et al. 1993a). However, when applied during an early developmental stage, cytokinins allowed progress beyond the torpedo stage but without improving the final embryo conversion rate (Goebel-Tourand et al. 1993). Addition of exogenous ABA to the medium often inhibited somatic embryo germination (Rajasekaran et al. 1982, Takeno et al. 1983, Gray 1989, Perl et al. 1995). On the other hand, it has been pointed out that this treatment can prevent precocious germination and may slightly help in promoting normal regeneration into plants (Goebel-Tourand et al. 1993, Faure et al. 1998).

Addition of gibberellin (GA_3; Mullins and Srinivasan 1976, Rajasekaran and Mullins 1979, Zlenko et al. 2002) and application of dehydration proved effective; moreover, the latter strategy has been cited as allowing embryo germination in a synchronous pattern of root to shoot emergence similar to a

seedling (Gray 1987, 1989). Strictly speaking, dehydrated embryos that germinate directly after imbibition are better described as quiescent rather than as dormant: the term 'quiescence' designates a resting state reversed solely by the addition of water, whereas 'dormancy' refers to rest where resumption of growth is dependent on specific treatments or conditions in addition to water, as defined by Gray (1987).

Alternatively, several authors have obtained germination of somatic embryos and subsequent conversion into plants, with differing degrees of efficiency, by culturing the embryos on plant growth regulator-free media (Nakano et al. 1997, Salunkhe et al. 1999, Nakajima et al. 2000, Carimi et al. 2005). Frequent transfers on a growth regulator-free medium (Coutos-Thevenot et al. 1992a), cotyledon removal (Mauro et al. 1986) or increased sucrose concentration (90-150 g l^{-1}; Compton and Gray 1996) have also been reported as treatments promoting embryo germination.

No definitive answer to the problem of germination failure has yet been given. A variety of factors are probably involved and a general pattern has not been defined. Researchers have reported different situations in their embryo cultures and the improvement of conversion rates is probably related to the optimization of conditions during embryogeny induction and expression.

3.3. Morphological and physiological alterations

Apart from the dormancy state, embryo teratologies have proven to be crucial in preventing germination of grape somatic embryos. Indeed, germination has been stated to occur only in white, well-shaped, well-polarized embryos with root and shoot axes, a hypocotyl and two cotyledons (Fig. 4a; Martinelli et al. 1993a). Remarkably, when optimal developmental and physiological status have been reached, somatic embryos are able to germinate and to recover plantlets even after directly seeding in sterilized soil (Jayasankar et al. 2001).

When lack of conversion into plantlets results from morphological abnormalities, embryo development has been observed to be blocked at an early stage (globular and heart-shaped stages; Couthos-Thevenot et al. 1992a) or following the torpedo stage (Bouquet et al. 1982, Faure 1990, Faure et al. 1996b, 1998). This fact has been well documented on somatic embryos with morphological abnormalities, mostly giant structures (Goebel-Tourand et al. 1993, Faure et al. 1996b). In these cases, block in the shoot development was observed, while root apexes were functional. Abnormal or missing shoot apexes (Favre 1977, Krul 1985, Lebrun and Branchard 1986, Faure 1990, Goebel-Tourand et al. 1993) or loss of meristematic characteristics of the shoot apex as embryogenesis proceeds (Faure et al. 1996b) have been documented. The latter

case is frequently associated with pronounced teratological behavior where the embryo exhibits continuous growth leading to abnormal structure and function of the shoot meristem (Faure et al. 1996b, 1998). This anomalous development strongly resembles 'precocious germination', an event already described for zygotic embryos during *in vitro* culture (Finkelstein and Crouch 1984), probably due to a breakdown in the mechanisms that regulate the germination of mature embryos (Gray 1989), such as insufficient accumulation of the growth regulators supporting normal plant development (Faure et al. 1998).

Enhanced germination efficiency and early plant development in Chardonnay has been described as being related to a peculiar abnormal embryo morphotype, i.e. complete absence of one cotyledon (Jayasankar et al. 2002). In embryo cultures, authors have frequently (10%) found a monocotyledonous morphotype comparatively larger and with a much broader area of meristematic cells comprising the shoot apical meristem than in canonical somatic embryos. The more rapid shoot emergence and plant development was ascribed to the larger apical meristematic region. Since histological observation has proven that the vascular strands of the absent cotyledon terminate in the meristematic region, a possible increase of nutrient supply was presumed to account for this enhanced performance. In this study, embryos were cultured on growth regulator-free medium, excluding that these compounds could have caused aberration of embryo cotyledon number.

Abnormal metabolic activity and unusual accumulation of specific compounds have been detected in somatic embryo cultures with low or no germination activity. In somatic embryos of *V. vinifera* cv Grenache noir large accumulation of reserve products (starch and lipids) was found, without subsequent hydrolysis and utilization (Faure and Aarrouf 1994). The low germination rate of somatic embryos has also been correlated with high free polyamine content and/or low putrescine/spermidine ratio (Faure et al. 1991) when compared to zygotic embryos.

3.4. Culture conditions

Culture conditions can also block somatic embryo development. It has been suggested that high humidity during *in vitro* culture greatly reduces the efficiencies of embryo conversion into plants in *Vitis rupestris*, where profound alterations in the embryo morphogenesis, such as an impermeable suberization of surface cell layers, have been documented (Faure 1990). In liquid cultures, the presence of products of metabolic activities in the media proved to have an inhibitory effect on culture development. Indeed, daily embryo subcultures to a fresh medium at low population density has been recommended for promoting embryo germination (Coutos-Thevenot et al. 1992a).

Also, the effect of extracellular proteins on grape somatic embryogenesis has been documented and an extracellular proteolytic mechanism has been proposed (Coutos-Thevenot et al. 1992a, b, Maës et al. 1997). In general, liquid media, which are often used for recurrent embryogenic suspension cultures, seem to be less favorable for the conversion of embryos into plantlets (Goebel-Tourand et al. 1993, Jayasankar et al. 1999). In addition, cell concentration and initial cell population density were also proven to have an influence on embryo development, possibly related to the above mentioned level of extracellular protein accumulation that seems to be implicated in the early inhibition of embyogenesis and in the stimulation of secondary embryogenesis (Maës et al. 1997).

4. ADVANCES IN UNDERSTANDING AND CHARACTERIZING GRAPE SOMATIC EMBRYOGENESIS

Although in model plants considerable research aiming at understanding the molecular and genetic mechanisms of somatic embryogenesis has been carried out (de Vries 1998, Chugh and Khurana 2002), yet basic studies on morphogenesis in the *Vitis* genus are rather limited. Some interesting research has been accomplished on ontogenesis and differentiation of somatic embryogenesis, but the biochemical and molecular mechanisms leading to somatic embryos and regulating their development remain largely unknown for grape. Thus, in this chapter all the available information is presented with a historical perspective.

4.1. Ontogenesis and differentiation of somatic embryogenesis

4.1.1. Characterization of somatic embryo origin

Several histological studies have been implemented for assessing the somatic or gametic origin of anther-derived plantlets, as these male organs have been the most commonly used starting material (Table 1). In fact, potentially dihaploid plantlets can be produced when morphogenesis arises from pollen grains. Only in one case, i.e. in the local Chinese cv Shengli, regeneration of putative haploid plants from grape anther cultures was reported (Zou and Li 1981). Here, within varying degrees of ploidy levels (di- tri- and tetra-ploidy), haploid chromosome sets (n =19) were found in the root tip cells of regenerated plantlets. A mixoploid population of cells was also found in anther-derived plantlets of *V. latifolia* (Salunkhe et al. 1999).

With these exceptions, further histological studies have excluded any haploid origin of anther-derived somatic embryos. Embryos were found to

regenerate from the diploid cells of the connective tissues of the anther filaments or loculi in two grape hybrids (Rajasekaran and Mullins 1983) and from the abaxial side or the lateral walls of the anther, the filament and all connective tissues between the loculi in *V. rupestris* du Lot (Newton and Goussard 1990), where endothecium was the only anther tissue not participating in callusing (Altamura et al. 1992). Involvement of the endothecium in *V. vinifera* cv Grenache noir during direct proembryo development has been described and microspore degeneration has also been documented during callus culture progression (Faure et al. 1996a). Caryotypic characterization of calli and the biochemical and ampelographic parameters measured in the subsequently regenerated plantlets also provided evidence for a somatic origin of callusing (Rajasekaran and Mullins 1983).

Since regeneration of haploid plants from anthers has generally failed, microspores have been used as explants in an attempt to completely exclude somatic anther tissues from the cultures. Production of haploid calli (Gresshoff and Doy 1974) and the development of globular structures, although not producing viable embryos, has been documented from microspores of *V. rupestris* du Lot (Altamura et al. 1992) and of several *Vitis vinifera* genotypes and *Vitis* interspecific hybrids (Sefc et al. 1997).

Apart from anthers, ovaries have also been proven to be suitable material for establishing somatic embryogenesis (Table 1). The origin of adventitious embryos and the subsequent developmental steps induced from immature ovule cultures of the *V. vinifera* cv Neo Mat have been identified by histological examination (Nakano et al. 2000). Callus was produced from the receptacle parenchyma tissues whereas cell division was never observed in any of the tissues of the ovule including embryo sac, inner and outer integuments, and nucellar tissues. The shift to morphogenesis was characterized by cells with starch grain accumulation, a prominent nucleus, dense cytoplasm and thick cell walls. During this process, ovules continued to degenerate thus proving the somatic origin of embryos.

Although, according to the research presented here, somatic origin of anther-derived embryos has been proven in most cases, participation of haploid pollinic cells has also been reported (Zou and Li 1981, Rajasekaran and Mullins 1983, Altamura et al. 1992). Furthermore, while somatic origin has been identified when ovules have been used (Nakano et al. 2000), callusing has also been described as having nucellar origin (Srinivasan and Mullins 1980). Given these controversial findings, which may be due to differences in genotypes and the media used, participation of sexual cells in regeneration when using flower explants can never be *a priori* fully excluded. Thus, potential recombination events are to be expected and need to be accurately monitored.

Molecular typing based on simple sequence repeats (SSR) was

successfully applied in evaluating floral regeneration products in *V. vinifera* cv Chardonnay, the rootstock Kober 125AA and the accession *V. rupestris* du Lot (Martinelli et al. 2004). A genotype assessment at a few SSR loci (VVS2, VVMD5, VVMD7, VVMD27, ZAG62 and VrZAG79, This et al. 2004) was carried out on somatic embryos and plantlets regenerated from embryogenic calli of both anthers and ovaries. In none of the selected samples were recombination events found to occur in the cells involved during the morphogenetic process from anthers and ovaries, thus excluding the participation of cells of the sexual lines in this event.

4.1.2. Embryogenic callus characterization

In most explants, somatic embryogenesis is generally indirect, i.e. it takes place after completion of callusing induction (Fig. 1); however, a primary embryogenic process arising directly either from the midvein of the leaves (Stamp and Meredith 1988a), from the anther endothecium (Faure et al. 1996a) or from zygotic embryos (Stamp and Meredith 1988b) has been described. Even within the wide variation observed among the different genotypes, explants and protocols (Table 1), the shift from an undifferentiated callus to an embryogenic callus generally takes place after several months of induction. Once induced, however, embryogenic competence is retained for long periods, as much as several years.

The embryogenic callus is typically granular and white, and is composed of clusters of small isodiametric cells, interspersed with larger vacuolated cells (Gray and Mortensen 1987, Nakano et al. 2000, Martinelli et al. 2001a); it is usually associated with a dark callus (Fig. 1) which, after biochemical analysis, has proven to be non-viable, autolyic tissue (Gianazza et al. 1992). The relationship between necrotic cells and somatic embryo initiation is not clear, and it has yet to be shown whether embryogenesis is the cause or the result of cell lysis (Krul and Worley 1977, Newton and Goussard 1990, Gray 1992, Robacker 1993).

In the callus, embryoid formation involves both the internal (deep genesis) and peripheral layer (superficial genesis) of single cells (Altamura et al. 1992). Embryogenic cells are easily identified by their prominent nuclei and nucleoli, their small size and a dense cytoplasmic content (Krul and Worley 1977, Newton and Goussard 1990, Altamura et al. 1992, Faure et al. 1996a and 1996b, Martinelli et al. 2001a).

A strong correlation between the genesis of somatic and zygotic embryos has been well documented in *V. rupestris* du Lot (Altamura et al. 1992) and in Grenache noir (Faure et al. 1996a and 1996b); by the time of the first division of the proembryonic cell, polarity is established with the formation of two cells, homologous to the basal and the apical zygotic ones, giving rise to the suspensor and to the *sensu stricto* embryonic cells respectively. Moreover, a further Asterad type development occurs and the subsequent progression of somatic embryos

follows the canonical phase order (globular, heart-shaped, torpedo) up to their conversion into plants.

4.2. Molecular tools for characterization of somatic embryogenesis

4.2.1. Protein analysis

The biochemical changes associated with morphogenesis, from explant to plantlet, were analyzed in *Vitis rupestris* (Martinelli and Gribaudo 2001). The analysis of the electrophoretic patterns of total proteins (Gianazza et al. 1992) and specific enzymes (acid phosphatase, alcohol dehydrogenase, esterase; Martinelli et al. 1993b) proved to be effective in characterizing the main steps in somatic embryogenesis. Comparison of the different stages leading the callus to embryogenic callus phases, embryo development and finally to plantlet formation, revealed typically different two-dimensional electrophoretic patterns.

More specifically, dramatically different patterns were found in the non-embryogenic and embryogenic calli, suggesting a massive shift in gene expression during the development of embryogenic competence in the callus. These results also suggest that expression of several genes is necessary for embryogenesis during the callus culture, and therefore that both the low frequency and the slow progression (several months) from the initial callus to the embryogenic callus is justified (Martinelli et al. 1993a). Furthermore, during embryo development and plantlet formation, a simplification of the two-dimensional protein pattern occurred, suggesting a less radical change in gene expression; accordingly, the latter developmental steps progressed much faster.

The zymograms obtained for alcohole dehydrogenase, acid phosphatase and esterase were also different, either in the specific activity or in the number and isoelectric point of the expressed isoforms (Martinelli et al. 1993b). The differences detected were always related to the subsequent steps of embryogenesis, i.e. the enzymatic changes observed in embryogenic calli were always retained by the developing embryos. Moreover, typical isoenzyme levels and types of embryo status were not retained in the calli differentiated from somatic embryos, suggesting differences in gene expression.

A recent comparison between total proteins extracted from embryogenic and non-embryogenic calli of *V. vinifera* cv Thompson seedless performed with two-dimensional gel electrophoresis coupled to mass spectrometry, identified several proteins whose expression differed during somatic embryogenesis (Marsoni et al. 2008). Among 31 detected proteins that were recognized as involved in cellular metabolism and protein processing, one protein was identified as probably implicated in morphogenesis regulation (regulatory sub-unit A, beta isoform of a protein phosphatase 2A). In addition, analysis of

differentially expressed proteins in the two types of calli suggested that embryogenic status is related to an enhanced activity in controlling oxidative stress.

Furthermore, histone proteins in the rootstock 41B characterized early stage somatic embryogenesis (Redon et al. 1996, 1999). In the young embryogenic clusters and in the globular embryos, five H1 histone variants were distinguished on the basis of molecular weight and the relative ratio determined. Both the qualitative and quantitative shifts of these proteins assayed during the subsequent heart-stage development suggested major changes in gene expression related to embryo morphogenesis.

4.2.2. Growth regulators

Endogenous hormone regulation is an important aspect of morphogenesis and some studies have been carried out to assess hormone levels during the crucial steps of embryognesis.

A radio-immunological analysis of several endogenous hormones was performed on callus cultures with different embryogenic potential of Seyval blanc, Riesling and Trollinger (Jiménez and Bangerth 2000). Among the several hormones assessed, only ABA levels correlated with the morphogenic capacity of the cultures since higher levels of this hormone were detected in embryogenic-competent callus lines. ABA contents were associated with embryogenic callus proliferation through secondary embryogenesis and with the prevention of precocious embryo germination, as previously reported (Faure et al. 1998). Furthermore, IAA levels were more dependent on genotypes than on callus types, while gibberellin's role was found to be non-determinant in morphogenesis and cytokinins were more related to cell division than to the embryogenic process. Surprisingly, after a two-year analysis, endogenous IAA and BA concentrations were found to exhibit cyclical behavior during the different seasons of the year, despite the constant environmental parameters of the growth chambers. On the other hand, in a different study (Faure et al. 1998), a decrease in IAA levels correlated with the expression of somatic embryogenesis.

Among the endogenous hormones, polyamines proved to be key factors during morphogenesis of grapes, and the putrescine/spermidine ratio was found to be a good marker of somatic and zygotic embryo development (Faure et al. 1991). The polyamine balance associated with specific steps of grape somatic embryogenesis was assessed in *Vitis vinifera* cvs Chardonnay and Brachetto a grappolo lungo (Bertoldi et al. 2004). The content of putrescine, spermidine and spermine and the activity and transcription of the principal enzymes involved in polyamine metabolism (ornithine decarboxylase, S-adenosylmethionine decarboxylase, arginine decarboxylase) were measured in samples of calli,

embryogenic calli and embryos at different stages and germinated plants. Total polyamine content was higher in the cv Brachetto g.l. than in Chardonnay and, in all samples of both cultivars, putrescine was the more abundant polyamine, as previously described in the cv Grenache noir (Faure et al. 1991). These data showed that the levels of polyamines are important factors during grapevine somatic embryo germination since a consistent decrease in the free polyamine fraction and a high polyamine conjugation process was observed during germination. Interestingly, this fact was associated with the high efficiencies obtained for embryo conversion into plantlets. Moreover, expression levels and enzyme activities of the three enzymes assessed were highest in the germinated plant samples. Accordingly, a much lower conversion efficiency was obtained in the cv Grenache noir, where a high free polyamine content and/or a low putrescine/spermidine ratio was measured in teratological embryos with difficult and abnormal germination (Faure et al. 1991). In the somatic embryos of two seedless cultivars (2A-Clone and Crimson Seedless), higher levels of cellular putrescine in pro-embryogenic masses correlated with maturation and germination efficiencies. Accordingly, an exogenous supply of putrescine was suggested for enhancing germination percentages (Nookaraju et al. 2008).

5. EXPLOITATION OF SOMATIC EMBRYOGENESIS POTENTIAL IN GENETICS, BREEDING AND SANITARY IMPROVEMENT OF GRAPE

5.1. Genetic improvement

Plant regeneration through somatic embryogenesis is undoubtedly a crucial and inevitable step during genetic engineering of grapevine, and the problems and successes of this biotechnological approach to grape breeding have been exhaustively reported in various reviews (Kikkert et al. 2001, Colova-Tsolova et al. 2001, Perl and Eshdat 2007).

Besides gene transfer, the exploitation of genetic modifications that potentially occur during dedifferentiation/differentiation processes has also been considered. Examples of variations in the morphological features of somatic embryo-derived plants have already been reported by Bouquet (1989), although the author could not establish the nature of variations. Later, selection pressure was applied to somatic embryos or embryo-derived plantlets, and lines able to grow in media containing fungal toxins (Soulie et al. 1993) or fungal culture filtrates (Jayasankar et al. 2000) were obtained. Somaclones of the cv Gamay cultivated in vineyard for 10 years showed lower yield, higher sugar content and improved maturity compared with the reference clone (Desperrier et al. 2003).

However, molecular analyses are required to ascertain if the somaclonal variations are of genetic or epigenetic origin. Until now, only a few studies have been aimed at characterizing grapevine somaclones by molecular techniques (Popescu et al. 2002, Bertsch et al. 2005, Leal et al. 2008, Gribaudo et al. 2009) and further research is needed for a better understanding of these issues.

Ploidy level analyses of somatic embryo-derived grapes have been already discussed. Mutagenesis was induced through gamma irradiation (Kuksova et al. 1997) or colchicine treatments (Yang et al. 2006) applied to calli or young embryos, and in both cases tetraploid plants were obtained.

Spontaneous somatic variants also represent a key resource in functional genomic studies in perennial plant species where mutants are difficult to generate. The phenotypic and molecular characterization of six grapevine somatic variants with abnormal flower development patterns has been recently carried out (Chatelet et al. 2007).

Another contribution that somatic embryogenesis makes to the elucidation of certain aspects of grapevine genetics is in the separation of chimeras. Somatic embryos were obtained from floral or nodal explants of chimeric Pinot meunier (Franks et al. 2002), Pinot gris (Hocquigny et al. 2004) and Chardonnay (Bertsch et al. 2005) clones. The cell layers of these periclinal chimeras were separated and the regenerated plantlets showed a diallelic genotype for the examined microsatellite loci. Regeneration through organogenesis, conversely, maintained the genetic chimerism of the mother plant (Bertsch et al. 2005).

5.2. Germplasm storage

Germplasm preservation is important in order to preserve biodiversity and potentially unknown useful genes or gene families. In the case of non-zygotic clonal germplasm, where seed storage is not possible, field collections are usually employed, although this method is hindered by high costs and climate conditions. Alternative strategies such as storage of dormant bud-wood (Esensee et al. 1990) or *in vitro* shoot tip cultures in cool climate rooms (Skene et al. 1988) have been used, although both procedures are time- and space-consuming.

Cryopreservation of embryogenic cell suspensions based on encapsulation-dehydration or encapsulation-vitrification methods has been attempted in some *V. vinifera* cultivars and rootstocks, with varying degrees of success, and has shown the interesting ability of cultures to retain their potential for regrowth and plant regeneration (Wang et al. 2004).

Synthetic seed technology based on mature somatic embryo encapsulation and/or drying and refrigeration have been proposed as alternative

strategies able to provide clonal germplasm storage in seed repositories (Gray and Compton 1993, Jayasankar et al. 2005, Das et al. 2006), and in some *V. vinifera* cultivars they have proved to be simple and efficient methods for long-term embryo storage and plant recovery.

5.3. Virus eradication

Although breeding is undoubtedly the main application of somatic embryogenesis, this technique has also be proven to be highly effective in eradicating viruses from infected grapevines. No viruses were detected in plantlets regenerated through somatic embryogenesis from mother plants infected by phloem-limited viruses, such as GLRaV-1 and -3, GVA and GRSPaV (Goussard et al. 1991, Gambino et al. 2006, Gribaudo et al. 2006). Somatic embryogenesis alone (Gambino et al. 2009) or combined with thermotherapy (Goussard and Wiid, 1992) allowed also elimination of GFLV. The distribution of some phloem-limited viruses in flower explants, embryogenic and non-embryogenic calli, single somatic embryos, and plants regenerated from embryogenic cultures was investigated (Gambino et al. 2006). Viruses were detected both in anthers and ovaries; four months following culture initiation most calli were still infected while no viruses were detected in calli tested 8 months later, nor in embryos or plantlets. Accordingly, somatic embryogenesis proved to be a promising technique for the elimination of certain viruses, although it is technically more difficult, more time-consuming and more cultivar-dependent than traditional sanitation procedures.

Grapevine somatic embryos have also been proposed as a source of hypocotyl explants to be used as cuttings for apex micrografting with the aim of virus eradication (Torres-Viñals et al. 2004).

6. CONCLUSIONS

Starting from the 1970s, when the first attempts to obtain somatic embryogenesis in the *Vitis* genus are dated, a nearly forty years research led to the development of suitable protocols for somatic embryogenesis induction and further plant recovery for a considerable number of genotypes. However, for various agronomically important cultivars, this technique is not yet a routine and efficiencies need to be improved.

Several strategies have been assessed for overcoming the problems affecting somatic embryogenesis, for enhancing efficiencies and for replacing the costly ‘trial and error’ procedures. After the morphological approach that has mainly been adopted, recently some basic studies aimed at understanding

tissue competence and molecular changes associated to morphogenesis on grape have been conducted. The increasing interest for grape biotechnology would hopefully address advanced research to understand grape morphogenesis. We believe, in fact, that it is necessary to develop molecular tools for a better understanding of the key steps in regeneration and for identifying markers and sets of specific marker genes.

At this time point, we can conclude that the earliest phases of somatic induction from plated explants seem to be the limiting step in the overall protocol for efficient somatic embryogenesis in grape, as the protein analysis has shown activation of many genes to be necessary. The later developmental phases, from mature somatic embryo to plantlet, seem to proceed more efficiently, although teratology, low conversion rate and dormancy are significantly problematic aspects.

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18

RECENT TRENDS IN GRAPEVINE GENETIC ENGINEERING

G.M. Reustle & G. Buchholz

RLP-AgroScience GmbH, AlPlanta, Institute for Plant Research, Breitenweg 7, D-67435 Neustadt/W. GERMANY

1. INTRODUCTION

In the former edition, genetic engineering was described as a powerful tool for plant breeding and research. In two chapters (Colova-Tsolova et al. 2001, Kikkert et al. 2001), applications to grapevine breeding and genetics, transformation methods, selection and regeneration systems for transgenic grapevines and the current status of grapevine transformation projects were comprehensively described. In addition strategies for disease and stress tolerance, regulatory issues and public perception were presented.

In this Chapter, we have attempted to present the progress made in the recent years, focusing on the improvement of transformation protocols, state-of the-art of transgenic grapevines, recent results from field experiments and new applications of the transgenic approach.

2. IMPROVEMENT OF TRANSFORMATION PROTOCOLS

Since the first report on genetically engineered grapevines, knowledge and experience in this field have increased enormously. Several ongoing projects aim to improve transformation efficiency, thus allowing to be used as a standard strategy for various purposes. The great interest for the transgenic approach is due to the possibility to establish disease tolerance or resistance in elite grapevine varieties and rootstocks without changing other genotype specific characteristics. Progress in grapevine genomics resulting in huge amounts of expressed genes and in the availability of the genome sequence of Pinot noir (Jaillon et al. 2007, Velasco et al. 2007) now makes the transgenic approach also interesting for basic research and analyses of gene functions.

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The limiting factors restricting from routinely using grapevine transformation are: (1) the complexity of the grapevine transformation procedure, and (2) the low transformation efficiency. Thus, experimental work has been focused on the improvement of transformation efficiency and improvement of the different steps of the transformation procedure. Detailed protocols have been published by Bouquet et al. (2006) for *Agrobacterium* mediated transformation and by Kikkert et al. (2005) for biolistic transformation.

2.1. Target tissue for transformation

Except of one group (Mezzetti et al. 2002), who have used meristematic cell clusters, different types of embryogenic tissue have been used as target cells for grapevine transformation. The production and establishment of the latter follows standard procedures starting from anthers and/or ovaries, whereas for some genotypes leaves can be used as donor tissue. As an alternative strategy to induce embryogenic callus, Agüero et al. (2006) successfully used inflorescence primordia instead of anthers as starting tissue. A recent report (Gambino et al. 2007) describes a facilitation of anther preparation by cultivation of the whole flowers instead of isolated anthers, which improved cell culture establishment Detailed description of somatic embryogenesis protocols have been presented by Martinelli and Gribaudo (2001) and in Chapter 17 in this Book.

Multiplication of embryogenic callus and maintenance of transformation and regeneration competence are prerequisites for continuous transformation experiments. Agüero et al. (2006) found variety specific response on different media. Furthermore, cultivation on ER (Emershad and Ramming 1994) or GS1CA (Franks et al. 1998) culture media induced gradual depletion of pre-embryogenic callus as a result of embryo differentiation.

Ben Amar et al. (2007) used conditioned media to establish embryogenic cell suspensions from solid embryogenic cell cultures. Although the maintenance of embryogenic cell suspensions is time and labour consuming due to the necessary frequent regular transfer to fresh medium, cell suspensions are highly standardized and may render possible reproducible transformation results. Nevertheless, it is possible to use either pre-embryogenic callus or somatic embryos (from globular to torpedo stage) from solid culture or embryogenic cell suspension for transformation. However it is necessary to be aware that the type of tissue very much requires a suitable transformation and selection procedure.

In our projects, at RLP AgroScience, after years using embryogenic callus grown on solid medium, we switched to the homogeneous embryogenic cell suspensions as donor tissue for transformation. As a consequence, we had to adjust the selection regime due to the high competence of these cells for regeneration to avoid the production of escapes or chimeras.

2.2. Agrobacterium mediated transformation

A key factor for efficient production of transgenic grapevine is the type of cells used as target tissue. The target cells must be competent for transformation at the point when DNA is transferred either by *Agrobacterium* or by particle bombardment, and for subsequent regeneration. The reasons for the different efficiencies are complex and data for optimal target tissue, used for transformation, are conflicting as they may depend on genotype, media used for maintenance and physiological state of cell at the time of transformation. Torregrossa et al. (2002) found a strong effect of the medium used for maintenance of embryogenic callus on transformation efficiency. Although C1P medium is routinely used to maintain friable embryogenic callus, pre-culture of the target tissue on GS1CA medium, which induces callus differentiation to globular and torpedo structures, resulted to a 3- to 4-fold higher transformation efficiency irrespective from the cultivar.

Wang et al. (2005) used embryogenic cell suspensions obtained from cryopreserved embryogenic cells. They found no difference in transformation efficiency between cryopreserved and non-cryopreserved cells, however conversion of transformed cells to embryos and plantlets were significantly improved by using cryopreserved embryogenic cell suspensions. In histological analysis they found that only cells with meristematic feature were able to survive freezing and thawing, whereas all differentiated cells were killed or severely damaged by the cryopreservation process. Thus homogeneous embryogenic cell suspensions with high regeneration frequency could be obtained from cryopreserved cells, resulting in improved embryo formation and plant regeneration.

Li et al. (2006) optimized the procedure of transformation and selection, using somatic embryos as target tissue (Fig. 1). Pre-culture of embryos for 7 days and addition of antioxidant (DTT) to the washing step following the co-cultivation, reduced browning of the *Agrobacterium* treated tissue. No difference was found in the transient transformation efficiency; however, stable transformation efficiency increased dramatically. Iocco et al. (2001) could reduce the necrotic effects after *Agrobacterium* co-cultivation by washing the bacterial culture overnight with liquid culture medium and adjusting the OD_{550} to 0.3. Thereby the use of anti-oxidants was avoided.

The most frequently and successfully used *Agrobacterium* strain for production of transgenic grapevine plants was the LBA 4404. In comparative experiments Torregrosa et al. (2002) confirmed former results (Franks et al. 1998, Iocco et al. 2001), that EHA strains are more effective in transforming several *V. vinifera* cultivars compared to strains GV3101, AGL0, AGL1, LBA4404, *A. vitis* K252 and *A. rhizogenes*. Agüero et al. (2006) found increased transforma-

tion efficiency with increasing *Agrobacterium* cell densities (from 10^7 to 10^9 cells /ml) used for co-cultivation.

A different strategy to create transgenic grapevines was developed by Mezzetti et al. (2002). They used meristematic bulk tissue produced form the apical dome of shoots by N6-benzyl-adenine (BA) treatment. Slices prepared from the meristematic bulk were used for *Agrobacterium* mediated transformation. Thus, the transgenic grapevine plants could be regenerated *via* organogenesis. The advantage of the system is, as described by the authors, the efficient shoot regeneration from the *Agrobacterium* treated slices and, in combination with a progressively increase of kanamycin, a limited number of escapes. However, the critical aspect of the system seems to be the slicing of the meristematic bulk, producing tissue composed of cells having a high regenerative capacity and a large number of damaged cells. This latter population of cells is known to produce substances that activate the mechanism of T-DNA transfer from *Agrobacterium* (for review see Citovsky et al. 2007).

Fig. 1. Plant regeneration from genetically engineered grapevine somatic embryos.

2.3. Biolistic transformation

Lots of efforts and success have been made by the group of Bruce Reisch to improve and optimize biolistic transformation (Kikkert et al. 2005). The technical improvements, compared to the previous biolistic transformation system for grapevine (Kikkert et al. 1996) included: (1) replacement of tungsten by gold particles and heating them up to 180°C before coating with DNA, (2) using 10-15 mg/L kanamycin for stepwise selection instead of 25-50 mg/L, and (3) incubating cell cultures at 27°C instead of 24°C for embryo induction during selection.

Furthermore co-bombardment of the selectable marker gene and gene of interest on two separate plasmids was performed (Vidal et al. 2003). The system

facilitated combination of different expression cassettes without cloning the cassettes into the same plasmid. As a step towards a 'clean gene technology', Vidal et al. (2006a) reported on the use of minimal cassettes consisting of the expression cassettes without plasmid backbone sequences.

2.4. Selection

Selection strategy plays an important role in transformation efficiency and subsequent plant regeneration. For grapevine transformation, the most widely used selectable marker gene is *NPTII* (neomycin phosphotransferase II) conferring resistance to kanamycin, paromomycin and some other aminoglycoside antibiotics. Regime of selection and different effects of the selective agents on embryogenic callus of grapevine have been controversial. Wang et al. (2005) found a more stringent selection with 20mg/L paromomycin compared to 80mg/L kanamycin. They postulated the much faster effect of paromomycin on cell death compared to kanamycin, as the reason for the higher efficiency. However, to our experience, depending on the vitality of the embryogenic callus, high selection stress directly applied after co-cultivation, can result in strong browning and necrosis even of transformed cells and finally of the complete callus. A recreation step for the co-cultivated cells at about one week before starting the selection reduces stress and helps to bridge this critical phase. Bouquet et al. (2006) recommended to start selection regime with a low antibiotic concentration with a gradual increase up to the final concentration.

An alternative to the *NPTII* gene, is the *HPTII* (hygromycin phosphotransferase II) gene conferring resistance to hygromycin. This however is rarely used in grapevine, probably due to its high toxicity and higher price. To our experience, selection by hygromycin is more stringent compared to that by kanamycin.

As a result of the controversy about the use of antibiotic resistance as selectable marker gene, our group focused on the development of alternative selection strategies. The use of the *BAR* (phosphinothricin actetyl transferase) gene conferring resistance to phosphinothricin (PPT) resulted in transgenic rootstocks (Reustle et al. 2005) and transgenic lines of *V. vinifera* cv Arich dressé (Jardak-Jamoussi et al. 2008). In both cases, embryogenic callus from solid culture being at the globular to torpedo stage was used as target for transformation. However, selection corridor is narrow and the risk of escapes and chimeras regeneration is high. Furthermore, in our experiments successful PPT selection was not possible, when embryogenic cell suspensions were used as target tissue. Attempts to use the phoshomannose-isomerase (*PMI*) gene as selectable marker were shown to be unsuitable for grapevine (Reustle et al. 2003, Kieffer et al. 2004).

A bifunctional fusion marker containing the genes for enhanced green fluorescent protein (*eGFP*) and *NPTII* was developed by Li et al. (2001). The dual functionality of this marker permits assays for transgene expression at the cellular level and is a helpful tool to establish and optimize transformation protocols. In a recent report Dhekney et al. (2008) could establish a protocol for *Vitis rotundifolia* Michx. transformation using the *GFP*/*NPTII* fusion marker.

In a two-step procedure, Franks et al. (2006) transferred three sorghum genes for secondary metabolite (dhurrin cyanogenic glucoside) biosynthesis to grapevine hairy roots. In the first step, whole plant transformation with two cDNAs encoding cytochrome P450 enzymes that catalyze the first reaction of the dhurrin biosynthetic pathway was carried out. In the second step, a transgenic line in which transcripts of both cDNAs accumulated, were inoculated with *A. rhizogenes* carrying a binary construct with the UDPG-glucosyltransferase-encoding cDNA. In that way, hairy roots were produced that expressed all three sorghum genes for dhurrin cyanogenic glucoside biosynthesis and accumulated the secondary metabolite. Despite numerous reports on hairy root and whole plant transformation of *Vitis* spp. this is the first to involve transfer of a multigenic trait.

3. STATE OF-THE-ART OF TRANSGENIC GRAPEVINES

Beside and in combination with experiments to establish or improve grapevine transformation systems, the transgenic approach has been used to establish virus resistance, fungal resistance, bacterial resistance and for the improvement of physiological traits. Although similar information is presented in the Chapter 19 of this Book, an overview is also presented below.

3.1. Virus resistance

The list of cultivars and rootstocks that have been genetically engineered for virus resistance is steadily increasing. Target viruses for resistance are the nematode-borne Arabis mosaic virus (ArMV), Grapevine fanleaf virus (GFLV) and Raspberry ringspot virus (RpRSV), the scale insect and mealybug-borne Grapevine leafroll-associated virus 3 (GLRaV-3), Grapevine virus A (GVA), and Grapevine virus B (GVB), as well as Grapevine leafroll-associated virus 2 (GLRaV-2) and Grapevine rupestris stem pitting-associated virus (GRSPaV) for which no vector is known (Fuchs 2003).

The favoured approach is the pathogen-derived resistance. Coat protein and movement protein genes or its fragments are the most commonly used sequences and are engineered as translatable, untranslatable or antisense versions.

Molecular analysis of transgenic lines of *V. vinifera* cvs Nebbiolo, Blaufränkisch, Lumassina and Russalka confirmed transgeneity of the regenerated lines. Different expression levels of the transgenes were discussed in the context of methylation or silencing on the transcriptional and post-transcriptional level (Gambino et al. 2005, Maghuly et al. 2006).

Results with model plants (Baulcombe 2004) showed that silencing of viral genes by the use of inverted repeat constructs is the most efficient strategy to induce virus resistance. Jardak-Jamoussi et al. (2003) evaluated inverted repeat constructs for their potential to confer resistance against nepoviruses (GFLV, ArMV, RpRSV). Proof of concept with *Nicotiana benthamiana*, as a model, was promising (Reustle et al 2006, Winterhagen et al. 2006); however, transformation of grapevine rootstocks, molecular characterization of the transgenic lines and evaluation of virus resistance is still ongoing.

Evaluation of virus resistance of transgenic grapevines is challenging. Valat et al. (2006) used protoplast electroporation for GFLV inoculation to investigate virus accumulation in transgenic rootstock 41 B expressing either the coat protein or the movement protein. A number of transgenic lines have been shown to inhibit or reduce the accumulation of challenge viral proteins at the protoplast level. Winterhagen et al. (2007) developed an *in vitro* dual culture system for grapevine and nematode *Xiphinema index*, the natural transmitter of GFLV, to evaluate transgenic rootstock lines. Furthermore inoculation with a GFP sensor construct to identify GFLV specific silencing in the transgenic grapevines and grafting experiments were conducted. However, results obtained were inconsistent and need further confirmation.

3.2. Fungal resistance

Most of the commercially important grapevine varieties are highly susceptible to fungal diseases. The transgenic approach offers the possibility to improve tolerance of elite varieties without changing the varietal specific characteristics. Initial attempts were carried out at Cornell University and JKI Geilweilerhof, Institute for Grapevine Breeding in Germany to express antifungal enzymes [endochitinase and chitinase, glucanase, and ribosome inhibiting protein (RIP)] in transgenic grapevines. Transgenic plants of *Vitis vinifera* cv Seyval blanc expressed chitinase and RIP; however, in field experiments, no different reaction on powdery and downy mildew infection compared to the non- transgenic control plants could be found (Bornhoff et al. 2004). Expression of a fungal endochitinase gene in cv Chardonnay plants showed reduced symptoms to powdery mildew and *Botrytis* bunch rot (Reisch et al. 2003).

The same group (Vidal et al. 2006b) found only slight effects on powdery mildew when anti-microbial peptides (natural and synthetic magainin) were ex-

pressed in transgenic Chardonnay. Hinrichsen et al. (2005) from the Chilean Institute for Agriculture Research in Santiago, developed transgenic table grapes expressing endochitinase from *Trichoderma harzianum* and an anti-microbial peptide describing increased tolerance to *Botrytis* infection. Legrand et al. (2003) evidenced that the *Vr-ERE* gene is an efficient candidate to confer resistance to eutypin (4-hydroxy-3-[3-methyl-3-butene-1-ynyl] benzaldehyde), a toxin produced by *Eutypa lata*, the causal agent of eutypa dieback of grapevines. Expression of *Vr-ERE* gene in transgenic rootstocks (Richter110) could increase detoxification capacity and opens new opportunities to study the role of the toxin in the development of the disease. Coutos-Thévenot et al. (2001) transformed 41B rootstocks with the *Vitis stilbene synthase* 1 (*VSTS1*) gene under the control of the pathogen-inducible promoter PR10. Some transgenic lines accumulated resveratrol 5- to 100-fold higher compared to the non-transgenic control. Infection assays with transgenic *in vitro* plants showed a decrease of *Botrytis* infection in lines with high resveratrol accumulation.

3.3. Bacterial resistance

Crown gall disease caused by *Agrobacterium vitis* and Pierce's disease caused by *Xylella fastidiosa* are the most severe bacterial diseases for grapevine. Expression of anti-microbial peptides (magainin) resulted in a reduced symptom severity after artificial crown gall infection (Vidal et al. 2006b). Expression of lytic peptides in transgenic grapevines conferred resistance to the Pierce's disease causing bacterium (Gray et al. 2005). This strategy to control the disease capitalizes on the bacteria's dissemination through the plant xylem. The engineered rootstocks produce lytic peptides which can, like the bacteria, travel in the xylem throughout the plant (Dutt et al. 2007). The elite scion itself is not transgenic.

Agüero et al. (2003) expressed a polygalacturonase inhibiting protein (PGIP) from pear in transgenic grapevines (*V. vinifera* cvs Thomson seedless and Chardonnay) and found a delayed symptom development by several weeks after artificial inoculation with *X. fastidiosa*.

3.4. Physiological traits

An increased fecundity (average number of inflorescences per shoot) was obtained by an auxin synthesis-enhancing transgene in *Vitis vinifera* cvs Silcora and Thomson Seedless. The ovule-specific auxin synthesizing (*DEFH9-iaaM*) transgene increased indole-3-acetic acid content in grape transgenic berries (Costantini et al. 2007).

Tesnière et al. (2006) investigated the functional role of alcohol dehydro-

genase (ADH) in regulating susceptibility to abiotic stress and the synthesis of secondary metabolites in over- and underexpressing *ADH*-transgenic grapevine plants. Plants transformed with either the construct in antisense orientation or the *ADH*-less construct displayed a low but detectable constitutive ADH activity, whereas plants transformed with the sense-expressed transgene showed a significantly higher (100-fold) ADH activity than the control. Grapevine plants overexpressing ADH displayed lower sucrose contents, a higher degree of polymerization of proanthocyanidins, and a generally increased content of volatile compounds, mainly of carotenoid- and shikimate-derived volatiles. In general, no significant differences between sense/antisense transformants were observed with regard to carotenoid and chlorophyll contents, suggesting a strong metabolic regulation of the synthesis of these compounds.

4. RESULTS FROM FIELD TRIALS

Information from field trials with transgenic grapevine plants is rather limited. Field trials were performed or still ongoing for virus resistance (Fuchs 2003). However although the first results are promising, information about resistance is still limited. The outcome of recombination events between viral transgene transcripts and RNAs from indigenous virus population was investigated in a field trial by Vigne et al. (2004). In these studies the transgenic grapevines did not show GFLV titers during the trial period.

Transgenic *Vitis vinifera* cvs Silcora and Thomson Seedless expressing the auxin synthesizing (*DEFH9-iaaM*) transgene allowing enhanced auxin synthesis were cultivated in the field. Plants were phenotypically homogeneous and did not show any morphological alteration in vegetative growth. The average number of inflorescences per shoot in transgenic Thompson Seedless was doubled compared to its wild-type control. Berry number per bunch was increased in both transgenic cultivars. Thereby, the quality and nutritional attributes of transgenic and non-transgenic grape fruit were found to be substantially equivalent. Finally, it is not clear if these results were based on a direct or indirect effect of enhanced auxin levels.

A group at the Chilean Institute for Agricultural Research, headed by Patricio Hinrichsen (DeFrancesco and Watanabe 2008), performed field trials with more than 700 transgenic table grapes with putative enhanced fungal resistance. After three seasons, fruits were obtained from some of the transgenic lines, though the yields were highly variable. No data are available about expressed resistance.

Field trails were carried out at JKI Geilweilerhof (Harst et al. 2006) to investigate pollen dispersal and outcrossing events of transgenic grapevines. Out-

crossing occured at a low frequency of 2% in a radial distance of 10m, and at 2.7% in the sectoral part of the main wind direction up to 20m distance from the pollen donor plants.

5. NEW APPLICATIONS OF GRAPEVINE TRANSFORMATION

Transient expression of genes using *Agrobacterium* is a powerful tool for the analysis of gene function in plants. Leaf infiltration with *Agrobacterium* suspensions were found to be an easy technique (Wroblewski et al. 2005), which has been employed in different plant species (Kapila et al. 1997) in order to study silencing mechanisms of resident genes (Schöb et al. 1997), for promoter and transcription factor analysis, and intracellular localization and trafficking studies (Batoko et al. 2000). Also, studies on Avr/Cf-mediated recognition and responses were made by infiltrating *Agrobacterium* harbouring both, the avirulence and the matching resistance (*R*) genes into tobacco leaves (van der Hoorn et al. 2000). The infiltration of *Agrobacterium* into leaf tissue was found to be applicable for a series of plant species; however, grapevine is considered as recalcitrant to leaf infiltration procedures.

Zottini et al. (2008) used a syringe for gentle pressure infiltration through the stomata of the lower epidermis of *in vitro* grapevine plantlets. With this method, the intracellular localization of transiently expressed auto-fluorescent proteins (GFP, RFP) fused to target sites for chloroplasts, mitochondria and the endoplasmatic reticulum, together with the cytoplasm localized, non-targeted control could be demonstrated. The efficiencies of infiltration and transformation, however, were found to be strongly dependent on the cultivar and the tissue culture conditions used in the experiments.

Santos-Rosa et al. (2008) developed transient transformation by vacuum infiltration of *Agrobacterium* into detached leaves of *in vitro* grapevine plantlets. This method was used to transiently overexpress stilbene synthase in grapevine leaves, in order to study the influence of stilbenes on downy mildew infection. As *Agrobacterium* infiltration itself induces synthesis of stilbenes, the effect of additional stilbene synthase activity by the transgene could not be identified.

Our investigation on the establishment of a reproducible low pressure infiltration procedure showed very clearly that the success for an effective infiltration depends on the chosen cultivar and the leaf age. Addition of non-ionic spreading reagent lowering the surface tension of the water at a concentration not being harmful neither to the plant leaf tissue nor the Agrobacteria, resulted in reproducible infiltrated leaf areas of almost 90-100% using greenhouse

grown shoots in the four- to six-leaf stage. The competence of the leaf cells for transformation, however, is not necessarily linked to an effective infiltration. As already shown, by Zottini et al. (2008), we found that the transformation efficiency depended on the cultivar and also on other, so far unknown, factors. Therefore using the system for functional gene analysis, markers to rate transformation efficiency are necessary, for a quantitative interpretation of the results.

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PROGRESS IN GENETIC ENGINEERING OF GRAPEVINE FOR DISEASE & STRESS TOLERANCE

V. Colova-Tsolova[1], A. Perl[2], S. Krastanova[3], S. Samuelian[1] & A. Atanassov[4]

[1] Center for Viticulture and Small Fruit Research , College of Engineering Sciences, Technology and Agriculture, Florida Agricultural and Mechanical University – Tallahassee, FL 32317, USA

[2] Department of Fruit Tree Breeding and Molecular Genetics, Institute of Horticulture, Agricultural Research Organization, The Volcani Center, P.O. Box 6, 50250 Bet-Dagan, ISRAEL

[3] Department of Plant Pathology, Cornell University, New York State Agricultural Experimental Station 14456 Geneva, NY, USA

[4] AgroBio Institute, 8 Dragan Tsankov Blvd, Sofia 1164, BULGARIA

1. INTRODUCTION

In 2007, the scientific community and wine world welcomed the complete genome sequencing of *Vitis vinifera* L cv Pinot Noir (Valasco et al. 2007, The French-Italian Public Consortium for Grapevine Genome Characterization, 2007), which made grape the first fruit and second commercial crop after rice to be fully sequenced. The development of high-throughput analytical techniques for analyzing the genome, proteome and metabolome resulted in the accumulation of large quantities of biological data for the living organisms and grape in particular, which currently is recognized as a 'systems biology' approach.

It was logical to expect that genetic engineering of grape will benefit from the fast pace of grape genomics research and will become a propeller for shifting the achievements of these tools into great benefits to the industry and the market. If this will be the valid scenario, the question is whether or not we are ready to accept the genetically modified (GM) grapes. Nonetheless, over the past ten years the negative campaign against transgenic plants was on a rise and it was the main reason for the serious backlash and discontinuation of many research programs in Europe and elsewhere (Pretorius 2005, De Francesco 2008).

However, field trials with transgenic grapes have been ongoing world-

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wide and altogether, since 1998, US Animal and Plant Health Inspection (APHIS) has granted 43 permits, 11 of which in the past two years alone (De Francesco, 2008).

Due to the overall negative social cartography and public scrutiny toward grape genetic engineering, there were limited numbers of published papers in that area lately. In this Chapter we tried to incorporate all the newly released achievements with the core information discussed in our previous review (Colova et al. 2001), on the progress to achieve grape improvement by using the recombinant DNA technology directed toward integration of well-defined genes, which may confer improved tolerance towards biotic (pests and diseases) and abiotic (cold, drought, salinity) stresses.

2. GRAPE BIODIVERSITY

In 1936, Negrul (1936) suggested a concept for the distribution of forms of immunity in *Vitis,* in the chapter 'Variation and Inheritance of Immunity' of his book 'The Genetic Basis of Grape Breeding'. The idea was based on conclusions made previously by Planchon and Viala and confirmed by his own studies. This concept reflects on a direct dependency of the existing biodiversity and development of forms of immunity in *Vitis,* with the center of geographical distribution of the major pathogens and pests of grape. The most common diseases, powdery mildew (*Uncinula necator*) and downy mildew (*Plasmopara viticola*) and the most damaging pest, phylloxera (*Dactylosphaera vitifoliae* Fitch) emerged after the exploration of new continent of America, and so-called old World grape varieties were introduced into the new World and *vice versa*.

Thus, since ancient times environmental extremes, pests and diseases accompanied by the social changes and the upheavals in the development of human civilization, and related mass human migrations have emerged as the key factors in resolving the puzzle of the present biodiversity of grape. As a result the main objective in current breeding programs worldwide is the development of grape cultivars, which combine improved fruit quality with disease and stress tolerances suitable to their area of cultivation.

Several factors limit the distribution of the European grape *Vitis vinifera* L. to latitudes between 20° and 50° in the north hemisphere and 20° and 40° in the south hemisphere. In the higher latitudes, north and south, these factors include the length of the growing season, which strongly affects the ripening of fruit and canes, and extremely low winter temperatures (below -21°C), which might cause serious damage, even over a short period (Alleweldt et al. 1990). At lower latitudes, they include the lack of sufficient chilling to induce dormancy and bud breaking, and the extremely high temperature during the growing sea-

son, which impair the normal pollination and the subsequent fruit set.

Other members of the *Vitis* genus, such as *Vitis riparia*, *Vitis amurensis* and *Vitis labrusca* can replace *Vitis vinifera* in the problematic growing area in the north, where they are the major alternatives for grape cultivars adapted to short season and cold hardiness. In the south, other species such as *Vitis aestivalis* cv Lincecumii, *Vitis aestivalis* cv Bourquiniana and especially *Vitis rotundifolia* (*Muscadinia rotundifolia*) provide cultivars having improved tolerance to heat and to an environmental background characterized by high pathogenicity (Raisch and Pratt 1996).

3. BASIC TERMS IN GENETICS OF HOST/PATHOGEN INTERACTION

The complexity of plant/pathogen interaction is mainly due to the fact that they involve interference between two genomes, that of the host and the pathogen. A process known as co-evolution, which represents a mutual reciprocal selective pressure during the evolution of pathogen and host genomes, defines, to a considerable degree, the complexity of most plant/pathogen interactions (Walton 1997). It has been said that plant disease is the exception rather than the rule (Lamb et al. 1989), and, in fact, only the interaction between a virulent race and a susceptible variety leads to disease development (Agrios 1988).

The concept of susceptibility and resistance is basic in plant pathology. If a specific pathogen can induce disease symptoms in a specific plant, that plant is defined as susceptible; if not, the plant is resistant to this particular pathogen. It should be mentioned here that when the term ‘resistance’ is considered from a purely economic point of view, another term is introduced, ‘tolerance’; this covers the host/pathogen interaction in which a crop plant may be susceptible at the biochemical level but yields are unaffected in spite of pathogen growth and damage. Such examples are anthracnose (*Elsinoe ampelina*) in *Vitis vinifera* grape, and Pierce’s disease (*Xylella fastidiosa*) in *Muscadine* grape. Specificity is also a central concept in plant/pathogen interactions; it covers both the extent to which a pathogen is limited to a specific plant-host and *vice versa*: extent to which the plant is or not susceptible to a specific pathogen.

Classical genetics work, earlier in this century, established that a single plant disease-resistance gene recognizes only to those biotypes of a pathogen species that harbor a corresponding or complementary avirulence gene (Flor 1942, 1956, Gabriel and Rolfe 1990). This gene-for-gene hypothesis has become a major working assumption in plant molecular pathology (Walton 1997). The specific genes in the pathogen genome, which define its pathogenicity,

match one-to-one with the specific genes in the host and establish its resistance, i.e. the phenotype of the specific host resistance is determined by the pathogen genotype on complementary basis and *vice versa*. The specific complementary pairs of genes are said to 'interact' but this must be genetically interpreted since the interaction does not necessarily occur on the biochemical level.

The elicitor-receptor model is commonly invoked to describe this relationship (Dixon and Lamb 1990, Keen and Dawson 1992). Avirulence genes have been cloned and characterized from viral, bacterial and fungal pathogens (Gabriel and Rolfe 1990, Keen 1990), and all the virulence genes known up to now are inherited as single, dominant alleles that encode single proteins.

Studies in several laboratories has shown that avirulence genes in a specific pathogen may result in the production of specific signal molecules, called elicitors, which are perceived by plants carrying complementary genes, and stimulate them to initiate a hypersensitive response (HR, Keen and Dawson 1992).

Genetic resistance that is effective in preventing the attacks only of certain races of a pathogen is called specific (or vertical) resistance. On the other hand, resistance effective in preventing attacks of all known races of a given pathogen is called general (or horizontal) resistance (Walton 1997). The known Mendelian resistance genes of higher plants are genes encoding a specific (vertical) resistance. Another large group of plant genes playing an important role in plant/pathogen interactions comprises the 'defense genes'; they are responsible for general (horizontal) resistance. Plant defense genes have been isolated not only on the basis of protein products but also through differential screening of cDNA libraries obtained from mRNA isolated from infected and non-infected plant species. Suitable examples of defense genes are the genes encoding for enzymes that participate in the biosynthetic pathway of phytoalexins (Collinge and Slusarenko 1987). The term Pathogenesis Related Proteins (PR) covers all antimicrobial plant proteins that are induced after a pathogen attack (Bowles 1990).

Constitutive resistance involves morphological or biochemical entities available in the host before the attack of the pathogen, for example, the plant cuticle, as well as a number of antimicrobial secondary metabolites which are constitutively produced by plants such as cyanogenic glucosides and saponins (Smith 1991). Induced resistance involves defense mechanisms, which are active only when an infection is present. This includes biosynthesis of low molecular weight antimicrobial secondary metabolites (phytoalexins), localized rapidly during cell death (the hypersensitive response, HR) and synthesis of novel proteins, which are directed to inhibit pathogens (PR proteins, Table1).

The above brief review does not cover entirely the complex and debatable terminology of the contemporary concepts for host/pathogen interaction genet-

ics. However it is not by chance that we, as authors, allowed ourselves to concentrate mainly on plant resistance, as this part of the question corresponds most closely to our general topic.

Table 1. Some of the cloned genes from the natural defense responses in grape (*Vitis vinifera* L.).

Gene	Gene product	Source	Reference
StSy	Stilbene synthase	*Vitis vinifera* L.	Sparvoly et al. 1994
VST 1	Stilbene synthase	*Vitis vinifera* L	Coutos-Thevenot et al. 1998
PAL	Phenylalanine ammonia lyase	*Vitis vinifera* L	Sparvoly et al. 1994
	1,3 beta Glucanase	*Vitis vinifera* L	Herrera et al.1999
VvChi4B	Class IV Endochitinase	*Vitis vinifera* L	Robinson et al. 1997
Cu/Zn SOD	Putative chloroplastic Cu/Zn Superoxide dismutase	*Vitis vinifera* L	Deng,M and McKersie BD (unpublished)
Vv-AMP1	Ripening induced antifungal peptide	*Vitis vinifera* L	de Beer and Vivier 2008

4. ADVANTAGES AND LIMITATIONS OF GENETIC TRANSFORMATION

Recombinant DNA technology comprises the removal of specific DNA fragments from one organism, the study of their functions and their subsequent incorporation into the existing genetic background of another organism, which may be a chosen plant species. This is comparatively a new technology (the first transgenic plants were obtained in the laboratory in 1983, field tests with transgenic tobacco and tomatoes were first performed in 1987) has become an important part of plant breeding and it is even differentiated in the so-called 'molecular breeding' (Logemann and Shell 1993). This methodology enables new genes encoding for new traits to be introduced without modifying the desired commercial attributes of target crop plants.

Indeed genetic transformation has become an efficient tool to express alien genes into the crop plants. It has a number of advantages: (1) The source of new genetic information (other plant species, animal cells etc.) that can be introduced into the plant genome is essentially unlimited, and (2) the gene of interest (when a particular gene has been isolated and reconstructed) can be used in a wide range of different crops; there are also a certain number of limitations. Because of that, genetic transformation cannot be assumed as a separate alternative of conventional plant breeding, but mostly as a complementary approach at

a later stage.

There are also certain ethical and safety limitations [regulation of Genetic Modified Organisms (GMOs)] that we are not going to discuss here. One essential limitation of pure methodological character is the number of transgenes that can be expressed in the same transgenic line, because of a process of co-suppression, 'transgene silencing' that can be activated and reduce the expression of transgene (Galun and Breiman 1997).

The availability of an efficient adventitious regeneration procedure pertinent to various cell and tissue culture system has significantly determined the development of genetic transformation procedures in grape. Somatic embryogenesis proved to be the regeneration protocol of choice, suitable for genetic transformation of grape. Comparison of regeneration through somatic embryogenesis with that achieved through organogenesis in grapes has demonstrated that the second one frequently leads to chimeras in transformed tissues (Mauro et al. 1995). Interesting work on grape transformation of two cultivars, Silcora and Thomson Seedless *via* organogenesis using regeneration of meristematic bulk tissue was presented by Mezzetti et al. (2002).

Much progress has been made in the last few years in the genetic transformation of grape (see reviews by Gray and Meredith 1992, Torregrosa 1995, Perl and Eshdat, 1998, Kikkert et al. 2001, Perl at al. 2004). *Agrobacterium*-mediated transformation has been the favored system for transformation of grape. A combination of particle wounding by bombardment followed by *Agrobacterium* transformation has also been reported (Scorza et al. 1995, 1996). Vidal et al. (2006) offered one improved biolistic transformation of grapevine using minimal gene cassette technology (see also Chapter 18 in this Book).

The efficiency of a transformation procedure depends on several factors, such as plasmid construct, optimal conditions for transformation, ability to regenerate plants from transformed tissue, proper selection and possibility of developing intact transgenic plants. The crucial requirement in the process of transformation is the availability of cells having both the ability to be transformed and subsequently to regenerate into plants. Thus, embryogenic cells in suspension are very promising candidates for successful transformation in grape (Fig. 1). The procedure for optimization and synchronization of grape embryogenic cell suspensions with high efficiency by cell fractionation through Ficoll density gradient centrifugation has been reported by Colova et al. (2007).

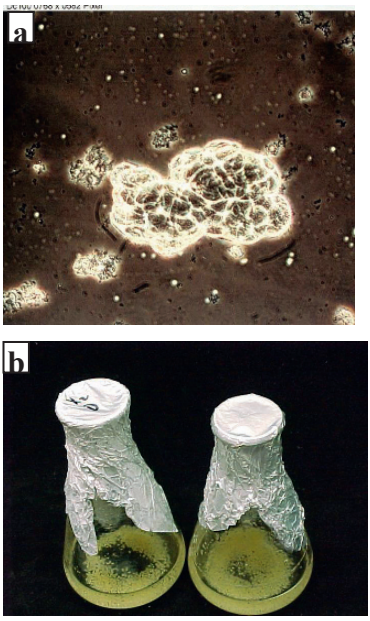

Fig. 1. Proembryogenic masses (PEMs) in grape embryogenic suspension (cv Sugarone): a) PEMs – light microscopy (faze contrast) X100; b) PEMs in liquid culture.

5. GENETIC TRANSFORMATION IN GRAPE FOR IMPROVED TOLERANCE TOWARD BIOTIC AND ABIOTIC STRESS

5.1. Viruses

In 1985 Sanford and Johnson for first time introduced theoretically the concept of 'pathogen derived resistance' against viral infection in transgenic plants. Viral coat protein, viral antisense RNA and modified viral replicase were proposed as possible approaches to viral resistance. A year later this concept was confirmed experimentally; Powel et al. (1986) reported that tobacco plants with expressed viral coat protein of tobacco mosaic virus (TMV) exhibited delayed expression of symptoms compared with non-transformed control plants. Soon afterwards, Kezayan et al. (1988) and Golembovski et al. (1990) confirmed the other two approaches by using antisense strategy and modified replicase.

It is not surprising that genetic transformation approaches to conferring viral resistance were the first and one of the most rapidly developing strategy in the new area of plant 'molecular breeding'. One of the main reasons for this de-

velopment arose from the absence of effective chemical treatments against viral infection and the almost total lack of sources of resistance against most of the major viral diseases in crop plants (Beachy 1993, Baulcombe 1994). The first successful report on grape (that was never repeated) was published in 1989. Hung et al. (1989) obtained seven transgenic plants by infecting apical meristem explants from over 300 cuttings of Cabernet Sauvignon, Chardonnay, Grenache and Riesling with an *Agrobacterium tumefaciens* strain carrying a modified Ti plasmid, pGV3850: 1103 neo, which conferred nopaline production and resistance to kanamycin.

Nepoviruses are an important group of phytoviruses characterized by isometric particles and transmission by soil-inhabiting nematodes: their genome comprises two separately encapsidated positive single-stranded RNAs. In grapevine, the most damaging and widespread nepoviruses are Grapevine fanleaf virus (GFLV) and Arabis mosaic virus (ArMV), both of which have a very important economic impact; they cause the disease known as infectious degeneration or 'court-noué' of grapevine. The disease can be controlled by soil disinfection with nematicides, but this procedure is forbidden in Switzerland, Germany, France, Italy and some states of USA, because of the high toxicity of the chemical (dichloropropene). Transgenic grapevines resistance to the virus may provide a possible alternative means to control the disease.

Krastanova et al. (1993) transformed two types of explants: embryogenic callus and fragments of embryo hypocotyls from grapevine rootstock 110R (*V. rupestris* x *V. berlandieri*) and *V. rupestris*. The *CP* gene of GFLV-F13 was cloned in binary vectors carrying the *NPT II* gene and the *GUS* gene. *Agrobacterium tumefaciens* LBA 4404 was used for transformation. The detection of integrated transgenes was performed by PCR and Southern hybridization. The expression of the product of CP gene was detected by ELISA and exhibited several fold increase. The stability of the insertion was proven by analysis of plantlets obtained after micropropagation of the transgenic plants in the absence of kanamycin selection.

Le Gall et al. (1994) obtained transgenic plants of grapevine rootstock 110 Richter (*V. rupestris* x *V. berlandieri*) from embryogenic cultures cocultivated with disarmed LBA4404 strain of *A. tumefaciens*, harboring a binary vector containing chimeric genes for hygromycin resistance (*HPT*), kanamycin resistance (*NPT II*), the *UID A* gene and the *CP-GCMV* (grapevine chrome mosaic nepoVIRus).

GCMV has a geographic distribution limited to several central European countries, but in regions where it is widespread, it is very damaging because of the severe symptoms it elicits: lack of vigor and unfruitfulness as severe as those caused by GFLV (Bouquet 1993, Le Gall et al. 1994). The best rate of transformation was obtained by selection of putative embryogenic tissue grown

on a medium containing 16-mg/l hygromycin. High levels of CP-GCMV expressions were detected by ELISA of somatic embryos, leaves and roots of transformed plants. Theoretically, resistant rootstock cultivars might confer full protection against infection by a soil-borne virus on the entire vineyard. On the other hand, a CP-mediated protection expressed in rootstock might not be sufficient to provide protection against the transmission of the viral infection to the scion part of the grafted vines especially in the field, where there may be a high pressure of secondary infection (Torregrosa et al. 1994).

The rootstock cultivars 110 Richter and *Vitis rupestris* were the target in the transformation studies by Krastanova et al. (1995) who utilized the CP of the most damaging GFL virus. All plantlets selected on a medium containing 50mg/l kanamycin stained positively for GUS expression. Nearly all leaflets harvested from these plants reacted positively in ELISA, but exhibited varying levels of expression of the *CP* gene. The stability of the insertions was proven by analysis of plantlets obtained after micropropagation of the transgenic plants in the absence of kanamycin selection.

A highly efficient transformation and regeneration system that gave rise to a high percentage of stable transformed cells, and therefore allowed the development of numerous intact transformed plants was described by Mauro et al. (1995). In this study, the two economically important rootstocks 41B (*Vitis vinifera* x *Vitis berlandieri*) and SO_4 (*Vitis berlandieri* x *Vitis riparia*) and the famous *V. vinifera* wine cultivar, Chardonnay were transformed with the *CP-GFLV*. The target cells for *Agrobacterium* mediated transformation was in an anther derived embryogenic suspension culture. The presence of the *CP* gene was tested by both PCR and Southern blots. One or two insertion bands were detected in most plants. ELISA assays were performed in 200 transgenic plants. Out of 200, 41.5% were positive, exhibiting a value of at least twice higher than that of the control. Seventeen plants showed level four to five-fold higher than that of the control.

Krastanova et al. (1996) utilized not only *CP* virus derived gene of GFLV but also replicase (RNA dependent RNA polymerase) to introduce resistance into grapevine rootstock cultivars. Two types of transgenic grapevine plants were regenerated: (1) Plants expressing the *GFLV-CP* gene, and (2) plants expressing a hybrid coat protein gene made of the 5'- end of the *GFLV-CP* fused to the 3'- end of the ArMV-CP. Six lines from 3309 (*V. rupestris* x *V. riparia*, Couderc), 12 lines from *Vitis rupestris* and 10 lines from 110 Richter (*V. berlandieri* x *V. rupestris*) or total 280 plants showed positive reaction in GUS staining. The integrated transgene was tested by PCR, followed by *in vitro* micrografting and green grafting.

In order to introduce resistance to nepoviruses, Spielmann et al. (1997, 1998, 2000) transformed *N. benthamiama* as well as several grape rootstock va-

rieties grown in Swiss vineyards [3309 Couderc (*V. rupestris x V. riparia*); Rupestris St. George du Lot (*V. rupestris*)]. In this work several constructs containing various encoding sequences from the GFLV or ArMV genomes were used, under the control of two constitutive promoters (CaMV 35S or soybean translation elongation factor). Expression of the transgenes was confirmed by RT-PCR, and ELISA or Western blots assaying accumulation of the CP product. Results with the *GFLV-CP* transgenes demonstrated that although the *CP*-RNA transcripts were produced in all plants tested, no CP could be detected by several immunological techniques. In contrast, results with the *ArMV-CP* transgenes showed that not only the *CP*-RNA transcripts were produced in all plants tested, but also in addition some transgenic lines expressed at very high level the ArMV-CP protein. In some cases, analyses by electron microscopy revealed the presence of virus-like isometric particles, mostly of the penetrated type, typical for empty virus shells. Moreover, in contrast to the results obtained for the transgenic *N. benthamiana*, Northern blot analyses of grapevine plants transformed with the *ArMV-CP* gene showed that the gene was expressed at lower level. The reasons for these differences in transgene expression and/or mRNA stability between grapevine and *N. benthamiana* is unclear, but the genetic state of the transgene(s) (homozygous in *N. benthamiana vs* hemizygous in *V. rupestris*) may have an effect on gene expression.

The list of genetically transformed grape genotypes for virus resistance is rapidly expanding. Gölles et al. (1997, 1998) reported the expression in embryogenic cultures of *Vitis vinifera* (self-pollinated cv Russalka) and 110 Richter, of sequences of CP of GFLV, ArMV, Grapevine virus A, and Grapevine virus B. Green-grafting of greenhouse-acclimatized transformants, micrografting of *in vitro* plants and transmission of the GFLV by its natural nematode vector *Xiphinema index* were used to screen the transgenic plants (Krastanova et al. 1995, Barbier et al. 1997).

Protection experiments involving grafting by both methods have been performed for a number of independent transgenic lines. Spielmann et al. (1997) presented preliminary data from infection experiments involving green-grafting performed on *V. rupestris* plants transformed with the GFLV replicase gene: one particular transgenic line was found to be resistant to GFLV infection. Courtois at al. (1997) and Mauro et al. (1998) performed not only micrografting but also electroporation of protoplasts derived from *in vitro* grown leaves of the 41B and from embryogenic cell suspension in order to evaluate resistance of the transgenic plants challenge with GFLV. These studies suggested protoplast electroporation as a rapid, efficient and reproducible screening assay for GFLV resistance.

Vitis vinifera cv Superior Seedless and *Vitis rupestris* transformed for resistance to viruses related to the rogose wood disease complex was reported also

by Martinelli et al. (1998). Grapevine virus A and B (GVA, GVB) cause stem grooving and corky bark, two diseases of grapevine rogose wood complex. Both these viruses encode for a 30-kD movement protein (MP), which is functional during the early stage of infection, facilitating cell-to-cell virus spread. No natural resistance to these diseases in grapevine is known. Preliminary results of molecular studies of these putative transformants (PCR and Southern blot) indicated that the MP constructs were successfully inserted into the grape genome.

Krastanova et al. (1998, 2000) performed transformation experiments with grapevine leafroll associated closteroviruses 2 (GLRaV-2) and 3 (GLRaV-3). The mealy bug transmits GLRaV-3, but there is no evidence of vector transmission of GLRaV-2. Development of rootstocks resistant to GFLV and GLRV should provide an alternative means of controlling these important VIRus diseases in grape. Early stage anther derived embryogenic calli of 3309 Couderc, Riparia gloire, MGT 101-14 110 Richter, Rupestris St George and 5C Teleki rootstock cultivars were co-cultivated with the *Agrobacterium tumefaciens* strain C58Z707 or LBA4404 containing various constructs of the *CP* VIRus gene. The gene constructs included translatable, antisense or non-translatable coat protein from GFLV, GLRaV-3 and GLRaV-2. A gene sequence in an open reading frame of GLRaV-3 was also used. Over 24 independent transformations were performed, and numerous putative transgenic lines were developed. Analysis of plants expressing the translatable *CP* gene of GFLV indicated various levels of expression. Resistance to GFLV is being evaluated by inoculation techniques including micrografting; heterografting with GFLV infected *Chenopodium quinoa*, *in vitro* green grafting and woody grafting. Results from greenhouse screening showed that some lines appeared to be resistant to the secondary infection.

Evaluation of the resistance to GFLV in grapevines usually relies on nematode-mediated GFLV transmission under field or greenhouse condition. A new study was reported by Valat et al. (2006) which evaluated the protoplast electroporation as an alternative rapid screening technique of transgenic grapevine clones expressing the *CP* or *MP* gene of GFLV and the accumulation of viral proteins at the cell level. Total of 42 independent transgenic grapevine rootstock 41B clones expressing the coat protein (*CP*) or movement protein (*MP*) gene of GFLV was assayed by protoplast electroporation. Two of the 26 transgenic clones expressing the *CP* gene did not support the accumulation of GFLV MP to detectable levels. Another 12 accumulated substantially lower levels of MP, and 12 accumulated equivalent levels of MP relative to protoplasts of non-transformed controls at 72 h post-electroporation. The inhibition of MP accumulation was achieved against virions but not viral RNAs, and was dependent on the inoculum dose. Interestingly, the authors reported that no interference was observed with the multiplication of *Arabis mosaic virus*, which is closely

related to GFLV. Also, one of the 16 transgenic clones expressing the *MP* gene significantly reduced the accumulation level of GFLV CP at 72 h post-electroporation, as demonstrated by DAS-ELISA with anti-GFLV γ-globulins.

Recently the group of Dr. Margit Laimer from BOKU University, Vienna published in two sequential works (Gambinno et al. 2005, Maghuly et al. 2006) the results of molecular characterization of significant pool (total of 180) of transgenic lines of *Vitis vinifera* L. cvs Russalka, Nebbiolo, Lumassina and Blofränkisch carrying the *NPTII* as a selectable marker gene and varIous sequences of the *GFLP CP* gene. Southern blot analysis revealed that the number of inserted T-DNA copy ranged from 1 to 6. More than 46% of the tested transgenic lines carried single copies of the T-DNA, which qualify them as promising candidates for further breeding experiments. In 15% of the lines tetracycline (*TET*) resistance gene was detected, which points that an integration of plasmid backbone sequence beyond the T-DNA borders occurred.

5.2. Fungal pathogens

The losses of crop plants from fungal attacks is a serious problem worldwide, amounting to annually losses of about 15% (Logemann and Shell 1993). In contrast to viral diseases, fungicides might successfully control fungal diseases. Fungicide application is a well established anti-fungal treatment, but it is known to have destructive environmental consequences. Several protective strategies have been described, which use alien genes expressed in plant genomes. These genes encode for proteins having antifungal activity and, therefore, enable the plant to protect itself against a fungal attack (Table 2).

Table 2. Agronomical important traits currently introduced into grape cultivars *via Agrobacterium* or the biolistic apparatus.

Target cultivars	Strain	Selection	Traits of interest	Gene utilized encoding for	Reference
I. *Agrobacterium* mediated transformation					
Chardonnay	LBA 4404	Par	GFLV resistance	CP	Mauro et al.1995
		Kan	Pierce's disease resistance	pPGIP	Agüero et al. 2005
Red Globe	EHA 101	Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Perl, unpublished
Sugarone	EHA 101, 105	Kan, Par	Fungal disease resistance	StSY, Rif, Gluc	Perl, unpublished
Superior Seedless	-	-	GVA, GVB resistance	MP	Martinelli et al. 1998
Rusalka	LBA 4404	Kan	GFLV, ArMV, GVA, GVB resistance	CP	Gölles et al.1997, 1998

Rusalka	LBA 4404	Kan	GFLV resistance	CP	Tsvetkov et al.1998
Thompson Seedless	EHA 101	Kan	TomRSV, bacterial disease resistance	CP, Shiva-1 lytic peptide	Scorza et al. 1996
V. rupestis 110 Richter	LBA 4404	Kan	GFLV resistance	CP	Krastanova et al. 1993
110 Richter	LBA 4404	Hyg, Kan	GCMV resistance	CP	Le Gall et al. 1994
SO_4	LBA 4404	Par	GFLV resistance	CP	Mauro et al. 1995
V. rupestris 110 Richter	LBA 4404	Kan	GFLV resistance	CP	Krastanova et al. 1995
St. George, 3309 Couderc	LBA 4404	Kan, Par	GFLV, ArMV resistance	CP, Oligo. synthase, Replicase	Krastanova et al. 1996, Spielman et al. 1997
110 Richter	LBA 4404	Kan	GFLV, ArMV, GVA, GVB resistance	CP	Gölles et al.1997, 1998
Vitis rupestris	-	-	GVA, GVB resistance	MP	Martinelli et al. 1998
Portan	EHA 105		Abiotic stress	ADH	Tesniere et al. 2006
Sultana		Kan	Phylloxera	CYP79A, Cyp71E1	Franks et al. 2006
5C Teleki, MGT 101-14, 3309 Couderc	LBA 4404 C58Z707	Kan	GLRaV-2, 3 resistance	CP	Krastanova et al. 1998, 2000
5 C Teleki, Riparia gloire	C 58Z707	Kan	Crown gall resistance	VIR E 2	Xue et al. 1999
Rusalka	LBA 4404	Kan	Cold resistance	Arf 11, 62, 75	Tsvetkov et al. 1998, Gothuranov et al. 2001
II. Gene Gun mediated transformation					
Concord		Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Kikkert et al. 1997
Chardonnay		Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Kikkert et al.1997 1998,
		Kan	Crown gall	magainin	Vidal et al. 2003, 2006
Merlot		Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Kikkert et al. 1997, 1998
Chancelor		Kan	Powdery mildew resistance	*Trichoderma* endochitinase	Kikkert et al. 1997, 1998

Abbreviations: *arf 11*, *62*, *75*, antifreezing genes; ArMV, arabis mosaic virus; CP, coat protein; Hyg, hygromycin; GCMV, chrome mosaic virus; GFLV, grape fanleaf virus; GLRaV, grape leafroll associated closterovirus; GVA, grape virus A; GVB, grape virus B; Kan, kanamycin; MP, movement protein; Oligo, synthase 2,5 oligoadenylate synthase; Par, paromomycin; Rip, ribosomal inhibitor protein; StSY, stilbene synthase; TomRSV, tomato ringspot virus; vst 1, stilbene synthase, .ADH, alcohol dehydrogenase.

Many microorganisms produce enzymes capable of degrading the cell wall of plant pathogenic fungi. Chitin is the main component of fungal cell walls and it predominates in hyphal tips, which therefore can be destroyed by exposure to chitinase. Also, chitinases, along with β-1,3 glucanases form the natural defense mechanism activated in a number of plants upon attack by pathogenic fungi (Logemann et al. 1992). Taking into consideration the concept for co-evolution between pathogen and host, as well as the 'gene-for-gene' hypothesis, the use of chitinases and β-1,3 glucanases from origin other than plants, is one attempt to break through this evolutionary set mechanism of host/pathogen complementary.

Other approaches improving the tolerance of plants to pathogens include: (1) Over-expression of phytoalexins, which are plant secondary metabolites toxic to bacteria and fungi (Darvil and Albersheim 1984, Hain et al. 1993); and (2) utilization of ribosomal inhibitor proteins (RIPs), which inhibit protein synthesis in fungi by RNA N- glucosidase modification of 28S rRNA and are not toxic to plants. RIPs are known in seeds of several cereals as a toxic to some predators and some fungi (Logemann et al. 1992).

The introduction of genes able to reduce the pathogenicity of fungal toxins or to detoxify pathogenic toxins is another attractive approach to obtaining plants genetically engineered for disease tolerance. The fact that resistance can be conferred in plants by introducing single dominant gene made this approach very promising (Yoneyama et al. 1993).

Fungal diseases are known to cause substantial crop losses in grape. For many years conventional breeding has approached the development of cultivars resistance to fungal disease on the basis of available sources of resistance in the genus *Vitis*. However, the main limitation of conventional breeding for disease resistance was that the resulting hybrid in most cases expressed also negative traits derived from the resistant parent and required many backcrosses in order to eliminate these characters.

The first report on the transgene approach for introducing in *Vitis vinifera* genotypes the chitinolytic enzyme, endochitinase that confers antifungal tolerance, was published by Kikkert et al. (1997, 1998). Genetic transformation was performed using the biolistic method. Embryogenic suspensions derived from anthers and ovaries from the cvs Merlot and Chardonnay, respectively, were bombarded with tungsten particles coated with plasmid pBin 19ESR - containing a cDNA clone of the *Trichoderma* endochitinase gene and *NPTII* selectable marker gene. A total of 124 Merlot and 93 Chardonnay putatively transformed plants were regenerated and evaluated for expression of chitinase using a fluorometric assay. PCR and Southern blot analysis were also used for confirming the transformation. All the chitinase-positive plants had an intact copy of the chitinase gene.

Coustos-Thevenot et al. (1998) reported gene transfer in 41B (grapevine rootstock) embryogenic suspension with the gene construct carried the pathogen inducible promoter sequence (cloned from alfalfa) and *VST1* (stilbene synthase gene) cloned from grape, as a strategy for improving grapevine tolerance to fungal disease, especially *Botrytis cinerea* and *Eutypa lata*. Results with the transgenic plants have shown increasing tolerance to *Botrytis* and delay to infection by *Eutypa lata*. Phytoalexin production has been checked in correlation with foliar symptoms developed on *in vitro* plants.

Successful application of the technique related with expression in defined plant genome of the detoxifying enzyme to a particular well defined toxin of pathogenic fungi in grapevine was reported by Roustan et al. (1998). Gene transfer was accomplished in *Vitis vinifera* cells only of a NADPH-dependent aldehyde reductase, which detoxifies eutypine, a toxin from *Eutypa lata* (Eutypa dieback currently one of the most serious disease of grapevine). A 36-kD enzyme, named VR-ERE (*Vigna radiata*-Eutypine Reducing Enzyme) was purified from a non-host plant for pathogen, which exhibited a very efficient detoxification activity. Transformed grapevine cells with the gene construct containing the *VR-ERE* cDNA were cultured successfully in the presence of eutypine, with reduced growth by 10%. The transgenic grapevines expressing a rice chitinase exhibited slight resistance against *Elsinoe ampelina* inducing anthracnose, resulting in a reduction of disease lesions (Yamamoto et al. 2000).

Leaf discs of grapevine cv Seyval blanc were transformed with gene constructs carrying genes for chitinase and RIP (ribosome-inactivating protein) in an attempt to improve fungal resistance (Bornhoff et al. 2005). The transgenic lines did not exhibit significant tolerance under field conditions for the major pests *Uncinula necator* and *Plasmopara viticola*. Finally, Vidal et al. (2003 and 2006) reported successful gene transfer of antimicrobial magainin genes in cv Chardonnay. Only two of the transgenic lines exhibited slight symptom reductions in response to *Uncinula necator*.

5.3. Bacteria

In spite of the fact that the bacterial diseases cause very serious losses to several major crops, there has been very little progress in obtaining transgenic plants resistant to pathogenic bacteria. Jainess et al. (1988) and Casteels et al. (1989) proposed the idea of using the genes that are usually expressed in insects, as a means of inducing resistance to bacteria in transgenic plants. These genes encode for peptides, which exhibit antimicrobial activity. Further investigations showed that such peptides play major role in defense against bacteria, not only in many insects, but also in mammals and plants (Boman 1995, Broekaert et al. 1995). They represent surface-active peptides, which bind

to and affect amphipathic surfaces, such as membranes and receptors (Destefano-Beltran et al. 1993). Among this family of compounds are cationic lytic peptides, which have been isolated from mammalian phagocytes, as defensins (Selsted et al. 1985), from insects, as the melitins (Haberman 1972), cecropins (Steiner et al. 1981), and sarcotoxins (Okada and Natori 1985), and also from amphibians, as the magainins (Zasloff 1987). These relatively short peptides (a few kilodaltons) are potent toxins to a very wide range of bacteria. They integrate into the outer bacterial membrane, causing lysis and subsequent death of the relevant bacterium (Boman 1995).

A newly designed antimicrobial peptide became available on the basis of natural antibacterial toxins. For example, Shiva-1 was designed to have significant differences in sequence with the natural cecropin B (46% homology) (Destefano-Beltran et al. 1993). A more advanced generation of lytic peptides is based on the synthesis of newly designed antimicrobial peptides instead of natural antibacterial toxin (Chen et al. 1988, Fink et al. 1989). Design of synthetic antimicrobial peptides with predetermined structures and properties should improve the stability of these gene products and their protection against proteases in the transformed plants.

Another prospect for conveying resistance against bacterial pathogens is a chimeric gene, expressing phage T4 lysozyme that is a very effective enzyme for lysis of a wide range of bacteria. (During et al. 1993). In grape, only Scorza et al. (1996) have reported the transformation with the lytic peptide, Shiva-1. Somatic embryos, derived from leaves of *in vitro* grown plants of Thomson Seedless were exposed to *Agrobacterium* harboring either the lytic peptide Shiva-1, or the tomato ringspot virus (Tom RSV) *CP* gene. Somatic embryos were either bombarded with gold microprojectiles and then exposed to *Agrobacterium tumefaciens* or were exposed to *Agrobacterium* without prior bombardment. Thirteen plants (obtained with or without pre-bombardment) survived kanamycin selection. PCR with *CP-Tom RSV* and *Shiva-1* primers or Southern blots with the *NTPII* gene as a probe, suggested that these 13 plants contained the predicted gene sequences.

Crown gall, caused by *Agrobacterium vitis* and, to a lesser extent by *A. tumefaciens,* is the most important bacterial disease of grape worldwide (Pearson and Goheen 1998). Many *Vitis vinifera* cultivars used for making quality wines and some rootstocks are highly susceptible to and may be killed by crown gall. *Agrobacterium vitis* survives systemically in vines and can be disseminated in symptoms- free propagation materials. Tumors usually form on the lower region of grape trunks, near grafting sites and are initiated following injuries caused by freezing temperatures (Burr and Katz 1984). No chemical controls are effective against grape crown gall. To facilitate the development of transgenic grapevines that would be resistant to crown gall, Xue et al. (1999)

developed system for rapid regeneration and transformation of the grape rootstocks 3309 Couderc, Riparia gloire, 5C Teleki, and 110 Richter. *A. tumefaciens*, strain C58 served as the source of the *VIR E2 del B*, which is a *VIR E2* derivative that encodes a protein lacking the 215 carboxyl-terminal amino acids of the wild type VIR E2 protein (Citovsky et al. 1994). The *VIR E2 del B* gene was amplified from C58 DNA. The presence of transgene in the different cultivars was verified (except for *VIR E2 del B* in Gloire) by PCR. At least five transgenic different lines were analyzed by PCR with transgene-specific primers. PCR products of the expected sizes were observed in all cases. Southern blots were done with three cultivars carrying *VIR E2 del B*. In all cases, evidence of stable gene insertion was verified. Transformation of 3309 and 5C with the *VIR E2 del B* gene resulted in multiple lines with different numbers of gene insertions.

In one attempt to control Pierce's disease (*Xylella fastidiosa*), Agüaro et al. (2005) reported on grape transgenic plants of cvs Chardonnay and Thompson Seedless expressing pear polygalacturonase protein (*pGIP* gene). The development of Pierce's disease was delayed in some of the transgenic lines. They had reduced leaf scorching, lower *Xylella* titres and better re-growth after pruning than the untransformed control lines.

Two US patent have been granted to Scorza and Gray (2001, 2006) for method producing transgenic grapevine cv Thompson Seedless; expression of the lytic peptide cecropin B and Shiva-1 provided resistance to the bacterium *Xylella fastidiosa*, the causative agent of Pierce's disease. Finally, in the reported above work of Vidal et al. (2006) against fungi, the transformed with the magainin genes Chradonnay lines exhibited significant reductions of crown gall symptoms. The authors suggested that the expression of magainin-type antimicrobial peptide in grapevines might be more effective approach for control of bacteria than fungi.

5.4. Nematodes and insects

For insect controls, two well-known approaches in transgenic plants have been successfully implicated: (1) The Bt approach uses proteins produced by *Bacillus thuringiensis*, which are endotoxins toxic to insects, and (2) the approach uses plant-derived inhibitors of protein digestion in insects, such as proteinase and amylase inhibitors (Aronson et al. 1986, Sanchez-Serrano et al. 1993.

Atkinson et al. (1995) reviewed the economic significance of plant parasitic nematodes and presented a detailed description of their biology and some contemporary approaches to their effective control. One of the basic ideas is that since the roots of the cultured plants are the primary target for most

parasitic nematodes, a transgene whose expression is limited to roots might provide a promising means of conferring tolerance in transgenic plants. Galun and Breiman (1997) suggested the combination of several root specific promoters (such as the *TOB RB7*) to provide several sequences able to confer nematode tolerance in transgenic plants.

It would not be realistic to consider that biological defense based on a genetic engineering strategy might replace the use of insecticides in modern intensive agriculture. However, the combination of breeding of plants harboring various genes that reduce their sensitivity to pests, with responsible use of environmentally acceptable pesticides, should make agriculture a more efficient and environmental- friendly industry (Logemann and Shell 1993).

In an attempt to target possible resistance to grapevine phylloxera (*Dactylosphaera vitifoliae*) a multigenic trait (biosynthesis of the secondary metabolite, dhurrin cyanogenic glucoside) was engineered *de novo* in grapevine hairy roots by using three genetic sequences from sorgum (Franks et al. 2006). The *in vitro* dual co-culture of the cyanogenic hairy root transgenic line and grapevine phylloxera didn't express enough evidence for protection of the plant tissue from the infection of the insect.

5.5. Abiotic stress

Environmental stresses severely affect the sustainability of agriculture (Logemann and Shell 1993). Oxidative stress imposes a constant burden of plants through the formation of toxic oxygen species, such as superoxide, hydroxyl radicals, hydrogen peroxide and other toxic species. These species are generated especially during photosynthesis and respiration and are enhanced under certain conditions, such as high light fluency, salinity and low temperatures (Galun and Breiman 1997, Gressel and Gallun 1994, Asada 1994, Skopelitis et al. 2006).

Several reviews have discussed the possible use of genetic transformation to protect the plants against oxidative stress (Bowler et al. 1992, Gressel and Galun 1994, Allen 1995). The most often strategy is over-expression in transgenic plants the genes encoding for various superoxide dismutases (SODs), such as the cytosolic Cu/Zn-SOD, the chloroplastic Fe-SOD, or the mitochondrial Mn-SOD (Beyer et al. 1991).

Rendering plants tolerant to cold (freezing) stress, which is an osmotic stress, has been based on studying the role of some osmoprotectants. Holmström et al. (1994) suggested that glycine betaine plays a major key in freezing tolerance in several bacteria, algae and higher plants. In grape, Tsvetkov et al. (1998) and Goturanov et al. (2001) reported the genetic transformation in *Vitis vinifera* cv Rusalka, with 3 different constructs harboring

the antifreezing gene sequences encoding for a protein isolated from an Antarctic fish. The PCR and Southern blot analyses with the *NPTII* gene as a probe, suggested that the regenerated transgenic plants contained the predicted gene sequences.

The functional role of alcohol dehydrogenase in regulating susceptibility to abiotic stress and the synthesis of secondary metabolites was investigated in transgenic grapevine plants over- and under-expressing *ADH* gene. Grapevine plants overexpressing *ADH* displayed a lower sucrose content, a higher degree of polymerization of proanthocyanidins, and a generally increased content of volatile compounds (Tesniere et al. 2006; see also Chapter 6 in this Book).

6. CO-TRANSFORMATION AS AN ADVANCED APPROACH FOR INTEGRATION OF MULTIPLE GENES TO CONFER FOR DISEASE TOLERANCE IN GRAPE

Krastanova et al. (unpublished data) used highly embryogenic line of the grape rootstock MGT 101-14 (*Vitis riparia* x *Vitis rupestris*) in experiments for co-transformation. Mixture of two *Agrobacterium* strains with the same selectable marker *NPTII*, were used for delivery of the genes of interest, that confer resistance to two VIRal and one bacterial disease. The preliminary results from the PCR assays showed different level of integration of the predicted sequences.

Colova-Tsolova et al. (2000) developed a highly efficient *Agrobacterium* transformation system in a grape embryogenic cell suspension, which facilitated co-transformation with two *Agrobacterium* strains carrying different selectable markers. The embryogenic cell suspension was developed from anthers of *Vitis vinifera* cv Sugarone. *Agrobacterium*-mediated co-transformation was performed by the simultaneous use of a mixture of two *Agrobacteria* carrying the *HPT* and *NPTII* selectable markers, respectively and three genes encoding for putative proteins that are involved in fungal disease's resistance. Selection was performed simultaneously in liquid germination medium supplemented with both paromomycin and hygromycin. PCR assays and Southern blots hybridization were used to confirm the co-transformation.

7. CONCLUDING REMARKS

Through the recent outcome of grape genomics the wine industry has just started to appreciate the power of biotechnology and the other 'omics' research. However, the fear of negative public perceptions still continues to hold back the grape genetic engineering and commercialization of transgenic grapes. There is the hope that by complementing the conventional breeding with molecular breeding and merging the 'green revolution' with the 'gene revolution will result in the 'evergreen revolution.

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MOLECULAR MAPS, QTL MAPPING & ASSOCIATION MAPPING IN GRAPEVINE

L. Costantini[1], F.M. Moreira[1], E. Zyprian[2], J.M. Martínez-Zapater[3] & M.S. Grando[1]

[1]Fondazione Edmund Mach IASMA Research Center, Genetics and Molecular Biology Department, Via E. Mach, 1, 38010 San Michele all'Adige (TN), ITALY

[2]Julius Kühn-Institut (JKI) Bundesforschungsinstitut für Kulturpflanzen, Institut für Rebenzüchtung, Geilweilerhof, 76833 Siebeldingen, GERMANY

[3]Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología, CSIC, Campus de la Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, SPAIN

1. LINKAGE MAPPING

Linkage mapping in grape is based on the pseudo-testcross strategy (Grattapaglia and Sederoff 1994). Starting from 1995 several linkage maps have been developed for grape (Table 1) with the goal of locating the genetic determinants of target traits and identifying markers to assist breeding. The first maps were mainly based on RAPD (Williams et al. 1993) and AFLP (Zabeau and Vos 1992) markers, which allow the rapid generation of Linkage Groups (LG), but do not easily permit their comparison. The increasing interest in the comparison of genes and QTLs detected in different crosses encouraged the development of microsatellite or SSR markers (Gupta et al. 1996), which are codominant, highly polymorphic and easily transferable across related *Vitis* species. The first large set (371 markers) was produced by the *Vitis* Microsatellite Consortium (VMC), a cooperative effort of 21 research groups in 10 countries that was coordinated by AgroGene S.A. in Moissy Cramayel, France.

Riaz et al. (2004) reported the first map based on 152 microsatellite markers, mostly from VMC, which has been adopted as a reference to harmonize LGs resulting from individual mapping projects (http://www.vitaceae.org). Two additional sets of microsatellite-based markers (169 VVI and 108 UDV) were developed by Merdinoglu et al. (2005) and Di Gaspero et al. (2005), respectively. One hundred twenty three VVI markers were positioned on the map

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Table 1. List and main features of the linkage maps developed for grapevine to date

Cross	Origin	Segregating traits	No. of progeny	Marker type	No. of LGs	Map length (cM)	Marker distance (cM)	Reference
Cayuga White x	Complex hybrid[a]	Disease resistance and others	60	RAPD, isoenzyme, RFLP	20	1196	5.6	Lodhi et al. 1995
Aurore	Complex hybrid[b]				22	1477	6.6	
Horizon x	Seyval x Schuyler[c]	Flower sex and fungal disease resistance	58	RAPD, SSR, AFLP, CAPS, phenotypic	20	1199	7.6	Dalbò et al. 2000
Illinois 547-1	*V. rupestris* x *V. cinerea*				20	1470	8.1	
MTP2223-2 x	Dattier de Beyrouth x Pirovano 75[d]	Berry size and seed content	139	AFLP, SSR, isoenzyme, RAPD, SCAR, phenotypic	22	767	5.7	Doligez et al. 2002
MTP2121-30	Alphonse Lavallée x Sultanina				23	816	6.7	
Moscato Bianco x	*V. vinifera*	Fungal disease resistance, Muscat flavour and flower sex	81	AFLP, SSR, SSCP	20	1639	9.3	Grando et al. 2003
V. riparia Mchx Wr 63	*V. riparia*				19	1518	6.9	
Regent x	Complex hybrid[e]	Fungal disease resistance, véraison time, berry size, leaf morphology and axillary shooting	153	AFLP, RAPD, SSR, SCAR/CAPS, phenotypic	20	1277	4.8	Fischer et al. 2004
Lemberger	*V. vinifera*				26	1158	7.0	

Regent x Lemberger	Extension of the previous map		144	AFLP, RAPD, SSR, RGA, SCAR, CAPS	19 (C)	1631 (C)	4.7 (C)	Welter et al. 2007
Riesling x	*V. vinifera*	Fruit morphology and flavour	153	SSR, EST	20	1469	10.5	Riaz et al. 2004
Cabernet Sauvignon	*V. vinifera*				21	1431	10.6	
Syrah x	*V. vinifera*		96	SSR	19	1172	6.5	Adam-Blondon et al. 2004
Grenache	*V. vinifera*				18	1361	7.6	
Riesling self	*V. vinifera*		96	SSR	19	1192	10.8	Adam-Blondon et al. 2004
D8909-15 x	*V. rupestris* x *V. arizonica* hybrid	Flower sex, resistance to fanleaf degeneration and Pierce's disease	116	AFLP, RAPD, ISSR, SSR	17	756	8.7	Doucleff et al. 2004
F8909-17	*V. rupestris* x *V. arizonica* hybrid				19	1082	11.7	
D8909-15 x F8909-17	Extension of the previous map		181	SSR, EST-SSR, EST-RFLP, phenotypic	18	865	5.5	Riaz et al. 2006
					19	1055	6.7	
Ramsey x	*V. champinii*	Flower sex, nematode resistance, lime and drought tolerance, vigour	188	SSR, phenotypic	19	1245	7.2	Lowe and Walker 2006
Riparia Gloire	*V. riparia*				18	1096	8.7	

MTP2687-85 x Muscat of Hamburg	Olivette Noire x Ribol[f]	Muscat aroma	174	SSR	19 19	935 1365		Doligez et al. 2006b
Dominga x Autumn Seedless	*V. vinifera* Calmeria x (Thompson Seedless x Muscat of Alexandria)	Berry size and seed content	118	AFLP, SAMPL, S-SAP, SSR, SCAR	19 19	1173 1131	3.3 3.4	Cabezas et al. 2006
Welschriesling x Sirius	*V. vinifera* Bacchus x Villard Blanc[g]	Magnesium uptake	92	SSR, RAPD	20 (C)		15.6 (C)	Mandl et al. 2006
Chardonnay x Bianca	*V. vinifera* Villard Blanc[g] x Bouvier	Resistance to mildew diseases	46	SSR, RGA	19 (C)	1210 1425	5.9 7.0	Di Gaspero et al. 2007
Cabernet Sauvignon x 20/3	*V. vinifera* Bianca x SK77-4/5[h]	Resistance to mildew diseases	46	SSR, RGA		1254 1418	5.8 6.9	
Ruby Seedless x Thompson Seedless	Emperor x Pirovano 75[d] *V. vinifera*	Berry size, seed content and phenology	144	SSR, AFLP, ISSR, SCAR, RAPD, phenotypic	19 (C)	906 1000		Mejía et al. 2007
Syrah x	*V. vinifera*	Phenology, fertility, metabolites	94	SNP, AFLP, SSR	19	1113	1.9	Troggio et al. 2007

Pinot Noir	*V. vinifera*				19	1204	1.9	
Merzling x	Complex hybrid[i]	Tolerance to fungal pathogens, resistance to *Phylloxera vastatrix*, bunch shape, metabolites	89	SNP, SSR, phenotypic	20	914	5.5	Salmaso et al. 2008
Teroldego	*V. vinifera*				21	1174	7.0	
Italia x	Bicane x Muscat of Hamburg	Phenology, yield, berry size, seed content, Muscat aroma	163	AFLP, SSR, EST, phenotypic, SCAR	19	1353	4.9	Costantini et al. 2008
Big Perlon	(Almeria x Cardinal) x Perlon				19	1130	5.4	

[a] From *V. aestivalis*, *V. labrusca*, *V. rupestris* and *V. vinifera*
[b] From *V. aestivalis*, *V. rupestris* and *V. vinifera*
[c] From *V. aestivalis*, *V. labrusca*, *V. rupestris and V. vinifera*
[d] Muscat of Alexandria x Thompson Seedless
[e] From *V. aestivalis*, *V. berlandieri*, *V. cinerea*, *V. labrusca*, *V. lincecumii*, *V. riparia*, *V. rupestris* and *V. vinifera*
[f] From Olivette Blanche x Alphonse Lavallée
[g] From *V. berlandieri*, *V. labrusca*, *V. lincecumii*, *V. rupestris* and *V. vinifera*
[h] From Kunbarát (*V. amurensis* x *V. vinifera*) x Traminer
[i] From *V. lincecumii*, *V. rupestris* and *V. vinifera*

Abbreviations:
AFLP = Amplified Fragment Length Polymorphism
BAC = Bacterial Artificial Chromosome
C = Consensus map, CAPS = Cleaved Amplified Polymorphic Sequence
EST = Expressed Sequence Tag
GC-MS = Gas Chromatography-Mass Spectrometry
ISSR = Inter Simple Sequence Repeat
LD = Linkage Disequilibrium
MAS = Marker Assisted Selection
RAPD = Random Amplified Polymorphic DNA, RFLP = Restriction Fragment Length Polymorphism, RGA = Resistance Gene Analog
SAMPL = Selective Amplification of Microsatellite Polymorphic Loci
S-SAP = Sequence-Specific Amplified Polymorphism
SCAR = Sequence Characterized Amplified Region
SSCP = Single Strand Conformational Polymorphism, SSR = Simple Sequence Repeat, STS = Sequence Tagged Site

of Syrah x Grenache (Adam-Blondon et al. 2004), which became the second international grape reference map, the first one representing the 19 chromosomes of genus *Vitis*.

The existence of common markers on distinct maps also enables their integration in a more comprehensive map filling in the major gaps present in individual maps and providing a better estimation of marker order and distance. Doligez et al. (2006a) reported an integrated genetic map of grapevine based on the segregation data from five mapping populations: MTP2223-27 x MTP2121-30 (Doligez et al. 2002), Riesling x Cabernet Sauvignon (Riaz et al. 2004), Syrah x Grenache, Riesling self progeny (Adam-Blondon et al. 2004) and Chardonnay x Bianca (Di Gaspero et al. 2005). This map provides the relative position of 502 microsatellite loci.

Considering the usefulness of microsatellites, Costantini et al. (2007) defined a set of SSR markers of general interest to develop and compare genetic maps for grape and to assess the consistency of major genes and QTLs identified in different genetic backgrounds. In the framework of a cooperative effort aimed to assist breeding programs in table grape (EU MASTER project), 298 microsatellites were tested for polymorphism in 13 table grape cultivars involved as progenitors in 7 intraspecific (*V. vinifera* L.) crosses. Based on polymorphism rate, amplification quality and available mapping information, a common set of 86 highly polymorphic and well distributed SSRs were chosen to match the homologous LGs in the different parental lines. Also, microsatellites were successfully used to anchor existing genetic maps on the physical maps of the grape genome based on Cabernet Sauvignon and Pinot Noir BAC libraries (Lamoureux et al. 2006, Troggio et al. 2007).

The subsequent step in the development of grape linkage maps was the introduction of markers related to transcribed regions of the genome, which have great potential for the identification of genes responsible for target traits. The first reports concerned the sporadic mapping of genes associated to specific traits (Dalbò et al. 2000, Grando et al. 2003, Fischer et al. 2004, Welter et al. 2007).

More significant efforts were described by Riaz et al. (2006; 60 EST-derived markers), Lowe and Walker (2006; 54 EST-derived markers), Troggio et al. (2007; 170 markers from ESTs, 313 from BAC-end sequences), Di Gaspero et al. (2007; 82 RGA markers), Salmaso et al. (2008; 139 markers from ESTs and BAC-end sequences), Costantini et al. (2008; 35 EST-derived markers). With 994 markers distributed onto 19 LGs, the Syrah x Pinot Noir map (Troggio et al. 2007) represents the most comprehensive grape linkage map based on a single cross. Around half of the markers analyzed in this progeny were included into a novel integrated reference grapevine map derived from three crosses: Syrah x Pinot Noir, Syrah x Grenache and Cabernet Sauvignon x Riesling (Vezzulli et al. 2008). It is comprised of 1134 loci (350 AFLPs, 332

BAC end sequences-based markers, 169 EST-based markers and 283 SSRs) spanning 1443 cM across 19 LGs, with a mean distance between adjacent loci of 1.3 cM. Grape genetic maps were applied in several cases for the detection of genetic factors controlling target traits.

2. TARGET TRAITS

2.1. Resistance to pathogens

Plants have the capacity to recognise and avoid pathogens and they respond to infection using a two-branched innate immune system. The first branch recognises and responds to molecules common to many classes of microbes while the second responds to pathogen-specific virulence factors through the gene-to-gene pathway (Kombrink and Schmelzer 2001).

The European grape *V. vinifera* is generally classified as susceptible to the majority of diseases caused by bacteria, fungi, mycoplasmas, insects, nematodes and viruses. During the 19th century several pathogens, such as powdery mildew (*Erysiphe necator*) and downy mildew (*Plasmopara viticola*), but also phylloxera (*Daktylosphaera vitifoliae*) were carried to Europe from North America and became major risk factors for viticulture. The cultivation of traditional varieties requires systematic spraying of chemicals, whose reduction would be of considerable benefit to environment, health and financial input.

Natural sources of disease resistance are normally found in geographic regions where populations of pathogens and host plants co-evolved. Many accessions of American and Asian *Vitis* species (*V. aestivalis*, *V. amurensis*, *V. arizonica*, *V. cinerea*, *V. labrusca*, *V. riparia*, *V. rupestris*, *V. yenshanesis*) and the closely related *Muscadinia rotundifolia* are partially or totally resistant to different pathogens and are regarded as valuable source of resistance genes for grapevine breeding (Eibach et al. 1989, Alleweldt et al. 1990).

2.2. Flower sex

Grapevine plants typically do not flower until their second or third year in the field. Marker-assisted selection for sex would be very useful in breeding programs incorporating disease resistance genes from native, dioecious *Vitis* into *V. vinifera* backgrounds for new fruiting varieties. The current model for sex inheritance in grape involves a single major locus with three different alleles, the allele for male flowers being dominant over the hermaphrodite type, and the latter being dominant over the allele for female flowers (Antcliff 1980). However, there is some evidence that this major gene is epistatically affected by

another locus (Carbonneau 1983).

2.3. Phenology

Phenological characteristics are the most important attributes involved in the adaptation of grapevine to its growing environment and to climatic changes. Moreover, the availability of early and late ripening varieties is desirable for staggering harvest along the growing season and for expanding production towards periods when the fruit gets a higher value in the market. Phenological traits are complex, which results from the interaction of various developmental quantitative characters such as flowering time, véraison and fruit ripening.

2.4. Seed content, berry size and yield

Seedless grapes are of interest for fresh fruit consumption and for raisin production. Two types of seedlessness have been observed in grape: parthenocarpy (fruit develops in the absence of pollination) and stenospermocarpy (pollination takes place but seed development fails because of early degeneration of endosperm; Ledbetter and Ramming 1989). Only stenospermocarpy can lead to berry sizes compatible with commercial requirements for fresh fruit marketing; however, an undesired negative correlation still exists between seedlessness and berry size. All traditional seedless grape varieties develop smaller berries than their seeded counterparts.

Stenospermocarpy exhibits qualitative and quantitative variation (Striem et al. 1992). The predominant genetic model to explain determinism of seed development (Bouquet and Danglot 1996) assumes the involvement of three independent recessive genes regulated by a dominant inhibitor locus, which was termed *SdI* (Seed Development Inhibitor) by Lahogue et al. (1998).

2.5. Aroma

Aroma plays an essential role in high quality winemaking and is also greatly appreciated for grape fresh consumption. The typical character of muscat grape varieties is related to the presence of C_{10} terpene molecules (monoterpenes), the most important being linalool, geraniol, nerol, α-terpineol and various forms of linalool oxides (Ribéreau-Gayon et al. 1975). Moderate concentrations of monoterpenes can be also found in aromatic but non-muscat varieties (i.e. Gewürztraminer, Riesling). On the contrary, in non-floral cultivars monoterpenes can either not be detected or are present in trace amounts and flavour, if noticeable, depends on different substances, like methoxypyrazines, C_{13}-

norisoprenoids and sulfur compounds (i.e. in Sauvignon Blanc, Cabernet Sauvignon, Cabernet Franc, Chardonnay, Merlot and Syrah). Plants synthesize the precursors of all terpenoids, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), by two independent pathways: the mevalonic acid (MVA) and the methylerythritol phosphate (MEP) pathway, which are localized in cytoplasm and plastids, respectively (Lichtenthaler 1999).

The predominant biosynthesis of monoterpenes *via* the plastidial pathway has been demonstrated in grape berries, but cooperation with the mevalonate pathway can not be excluded (Luan and Wüst 2002). Analyzing several crosses, Wagner (1967) concluded that the genetic control of muscat flavour involves at least 5 complementary genes plus a modifier gene. The highly significant correlation detected by Versini et al. (1993) between several monoterpenes in 40 muscat cultivars suggested that a large part of the genetic variability for muscat aroma might be due to pleiotropic or closely linked genes, but independent genes are probably also involved in the production of specific compounds.

3. MAPPING AND TAGGING OF MAJOR GENES

Up to now single major genes were mapped in grapevine for the following traits: powdery and downy mildew resistance, Pierce's disease resistance, flower sex, berry colour, berry flesh and seedlessness.

3.1. Powdery mildew resistance

3.1.1. *Run1*

A total resistance to powdery mildew derived from *M. rotundifolia* was demonstrated to be controlled by a single dominant locus by Bouquet (1986). This locus, called *Run1* (for Resistance to *Uncinula necator* 1), was introduced into the *V. vinifera* genome using a pseudo-backcross strategy aimed at the creation of new good quality grape varieties which are resistant to powdery mildew (Bouquet 1986). Pauquet et al. (2001) and Donald et al. (2002) used the Bulked Segregant Analysis (BSA) approach to identify a number of genetic markers, AFLPs and RGAs respectively, tightly linked to the *Run*1 locus and to develop a local map around the gene.

In a continuation of the previous work, Barker et al. (2005) performed physical mapping of the *Run1* gene using a BAC library constructed from a resistant *V. vinifera* individual carrying *Run1* within an introgression. BAC contig assembly enabled the identification of 20 new genetic markers closely linked to *Run1*, and the position of the resistance locus was refined, locating the gene between the SSR marker VMC4F3.1 and the BAC end sequence-derived marker

CB292.294 on LG12. This region contains two multigene families of RGAs. A comparison of physical and genetic mapping data indicates that recombination is severely repressed in the vicinity of *Run1*, possibly due to divergent sequence contained within the introgressed fragment from *M. rotundifolia* that carries the *Run1* gene.

3.1.2. *Ren1*

Another genetic resistance source to powdery mildew was investigated by Hofmann et al. (2008). *V. vinifera* Kishmish vatkana, a cultivated grapevine from central Asia, does not produce visible symptoms in response to natural or artificial inoculation with the fungus *Erysiphe necator*. A progeny of 310 plants from a Nimrang × Kishmish vatkana cross were scored for the presence or absence of visible conidiophores throughout two successive seasons. Phenotypic segregation revealed the existence of a single dominant allele termed *Ren*1 (for Resistance to *Erysiphe necator* 1). A BSA was carried out using 195 microsatellite markers uniformly distributed across the entire genome. The phenotypic locus was assigned to LG13, a genomic region in which no disease resistance had been reported previously. The closest markers, VMC9H4-2, VMCNG4E10-1 and UDV-020, were located 0.9 cM away from the *Ren1* locus.

3.2. Downy mildew resistance

A major determinant responsible for resistance against downy mildew was reported by Merdinoglu et al. (2003). Polymorphisms revealed with RAPD, ISSR and SSR markers were screened between two bulks produced by separately pooling the individual DNAs from the six most resistant and the six most susceptible plants. These individuals derived from a cross followed by two successive backcrosses between *M. rotundifolia* variety Dearing, as the donor parent carrying the resistance, and several *V. vinifera* cultivars as the recurrent susceptible parents. By analysis of variance, 1 RAPD, 4 ISSRs and 8 SSRs were shown to have a significant effect upon the level of resistance. Twelve of these markers were mapped on the same LG and covered a 45 cM long region, which accounted for 73% of the observed variation and 83% of the genetic variation. These results strongly suggest that a unique major gene is conditioning the Muscadine grape downy mildew resistance, which was named *Rpv1* (for Resistance to *Plasmopara viticola* 1). Moreover, *Rpv1* was tightly linked to the dominant gene conferring resistance to powdery mildew, *Run1*, on LG12.

3.3. Pierce's disease resistance

The inheritance of resistance to *Xylella fastidiosa* (*Xf*), the bacterium which causes Pierce's disease (PD) in grapevines, was evaluated by Krivanek et al. (2005) within a factorial mating design consisting of 16 full-sib families with resistance derived from *V. arizonica* interspecific hybrids. Complex segregation analysis strongly indicated the existence of a major gene for PD resistance, which accounted for 91% of the total genetic variance. Conversion of the quantitative data into qualitative resistance levels and evaluation *via* a chi-square analysis showed that 15 of the 16 families segregated in accordance with a single gene hypothesis with a dominant allele controlling PD resistance. These results were confirmed by Krivanek et al. (2006) which identified a major QTL controlling resistance to PD on *Vitis* LG14.

The same group reported in two subsequent works a more detailed mapping of the *PdR1* resistance gene (for Pierce's disease Resistance 1). First, an extended framework genetic map based on genomic DNA-derived SSR, EST-derived SSR, EST-STS and EST-RFLP markers was developed and the *PdR1* was mapped to LG14 between markers VMCNG3H8 and VVIN64, respectively located 4.3 and 2.7 cM away from *PdR1* (Riaz et al. 2006). Secondly, a refined genetic map of LG14 was obtained from three grape mapping populations having *V. arizonica/candicans* b43-17 as source of PD resistance. In the 9621 progeny (425 individuals) the resistance locus mapped as *PdR1a* between markers VvCh14-56/VvCh14-02 and UDV095 within a 0.6 cM genetic distance, while in the 04190 population (361 individuals) it mapped as *PdR1b* between markers VvCh14-02 and UDV095/VvCh14-10 within a 0.4 cM distance. Moreover, a major segregation distortion region on LG14 was observed, suggesting the involvement of gametophytic factors (Riaz et al. 2008).

3.4. Flower sex

Sex was scored as a male segregating marker after visual inspection of inflorescences during bloom and verification of fruit set in the fall. In Dalbò et al. (2000) a cross between a male and a hermaphrodite resulted in these two types segregating approximately 1:1, consistent with the single-locus hypothesis. The sex locus was mapped on LG14 of Illinois 547-1 close to the VVS3 marker (corresponding to LG2 of the reference map). Sex was found to segregate 1:1 as a single gene also in the Ramsey x Riparia Gloire population (Lowe and Walker 2006) and in a *V. rupestris* x *V. arizonica* progeny (Riaz et al. 2006), both derived from the cross between a female and a male genotype. In both cases the sex locus was placed on LG2 between markers VVIB23 (respectively 1.5 and 1.6 cM far away) and VVMD34, thus confirming the original map position re-

ported by Dalbò et al. (2000).

3.5. Berry colour

Berry colour was scored as qualitative character. Black, blue, purple or red were registered as the presence of coloration, yellow or green as absence. It was mapped on LG2 of the reference map by Doligez et al. (2002), Fischer et al. (2004), Lijavetzky et al. (2006), Doligez et al. (2006a), Mejía et al. (2007), Salmaso et al. (2008) and Costantini et al. (2008). Lack of berry colour was found associated to the presence of a retrotranposon (*Gret1*) insertion in the promoter region of the *VvmybA1* gene (Kobayashi et al. 2004), which has been involved in the regulation of anthocyanin biosynthesis. Lijavetzky et al. (2006) showed that colour co-segregated with the *VvmybA1* gene what was also confirmed by Salmaso et al. (2008). This locus was shown to be more complex due to the presence of a tandem duplication of at least two *VvMybA* genes (Walker et al. 2007).

3.6. Berry flesh

The *fleshless berry* (*flb*) mutation of cv Ugni Blanc (*V. vinifera*) impairs the differentiation and division of inner mesocarp cells that form the flesh in grapevine berries. This mutation reduces the fruit weight 10 times without any effect on fertility or seed size and number. In order to determine *flb* genetic inheritance and map position, five segregating progenies were created and classified based on ovary phenotype by Fernandez et al. (2006). Phenotypic segregation suggested the involvement of a single dominant allele. Based on BSA the *flb* locus was assigned to LG18 and its position was refined in a local map by analyzing the segregation of microsatellites in the target region. The *flb* locus was located at the extremity of LG18, 5.6 cM away from VMC2A3.

3.7. Seedlessness

Seedlessness was scored as a qualitative trait after classifying progenies as described by Bouquet and Danglot (1996). The Seed development Inhibitor (*SdI*) locus was mapped on LG18, close to VMC7F2, by Mejía et al. (2007) and Costantini et al. (2008).

4. QTL MAPPING

Most of the above traits show a quantitative distribution. The genetic factors responsible for their variation could be localized as QTLs on the basis of the association between quantitative phenotypic variation and specific alleles of molecular markers. Even if interval mapping is more powerful than single marker analysis, QTL identification used both methods because some traits did not follow a normal distribution, which is prerequisite for interval mapping but not for the non-parametric Kruskal-Wallis (KW) rank sum test. Refined interval mapping enabling the detection of multiple QTL regions in the grape genome (MQM or CIM) is now generally applied (Jansen and Stam 1994, Cabezas et al. 2006, Krivanek et al. 2006, Mandl et al. 2006, Welter et al. 2007). Common QTLs between different mapping experiments were identified, as well as QTLs specific to distinct genetic backgrounds.

4.1. Resistance to pathogens

Many long standing breeding programmes have been established in the past aimed at combining *V. vinifera* fruit quality traits with resistance traits, contributed by other *Vitis* species. This has, however, proved to be a difficult task mainly due to the quantitative nature of resistance determination.

The first QTL analysis of segregating resistance to some fungal diseases was carried out at Cornell University by Dalbò et al. (2000). Genetic linkage maps were created from a segregating population derived from the cross of two grapevine genotypes, Horizon (Seyval x Schuyler) and Illinois 547-1 (*V. rupestris* x *V. cinerea*), using the pseudo-testcross strategy. Maps were based mostly on RAPD markers plus SSR, CAPS (Staub et al. 1996) and AFLP markers. Data from 5 years of observation of this population for resistance to several diseases were used for QTL analysis. For black rot resistance four QTLs were detected, two in each parent (I-2, I-10, H-16, H18). A major QTL was found for powdery mildew resistance in the Illinois 547-1 (resistant parent) map (I-10) and two other QTLs with a smaller effect were found in the Horizon map (H-16, H-18). A single marker (CS25b) associated with the major powdery mildew QTL accounted for approximately 41% of the phenotypic variation. The same marker was present in *V. cinerea* B9, one of the parents of Illinois 547–1 and the likely source of resistance. Interestingly, resistance to powdery mildew and black rot was correlated in the Horizon x Illinois 547–1 progeny. The same map locations associated to powdery mildew resistance were also associated with black rot resistance but to different degrees. The results show that resistance to both diseases may be polygenic but major genes or gene clusters are involved.

The JKI Institute for Grapevine Breeding Geilweilerhof is another impor-

tant research center for grapevine breeding. The major focus is on localization and long-term molecular characterization of genes involved in pest resistance and fruit quality traits with the aim of understanding their complex genetic basis. Fischer et al. (2004) evaluated segregation of resistances to powdery and downy mildew in a F_1 population derived from the cross of Regent, a newly bred cultivar with multiple field-resistances to fungal diseases, and Lemberger, a traditional fungus-susceptible cultivar. Two parental maps using AFLP, RAPD, SSR and CAPS markers were constructed and twelve pairs of homologous LGs could be integrated into consensus LGs. Interval mapping reproducibly localized a QTL for resistance to *Erysiphe necator* on LG15, and QTLs for endurance to *Plasmopara viticola* on LGs 5 and 18 of Regent.

This genetic map was improved with microsatellite markers by Welter et al. (2007). RGA-derived markers and SCAR markers correlated with powdery and downy mildew resistance were also mapped. After 3 additional years of phenotypic evaluation one QTL with major effect was detected for both powdery and downy mildew (on LGs 15 and 18, respectively), in agreement with the earlier description of Fischer et al. (2004). For downy mildew resistance, one supplementary QTL with minor effect was identified on LG4, whereas the QTL identified on LG5 in the previous study was significant only in one year. Some RGA-derived markers were found co-localized in the region covered by the major QTL for resistance to downy mildew hinting at their putative functional relevance.

A study of *P. viticola* resistance segregating from *V. riparia* in a cross with Moscato Bianco using data for leaf resistance resulted in major QTLs identified on LGs 4 and 7 of *V. riparia* (Battilana et al. 2006, Madini 2007). A genotype of this species could be one of potential resistance donors involved several generations back in the breeding of Regent.

Researchers at the University of California studied other pathogens which damage the grapevine cultures, such as *Xylella fastidiosa (Xf),* the bacterium which causes Pierce's disease (PD), and *Xiphinema index (Xi),* the nematode which not only causes severe feeding damage to the root system, but also is the vector of grapevine fanleaf virus (GFLV). The QTL analysis performed by Krivanek et al. (2006) identified on a *Vitis* linkage map a major QTL controlling resistance to PD, confirming previous experiments carried out by Krivanek et al. (2005). Placement of this locus was accomplished by evaluating a family (9621) derived from a cross of two PD-resistant interspecific hybrids: D8909-15 [*V. rupestris* A. de Serres x b42-26 (*V. arizonica*)] and F8909-17 [*V. rupestris* A. de Serres x b43-17 (*V. arizonica*)].

A QTL accounting for 72% of the phenotypic variance in bacterial numbers was localized on LG14 of the male parent F8909-17. The approximate 95% confidence interval around the QTL peak extended 5.7 cM when using composite interval mapping. The other disease evaluation methods (leaf scorch and

cane maturation index, respectively) placed the resistance QTL to the same region on LG14, although at wider 95% confidence intervals (6.0 and 7.5 cM) and accounting for less phenotypic variance (59 and 42%). This was the first report of a *Xf* resistance QTL mapped in any crop species.

Recently, on the same population, Xu et al. (2008) studied the resistance to the dagger nematode *Xi*. In this report, the dynamics of nematode numbers, gall formation, and root weight loss were investigated using a variety of soil mixes and pot sizes over a 52-week period. The modified *Xi* resistance screening method was successfully applied to 185 individuals of the 9621 population, which segregates for a form of *Xi* resistance originally derived from *V. arizonica*. QTL analysis was carried out on both parental genetic maps of 255 markers. Results revealed that *Xi* resistance is controlled by a major QTL, designated *XiR1*, close to marker VMC5A10 on chromosome 19. The *XiR1* QTL explained 60% of the resistance variance in the mapping population. This has been an interesting result for grapevine rootstock breeding programs.

4.2. Magnesium deficiency

Magnesium deficiency symptoms are displayed by several cultivars, especially on light and acidic soils and sometimes due to high potassium fertilization (Grimme 1975). Low Mg^{2+} content in leaves reduces photosynthesis and consequently glucose production, resulting in reduced wine quality.

Mandl et al. (2006) investigated the segregation pattern of low magnesium uptake on a population derived from a cross between Welschriesling and Sirius. Welschriesling is one of the most important grapevine cultivars in Central and Eastern Europe (Ambrosi et al. 1994) and is known for its low and inefficient magnesium uptake. Sirius has been bred for fungal disease resistance at JKI IGB Geilweilerhof from the cross of Bacchus x Villard Blanc. The genetic map rendered possible to identify a major QTL (51-55% of explained variance) on LG11. This locus can be associated with visual symptoms of Mg^{2+} deficiency and the amount of Mg^{2+} concentration in the leaves of the grape zone. The SSR markers VVS2 and VMC6G1 showed the highest linkage to the core region of the Mg^{2+} uptake QTL.

4.3. Phenology

Two QTLs for the onset of berry ripening (véraison) were detected by Fischer et al. (2004) on LGs 7 and 8 and a third one was located on a Regent LG with no homology to the reference map back that time. Mejía et al. (2007) identified QTLs for ripening date on LGs 17 (one year, 19% of explained variance) and 18 (2 years, 14-32% of explained variance).

Costantini et al. (2008) reported the most exhaustive analysis for the genetic determinism of phenological traits to date. They suggested the involvement of three major regions on LGs 2, 6 and 16. On LG2 QTLs were identified in three years for flowering time (7-16% of explained variance), véraison time (6-13%), véraison period (16-44%), flowering-véraison interval (13-21%) and véraison-ripening interval (15-22%). On LG6 QTLs were found in at least two years for flowering time (13-21% of explained variance), véraison time (9-10%), ripening date (10-17%), flowering-véraison interval (8%) and flowering-ripening interval (9-15%). Finally, LG16 was found to control véraison, as revealed by the detection of two coincident QTLs for véraison time (21-45% of explained variance) and flowering-véraison interval (15-37%) in three years. Additional QTLs were reported on LG1 (flowering time) and 12 (véraison-ripening interval).

4.4. Seed content, berry size and yield

In the last few years different groups undertook the quantitative genetic analysis of seed content and berry size (Doligez et al. 2002, Fischer et al. 2004, Fanizza et al. 2005, Cabezas et al. 2006, Mejía et al. 2007, Costantini et al. 2008). Most of them were involved in a project based on the use of a common set of SSR markers for linkage map development in table grapes (Costantini et al. 2007) and on a common protocol for phenotypic evaluation. All these groups detected a major QTL affecting both seed content and berry weight on LG18 (Fig. 1). This co-localization might be due either to pleiotropic effects of a single QTL or to the existence of tightly linked QTLs. The hypothesis of pleiotropy is supported by the gibberellin production of the seeds that affects berry growth. However two experiments (Doligez et al. 2002, Cabezas et al. 2006) suggested the involvement of several linked QTLs on LG18. Further research is required to test both hypotheses. The major QTL on LG18 co-positioned with the *SdI* locus (Mejía et al. 2007, Costantini et al. 2008).

Several additional QTLs with small effects were identified in one or more progenies. Interestingly, some regions (on LGs 2, 3, 4, 6, 10, 14 and 16) were found to regulate seed content without affecting berry weight, which might allow to dissociate the unfavourable correlation between seedlessness and berry size in breeding programs.

Co-positioning of QTLs for seed content and phenological stages was reported by Mejía et al. (2007) on LG18 for seedlessness and ripening date and by Costantini et al. (2008) on LG2 for mean seed number, mean seed dry weight and flowering time. The authors suggested that these correlations might be due to the inhibitory effect of seed gibberellins on flowering and ripening.

Finally, Fanizza et al. (2005) investigated the genetic determinism of fruit

yield in the Italia x Big Perlon progeny by measuring vine yield, number of clusters per vine, cluster weight, number of berries per cluster and berry weight in three years. They detected different QTLs explaining a low percentage of the phenotypic variation for each trait. Their study showed that yield components have a low to moderate heritability and are significantly affected by yearly climate variations, especially in the case of the number of clusters per vine and the number of berries per cluster. Indeed differential genotypic sensitivity to yearly climate variations is one of the explanations suggested by the authors for the lack of QTL stability in different years. Correlations between traits were investigated as well. Interestingly, the negative phenotypic correlation observed between berry weight and the number of berries per cluster did not reflect any QTL coincidence. This might indicate that the genes controlling berry weight and the number of berries per cluster function independently of each other, thus offering the possibility of selecting seedless genotypes with large berries without affecting cluster weight.

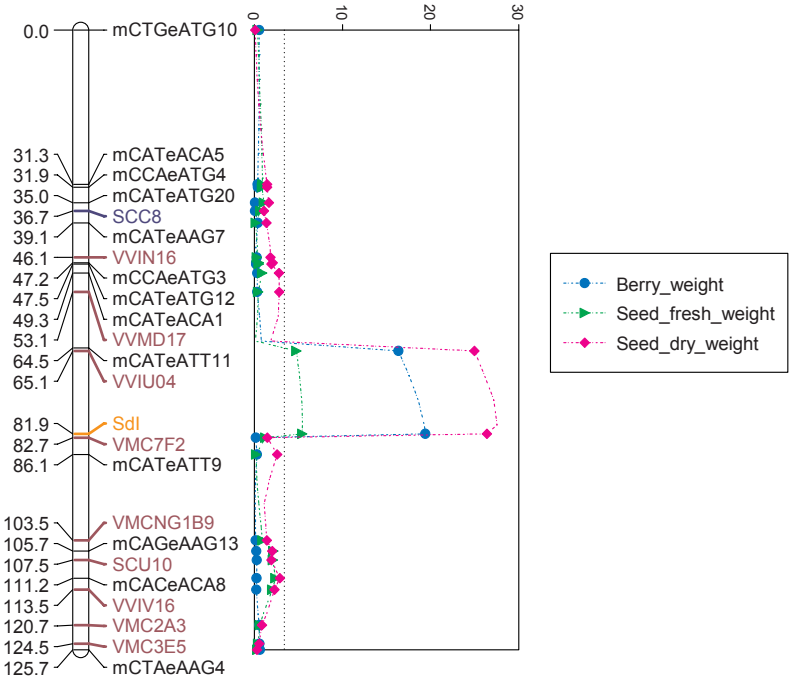

Fig. 1. Co-localization of the QTLs for berry and seed weight on LG18 of the Italia x Big Perlon map (Costantini et al. 2008). The horizontal scale indicates LOD values.

4.5. Aroma

QTL detection for muscat flavour was reported by Doligez et al. (2006b). A major QTL was found on LG5 in two years for the content of linalool (17-27% of the total variance), nerol (18-55%) and geraniol (28-55%) as measured with GC-MS. This locus co-located with one major QTL for muscat score as recorded by a tasting panel. Additional QTLs were identified on LG13 in one year for nerol and geraniol (9-15% of explained variance) and on LG2 in two years for linalool (20-48%). A linkage between the linalool QTL and both colour and sex genes was observed on LG2, which is potentially of great interest in MAS to break down undesirable correlations between these traits.

Similar results were obtained by the Italian group of IASMA (Costantini et al. 2005, Battilana et al. 2009) from the analysis of the Italia x Big Perlon and the Moscato Bianco x *V. riparia* progenies. These populations were phenotyped respectively in three and two growing seasons by quantifying the content of linalool, nerol and geraniol through GC-MS. In the Italia x Big Perlon progeny a major QTL for the amount of linalool (26-36% of the total variance), nerol (69-83%) and geraniol (69-84%) was detected on LG5 in three years, as in Doligez et al. (2006b). One additional QTL for linalool content (11-36% of explained variance) was found on LG10 in three years, as well as QTLs explaining a high percentage of the total variance for linalool/nerol and linalool/geraniol ratios. A major QTL for the content of the three monoterpenes was identified on LG5 also in the Moscato Bianco x *V. riparia* progeny in two years. It explained 64-72% of the total variance of linalool, 84-93% of nerol and 83-90% of geraniol content. Another QTL for linalool content (32% of explained variance) was found in one year on LG2, as in Doligez et al. (2006b). Finally, a QTL controlling the ratio between linalool and nerol (35% of explained variance) was detected on LG10 in one year.

The existence of common (on LG5) and linalool-specific (on LGs 2 and 10) QTLs in both works is in agreement with the significant correlation existing between these compounds (stronger between nerol and geraniol than between linalool and nerol or linalool and geraniol), which was reported by Versini et al. (1993).

4.6. Leaf morphology

Leaf morphology traits are discriminant parameters in ampelography contributing to the identification of unknown cultivars. Welter et al. (2007) detected in one season 27 QTLs affecting 18 leaf morphology descriptors and their ratios in the Regent x Lemberger population. These QTLs were found dispersed all around the genome on 12 of the 19 LGs, with a noticeable concentration on LG1. The percentage of explained variance ranged between 7 (length petiole si-

nus to lower leaf sinus) to 71 (length of vein N2/length petiole sinus to upper leaf sinus).

5. FROM QTLs TO UNDERLYING GENES

QTL analysis identifies regions of the genome that contribute to trait variation. The following step is to narrow down those regions to the point, where the effects can be ascribed to specific genes. To this purpose Costantini et al. (2008) adopted the candidate gene approach (Pflieger et al. 2001) at two levels. First, 34 'functional candidate genes' were mapped in the Italia x Big Perlon cross and tested for co-localization with QTLs. Most of them had a hypothetical role in muscat flavour determination based on Gene Ontology terms.

Map co-localization between candidate gene markers and QTLs was observed, which provides clues regarding the possible role of these genes in the regulation of aroma. The most interesting case concerned the marker DXS, which co-mapped with the major QTL for the content of linalool, nerol and geraniol detected on LG5 (Battilana et al. 2009). The *DXS* gene encodes for 1-deoxyxylulose-5-phosphate synthase, which is the first enzyme implicated in the non-mevalonate pathway of IPP biosynthesis.

Secondly, the complete sequence of the SSR markers underlying the main QTLs reported in Costantini et al. (2008) was used to identify the surrounding genomic sequence of Pinot Noir by alignment to predict putative genes. This strategy allowed the definition of three, positional candidate genes, a YABBY-like transcription factor for the flowering time, flowering-véraison interval, mean seed number and mean seed dry weight QTLs on LG2, a gene (*GRIP31*) encoding a putative ripening-related *V. vinifera* protein for the véraison-ripening interval QTL on LG12 and, finally, a gene encoding *V. vinifera* MADS-box protein 5 for the seed content and berry size QTL on LG18.

A similar strategy was adopted by Di Gaspero et al. (2007) and consisted in mapping RGAs, which are functional markers related to disease-resistance developed on DNA sequences that resemble plant resistance genes. Plant material consisted of two pseudo-testcrosses, Chardonnay x Bianca and Cabernet Sauvignon x '20/3' where the seed parents were *V. vinifera* genotypes and the male parents were *Vitis* hybrids carrying resistance to mildew diseases. RGA markers were found in 18 of the 19 LGs but most of them (83%) were clustered on seven LGs, namely groups 3, 7, 9, 12, 13, 18 and 19. Several RGA clusters mapped to chromosomal regions where phenotypic traits of resistance to fungal diseases such as downy mildew and powdery mildew, bacterial diseases such as PD, and pests such as dagger and root knot nematode, were previously mapped in different segregating populations.

6. MARKER-ASSISTED SELECTION (MAS)

6.1. Resistance to diseases

The first study to test the applicability of MAS in grapevine was carried out by Dalbò et al. (2001). These authors previously constructed genetic maps from the cross Horizon x Illinois 547-1 and identified a genomic region with a strong influence (41% of explained variance) on powdery mildew resistance (Dalbò 1998). On the same LG three QTLs for black rot resistance and a QTL for the production of resveratrol were also located. A RAPD and an AFLP marker linked to the major QTL for powdery mildew resistance were obtained by BSA and then converted into CAPS markers for testing in other segregating populations where the same source of resistance was used. In all crosses the markers were closely associated with resistance and allowed to reduce the percentage of susceptible individuals (from 24-52% to 2-18%) by selecting for resistant ones. An allele-specific amplified polymorphism marker associated with powdery mildew resistance was also developed from the sequence of the RAPD marker, but it was found more error-prone compared to CAPS markers and was not used for the segregation analysis. A similar approach was reported by other groups.

Pauquet et al. (2001) applied BSA to the identification of AFLP markers linked to *Run1*, the dominant locus controlling resistance to powdery mildew. Thirteen AFLP markers were selected and used to establish a local map around *Run1* in a population derived from *M. rotundifolia* after five pseudo-backcrosses. Out of them ten markers were found to co-segregate with the resistance gene. The thirteen linked markers were then tested in a set of resistant and susceptible genotypes for their usefulness in breeding programs. Three markers were found to be always present in resistant genotypes and always absent in susceptible genotypes and thus represent good candidates to select *Run*1 carrying resistant individuals.

Luo et al. (2001) applied a BSA to the identification of molecular genetic markers tightly linked to downy mildew resistance genes derived from *V. quinquangularis*. A RAPD marker was found 1.7 cM apart from *RPv-1*, a major gene for resistance to *P. viticola*, and was subsequently converted into a SCAR. Its usefulness for the rapid selection of *Rpv-1* carrying resistant genotypes was confirmed by analyzing five progenies from crosses between *V. quinquangularis* and *V. vinifera*, as well as wild grapes native to China. The SCAR marker produced a single band only in resistant plants.

Donald et al. (2002) utilized the RGA approach to investigate the genetic basis of resistance mechanisms in grapes. Twenty eight unique grapevine RGA sequences were identified and subdivided into 22 groups. Representatives from each group were used in a BSA strategy to screen for restriction fragment length

polymorphisms linked to the powdery mildew resistance locus, *Run1*. Three RGA markers were found to be tightly linked to the *Run1* locus. Of these markers, two (GLP1–12 and MHD145) co-segregated with the resistant phenotype in the progeny tested, whereas the third marker (MHD98) was mapped to a position 2.4 cM from the *Run1* locus.

Mahanil et al. (2007) cloned a total of 122 putative RGA sequences from *V. cinerea*, *V. rupestris*, and Horizon. Twenty-three STS and 3 CAPS markers developed from RGAs were checked for resistance segregation among 179 seedlings derived from Horizon x Illinois 547-1, and 18 showed goodness-of-fit using a chi square test. Marker stkVa011 correlated with segregation for downy mildew resistance in this population. These STS markers are currently being investigated for their potential in molecular breeding for disease resistance

Akkurt et al. (2007) developed SCAR markers from RAPD markers linked to the major QTL for powdery mildew resistance in the population Regent x Lemberger. The SCAR markers ScORA7-760 and ScORN3-R produced amplification fragments predominantly in resistant individuals and were found to correlate to disease. ScORA7-760, in particular, was suggested to be suitable for MAS for powdery mildew resistance and to facilitate pyramiding powdery mildew resistance genes from various sources.

Finally, Eibach et al. (2007) showed the first combined application of resistance-linked markers originating from *M. rotundifolia* and Regent in MAS. An F_1 progeny derived from a cross of VRH 3082-1-42 (a BC4-line of a hybrid of *M. rotundifolia* x *V. vinifera*) and Regent was screened with markers linked to *Run*1 and *Rpv*1 resistance from *M. rotundifolia* in combination with powdery- and downy mildew resistance linked markers from Regent. Phenotypic evaluation indicated that there is a clear correlation between the degree of resistance and the presence of the resistance-related marker alleles. Out of 119 individuals in the progeny four could be identified to carry a pyramidized combination of resistance regions from both parental types.

6.2. Seedlessness

The first attempt to perform marker-assisted selection for seedlessness was reported by Striem et al. (1996). They identified 12 RAPD markers correlating to seven subtraits of seedlessness in a progeny derived from the cross between Early Muscat (seeded) and Flame Seedless (seedless). A combination of seven markers allowed the exclusion of most of the seeded individuals, about 44% of the population.

The application of BSA in a progeny derived from the cross between two partially seedless genotypes lead Lahogue et al. (1998) to the development of a SCAR marker closely (0.7 cM) linked to *SdI*, which was named *SCC8*. All in-

dividuals homozygous for the *SCC8*$^{+}$ allele were completely seedless or had only small-sized seed traces, whereas all individuals with normally developed seeds were homozygous for the *scc8*$^{-}$ allele, except one recombinant. Heterozygous individuals showed a large variation in the degree of seed development.

This marker accounted for a large proportion (65-89%) of the phenotypic variation of seedlessness subtraits in the examined progeny. The efficiency of *SCC8* for selection was further tested by Adam-Blondon et al. (2001) in three additional full sib families segregating for seedlessness and in a set of table grapes. Its usefulness was found to be confirmed, but reduced by the type of cross (larger in partially seedless x partially seedless crosses than in seeded x seedless progenies) and by the existence of null alleles for this marker. BSA was applied also by Mejía and Hinrichsen (2003) to develop a SCAR marker (SCF27), which showed a strong (83%) association with seedlessness in the Ruby Seedless x Sultanina progeny.

Finally, Cabezas et al. (2006) tested the efficacy of two microsatellite loci (VMC7F2 and VMC6F11) closely linked to the locus *SdI* for seedlessness marker-assisted selection. Based on association analysis in the segregating progeny Dominga x Autumn Seedless and in a collection of 46 seeded and seedless genotypes they suggested VMC7F2 as a useful tool for seedlessness breeding.

7. ASSOCIATION MAPPING IN GRAPEVINE

Linkage mapping approaches described above generally have low resolution and the evaluation is limited to a low number of alleles. Low resolution results from the reduced number of recombination events available in the average 150 plants populations used. Furthermore, the maximum number of alleles per locus segregating in the F_1 population used for the pseudo-testcross strategy is four (Grattapaglia and Sederoff 1994). Association mapping, also known as linkage disequilibrium mapping, is a tool that could be efficient to reduce those limitations. Association mapping does not require the construction of mapping populations and is performed on cultivar collections. This has the advantage that there is no restriction on the number of alleles tested and therefore no limitations on the genetic diversity used. Furthermore, phenotypic information on the existent grapevine collections can directly be used in association mapping avoiding the time required to construct and evaluate additional progenies. Obviously, the resolution of association mapping will be related to the extent of linkage disequilibrium in the collections used and this will be inversely related to the number of recombination events accumulated in the collection.

In the case of association mapping, for two given markers, the number of recombination events is not only a function of genetic distance but also of the

number of meiotic generations separating genotypes from each other. This makes the genetic analysis more complex because historical factors such as population admixture, selection or genetic drift can bias the detected association. The power and resolution of association mapping depends on the extent of linkage disequilibrium in the mapping collection (Rafalski and Morgante 2004). A rapid decay of LD within a short genetic distance will allow a high resolution mapping of a given trait but will require large number of markers distributed all along the genome. On the contrary, the presence of large LD blocks will allow association mapping to given chromosomal regions with a small number of markers but with limited resolution.

In different European countries there are large collections of grapevine genetic resources. The largest one is maintained by INRA, in France. It contains 2262 unique genotypes (Le Cunff et al. 2008) from 38 different countries supposedly representing half of the existing grapevine cultivars (This et al. 2006). This collection has been genotyped at 20 unlinked SSR loci and phenotyped for 50 morphological traits and this information has been used to build nested core collections representing different levels of SSR allele diversity (Le Cunff et al. 2008). This collection can provide a starting tool for whole genome association mapping approaches once its structure is understood. This is very relevant information because many of the current grapevine cultivars are the result of spontaneous hybridizations along the history of grapevine cultivation and in many cases only one meiotic generation separates them. As an example, over 250 French cultivars are F_1 hybrids derived from fortuitous crosses between the cultivars Gouais and Pinot Noir (This et al. 2006).

Regarding molecular markers for association mapping, many sets of SSRs have previously been developed and mapped for grapevine, as described above. Furthermore, the availability of the genome sequence of cultivar Pinot Noir has identified circa two million SNPs in heterozygosity in this cultivar (Velasco et al. 2007) and has prompted the development of re-sequencing approaches to provide large number of suitable unbiased SNPs (Lijavetzky et al. 2007). Currently, further SNP development going on in different laboratories warrants the future availability of several thousand markers for association mapping in grapevine.

7.1. Linkage disequilibrium in grapevine

Availability of markers and collections has allowed the first analyses of linkage disequilibrium in grapevine samples. The analysis of LD for 38 SSR loci located on five LGs within a core collection of 141 cultivars maximizing the genetic diversity present in the INRA collection showed the existence of significant genotypic LD within LGs extending up to 16.8 cM as well as sig-

nificant haplotypic LD over 30 cM. Genotypic and haplotypic r^2 values were found to decline to around 0.1 within 5–10 cM (Barnaud et al. 2006).

These results suggest the accumulation of a limited number of recombination events since the initial domestication of grapevine, what is in agreement with the close genetic relationships observed between some cultivars and the low number of meiotic generations that have likely elapsed among them as a result of the major role of vegetative multiplication in cultivar propagation. These results also support the possibility of using association mapping as a strategy for low resolution mapping in grapevine although no results using this strategy have been published yet.

On the other hand, studying SNPs in over 200 gene sequences in a small selected sample of cultivated and wild grapevine genotypes, Lijavetzky et al. (2007) found indications of fast decaying linkage disequilibrium on the short range with r^2=0.2 at 0.3 kb. The discrepancy observed with respect to SSR results is due to a marker effect in such a way that more informative markers like SSRs are able to detect higher LD than less informative ones such as SNPs (Varilo et al. 2003).

7.2. Candidate gene approach

Linkage disequilibrium can be used at local level in specific genomic regions to reduce the size of QTL confidence intervals. This approach is based on the detection of LD between the phenotype affected by the QTL and molecular markers in the chromosomal region in a core collection of genotypes. So far, there is no published information on the success of this approach in grapevine. Core collections can also be used to test association between nucleotide sequence variation of candidate genes and predicted phenotypes.

This approach was used to show that berry colour was associated to nucleotide sequence variation at gene *VvmybA1* in a study involving over 200 grapevine cultivars with different colour berries (This et al. 2007). In spite of the fact that mutations in this gene could have been selected during the process of grapevine domestication, little extension of LD was observed at this locus with r^2=0.2 at 0.7 kb and later decaying rapidly.

As a conclusion, association mapping could be an efficient strategy for trait mapping in grapevine when considering monogenic traits and major effect QTLs. Investment in the phenotypic evaluation of germplasm collections and in the development of efficient markers and genotyping systems will be required in the future years, what can easily be developed within the context of international collaboration.

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MICROSATELLITE MARKERS FOR GRAPEVINE: TOOLS FOR CULTIVAR IDENTIFICATION & PEDIGREE RECONSTRUCTION

K.M. Sefc[1], I. Pejić[2], E. Maletić[2], M.R. Thomas[3] & F. Lefort[4]

[1] Department of Zoology, University of Graz, Universitätsplatz 2, 8010 Graz, AUSTRIA

[2] Faculty of Agriculture, University of Zagreb, Svetošimunska 25, HR-10000 Zagreb, CROATIA

[3] CSIRO Plant Industry, P.O. Box 350, Glen Osmond, South Australia 5064, AUSTRALIA

[4] Plants and Pathogens Group, Research Institute Earth Nature and Landscape, University of Applied Sciences of Western Switzerland, 150 Route de Presinge, 1254 Jussy, SWITZERLAND

1. INTRODUCTION

Microsatellites have become a favorite type of DNA marker for identification of grapevine cultivars, and their properties enable a wide range of applications from cultivar identification based on various parts of the grapevine plant to pedigree reconstruction and genome mapping (Sefc et al. 2001). Genetic markers have the advantage that the DNA of a certain plant is identical in all cells of any tissue at any stage of development and is not influenced by environmental or sanitary conditions of the plants. DNA can be obtained from every kind of plant tissue available, e.g. wood, leaves or berries, and analyses can therefore be carried out at any time of the year. Thanks to the PCR (Polymerase Chain Reaction) technology, minute amounts of DNA can be analyzed, which extends the usefulness of the method from plant tissue to grapevine products such as must and wine.

The typical microsatellite sequence consists of five to about one hundred tandem repeats of short, simple sequence motives composed of 1 to 6 nucleotides (e.g. (GA)n, (GATA)n (Fig. 1)). In eukaryotes, an estimated 10^4 to 10^5 microsatellite loci are scattered randomly throughout the genome. This abundance of microsatellite sequences in eukaryote genomes constitutes an almost unlimited source of polymorphic sites that may be exploited as genetic markers.

Thomas et al. (1993) first investigated the use of microsatellite DNA for

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identifying grapevine cultivars. They showed that microsatellite sequences were abundant in grapevine and very informative for identifying *V. vinifera* cultivars. Moreover primer sequences were conserved across other *Vitis* species and *Muscadinia* (Thomas and Scott 1993). It was also demonstrated through pedigree analysis that the microsatellite alleles were inherited in a co-dominant Mendelian manner (Thomas and Scott 1993) confirming their suitability for genetic mapping and investigation of genetic relatedness (Thomas et al. 1994).

As other groups throughout the world became interested in grapevine microsatellite genotyping, a large number of markers has been developed by individual groups (e.g. Bowers et al. 1996, 1999b, Sefc et al. 1999, Arroyo-Garcia et al. 2004, Adam-Blondon et al. 2004, Di Gaspero et al. 2005, Merdinoglu et al. 2005, Goto-Yamamoto et al. 2006) and in the framework of the Vitis Microsatellite Consortium (VMC, 1997-2004). The approach used for microsatellite marker development in these studies was the construction of a genomic library from a grapevine cultivar or rootstock, screening of the library with microsatellite probes, sequencing of microsatellite containing clones, design of PCR primers from the sequences flanking the microsatellite, optimization of PCR conditions and characterization of the microsatellite polymorphism.

Furthermore, public databases as those compiled from cDNA and EST (Expressed Sequence Tag) type data were recognized as a source of microsatellite loci, and a large grape EST dataset (Ablett et al. 1998) was employed for the development of microsatellite markers (Scott et al. 2000b). Over 100 microsatellites were readily identified, and the further investigation of some of these loci showed enough polymorphism to allow the discrimination of several grapevine and rootstock cultivars, and transferability of the primers to species of the related genera *Cissus* and *Cayratia*. To date, many microsatellite markers have been developed and mapped in *Vitis*, and locus information including primer sequences and mapping position can be retrieved from the UniSTS database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unists).

1.1. Microsatellite genotyping

Microsatellite genotyping requires the determination of the number of repeat units at a given locus in a given cultivar. This is achieved by electrophoretic sizing of the fragment containing the repeat region (the microsatellite allele), which was amplified by PCR with primers situated upstream and downstream of the microsatellite DNA (Fig.1). Methods to visualize and score these PCR products have been improved tremendously in the last 20 years, starting from radioactive labelling, silver or ethidium bromide staining of PCR products followed by manual size scoring (Thomas and Scott 1993, Scott et al. 2000b, Bowers et al. 1996, 1999b, Bowers and Meredith 1997), which was soon

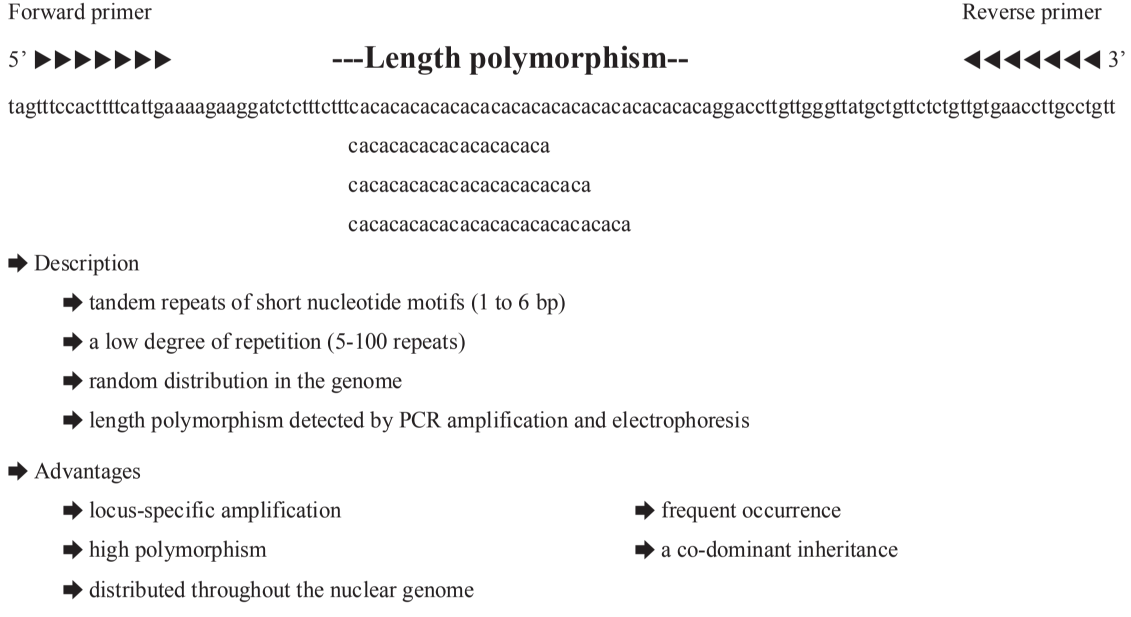
replaced by the use of primers tagged with fluorochromes and fragment analysis employing automated sequencers and fragment sizing software. Allele size calling between gels and different gel-based ABI sequencing machines has shown a high reproducibility, while differences in size calling are observed when results from different electrophoresis systems are compared. For example, an allele estimated to be 150 bp by silver staining may run at 148 bp in the Pharmacia system, while it may be called 153 by the ABI sequencer. These deviations, however, are constant within a certain locus, and can easily be determined by analysing a small set of genotypes in each of the systems. Later on, the allele sizes determined by different systems can be corrected for the observed deviation to one established standard system, and data from different sources become easily comparable again (This et al. 2004). Therefore, data obtained in different laboratories and by different methods can easily be combined into joint analyses and databases.

1.2. How many markers?

The extraordinary potential of microsatellite markers for grapevine and rootstock cultivar discrimination has been demonstrated in several studies (Thomas and Scott 1993, Thomas et al. 1994, Cipriani et al. 1994, Bowers et al. 1996, Sefc et al. 1998a, 1998b, 1998c, 1998d, 1999, Sanchez-Escribano et al. 1999). Theoretically, five unlinked markers each with five equally frequent alleles could produce over 700 000 different genotypes (Bowers et al. 1996). In reality, however, these ideal conditions are seldom fulfilled. In order to minimize the number of markers necessary for a reliable discrimination and identification of cultivars, the most informative loci have to be selected (Sefc et al. 2001).

The information content of a given marker may differ between cultivar collections from different regions, as allele frequencies vary between grapevine gene pools. On the whole, however, a marker set containing the most informative markers as defined in one cultivar collection will also yield a high level of discrimination power in other gene pools (Sefc et al. 2000). A marker set comprising six highly variable loci has been evaluated and recommended as a potential descriptor for grapevine cultivars (Cabello et al. 1999, This et al. 2004). Of course, many more markers are available and were shown to be highly polymorphic, such as UCH12 and UCH29 with 30 alleles each (Lefort et al. 2003b) or ssrVrZAG93 with 31 alleles (Sefc et al. 1999), in *Vitis* species. Due to the high discriminative power of microsatellite analysis, the finding of identical genotypes in two different plants is strong evidence that these plants in fact belong to the same cultivar. Therefore, microsatellite analysis can be used to determine cultivar identity and to identify plant material of unknown varietal ori-

Fig. 1. A dinucleotide microsatellite locus.informative for identifying *V. vinifera* cultivars.



gin by comparing the genotype obtained from the sample with reference genotypes of cultivars stored in a database.

In contrast to the relatively small number of markers needed to determine the identity or non-identity of two grapevine samples, a much larger number of markers is needed for the reconstruction of parentage and pedigrees among cultivars in order to avoid erroneous assignment of relationship. Most of the recent studies of parentage and kinship reconstruction employed more than 25, and sometimes more than 50 markers (see also below).

2. IDENTIFICATION OF CULTIVARS OF *VITIS VINIFERA* AND ROOTSTOCKS FROM *VITIS* SPECIES

2.1 Source and quality of DNA used for PCR amplification

DNA suitable for PCR has been successfully isolated from wood (cambium tissue scrapings from beneath the bark of vine trunks, whole wooded canes), green canes, petioles, young leaves, floral tissue, pre-véraison and post-véraison berries and the rachis of grape bunches. Bulky tissue such as canes and berries can be frozen in liquid nitrogen and while still frozen ground to a fine powder in an electric coffee grinder. Mature leaf tissue is the most difficult tissue for extracting good quality DNA. Canes and berries can be a source of very clean DNA and it is of interest to note that the original genomic library used for grapevine microsatellite isolation was made from DNA isolated from post-véraison berries of Sultana (Thomas and Scott 1993).

Recently, impressive progress has been made in the DNA extraction and genotyping from grape must and wine. Protocols for DNA extraction from must and/or wine differ from those used for grapevine plant tissue (e.g. Thomas et al. 1993, Lodhi et al. 1994, Wolf 1996, Lefort and Douglas 1999) and were developed by Siret et al. (2000), Faria et al. (2000), Garcia-Beneytez et al. (2002), Nakamura et al. (2007), and several extraction protocols were compared and evaluated by Savazzini et al. (2006), Baleiras-Couto and Eiras-Dias (2006), and Drabek et al. (2008). Microsatellite genotyping of wine DNA allowed the identification of cultivar-specific alleles in monovarietal wines (Savazzini et al. 2006, Baleiras-Couto and Eiras-Dias 2006) and even in experimental wine mixtures (Siret et al. 2002). Genotyping profiles of multivarietal musts were correlated to the proportion of each variety in the mixture (Baleiras-Couto and Eiras-Dias 2006). These encouraging findings suggest that, in the not so far future, it will be possible to apply DNA-based genotyping techniques to the control of commercial wines.

2.2. Identification of grapevine cultivars and rootstocks by using nuclear SSRs

Grapevines are propagated vegetatively, so that, except for somatic mutations, the individual vines of a cultivar are genetically identical to each other. In principle, the microsatellite data obtained from one individual represent the microsatellite profile of that cultivar. The results of microsatellite analysis can be reproduced and compared in different laboratories or at different periods, provided standardization is carefully performed (see above). Consistent microsatellite profiles were obtained for example using DNA of the same grapevine cultivars genotyped in different years (Botta et al. 1995) and different laboratories (Sefc et al. 1998, Grando and Frisinghelli 1998, Lefort et al. 2001).

The initial grapevine microsatellite study by Thomas and Scott (1993) at CSIRO Plant Industry, Australia, reported the DNA identification of 26 *Vitis vinifera* cultivars and 6 additional *Vitis* species as well as *Muscadinia rotundifolia*. Since then, researchers have accumulated microsatellite profiles of hundreds of grapevine cultivars from many different regions (Table 1). Many of the data have been made available in public databases (see below).

Some examples of practical applications of microsatellite based cultivar identification are outlined in the following. In the course of a project, which aimed to reduce virus contamination of grapevine material by *in vitro* meristem culture, thermotherapy and subsequent propagation of the treated material for the production of certified planting material, cultivar identity of the *in vitro* plantlets prior to the propagation steps was carried out by microsatellite analysis. Two samples per clone were analyzed at four microsatellite loci, and the resulting genotypes were compared to a reference database. This quality control step proved to be essential, as mistakes in cultivar assignment were detected (Sefc et al. 1998d).

Cases of misidentification of rootstocks Teleki 5C and SO_4 such as that reported for the University of California collection (Walker and Boursiquot 1992) can now be solved by DNA profiling (Thomas et al. 1994). De Andres et al. (2007) reported numerous misclassified accessions in European rootstock germplasm collections, which were resolved by microsatellite profiling. Similarly, evidence of false identities of rootstocks or cultivars in vineyards was found in Greece (Lefort and Roubelakis-Angelakis, unpublished). In a vineyard, rootstocks believed to be 1103 Paulsen and Ruggeri 140 appeared to be different from the reference plants of two ampelographic collections and remain unknown until now. Another example concerned 3 cultivars from the same vineyard where genotyping at nine SSR loci showed that if Liatiko was really Liatiko, a so-called Kotsifali proved to be an offspring of Liatiko and a so-called Kotsifaloliatiko was in fact a cultivar named Fegi.

Table 1. Examples of studies genotyping cultivars from specific wine growing regions. See last row for studies focusing on table grapes.

Region	References
Albania	Ladoukakis et al. 2005 (29 accessions)
Austria and Germany	Sefc et al. 1998 (66 grapevine and rootstock accessions)
Bulgaria	Hvarleva et al. 2004 (74 accessions)
Carpathian Basin	Halasz et al. 2005 (97 accessions)
China and Japan	Goto-Yamamoto et al. 2006 (6 accessions)
Croatia	Maletic et al. 1999 (22 accessions)
Cyprus	Hvarleva et al. 2005 (12 accessions)
Czech Republic	Moravcova et al. 2006 (51 accessions)
Greece	Lefort et al. 2001 (50 accessions)
Iran	Najafi et al. 2006 (136 accessions), Doulaty Baneh et al. 2007 (69 accessions, 63 wild vines)
Italy : Aosta Valley	Labra et al. 2002 (19 accessions)
Italy: Apulia	Zulini et al. 2002 (38 accessions)
Italy: Campania	Costantini et al. 2005 (114 accessions)
Italy: Reggio Emilia	Boccacci et al. 2005 (32 accessions)
Italy: Southern Italy	Pellerone et al. 2001, Reale et al. 2006
Italy: Verona	Vantini et al. 2003 (12 accessions)
North and South America	Milla Tapia et al. 2007 (79 accessions), Agüero et al. 2003 (accessions from Argentina), Martinez et al. 2006 (25 accessions from Peru and Argentina), Pollefeys and Bousquet 2003 (French-American hybrid grapevines, 16 accessions)
Portugal	Almadanim et al. 2007 (51 accessions), Lopes et al. 1999 (49 accessions), 2006 (46 accessions) , Pinto-Carnide et al. 2003 (12 accessions)
Russia, Ukraine, Moldova	Lefort et al. 2003
Spain	Martin et al. 2003 (176 accessions) Ortíz et al. 2004 (621 accessions) Reale et al. 2006
Spain: Comarca del Bierzo	Gonzales-Andres et al. 2007 (79 accessions)
Tunisia	Snoussi et al. 2004 (80 accessions)
	Selli et al. 2007 (31 accessions), Karatas et al. (2007)
Table graphs	Crespan et al. 1999 (20 accessions), Sánchez-Escribano et al. 1999 (43 accessions), Costantini et al. 2007 (13 accessions)

In a survey of table grapes for sale in various supermarkets and market places in Austria (Sefc et al. 1998b), only about two thirds of the samples were shown to be correctly labelled. This inspection of the correctness of marketed

table grape labels, though on a small scale, revealed a substantial degree of inaccuracy in information provided to the consumer.

Microsatellites can also be used as a help tool for screening the variability collected from old vineyards (Lefort and Roubelakis-Angelakis 2002).

3. SYNONYMS

Microsatellite analysis can further be applied to confirmation and definition of synonyms, i. e. identical genotypes known under different names. The identification of duplicates is of particular importance in germplasm collections when maximum genetic variability should be maintained while keeping the number of specimens at minimum. For several cultivars, synonymy had been suspected or assumed based on ampelographic observations and could be confirmed by microsatellite analysis, whereas in other cases, unsuspected synonymy was discovered by comparisons of microsatellite profiles (Table 2).

Often, synonymies result from cultivars being assigned different designations in different wine growing regions, such as the use of the name 'Burgunder' in German speaking regions for the Pinot cultivars. Interestingly, identical vine genotypes have sometimes received different denominations in a geographically narrow wine producing area. The names are however often related, e.g. Biancaccia and Biancazza, Vernaccia and Vernazzola or have similar references eg. Schiava Grigia (Schiava gray) and Cenerina (Cinereo, ashen) (Grando et al. 2000). Concerning rootstocks, 5A Teleki and 5BB Kober have been shown to have the same DNA profile indicating that the two different names were mistakenly given to the same rootstock in the past (Thomas et al. 1994).

In contrast, putative synonymy is sometimes rejected by microsatellite analysis. The Croatian cultivar named Hrvatica was supposed to be identical to the Italian cultivar Croatina, as both names mean 'Croatian girl'. However, it was shown that Hrvatica and Croatina differ at several loci and thus represent two distinct cultivars (Maletic et al. 1999). Likewise, synonymy of the Hungarian cv Keknyelu with the Italian cv Picolit was indicated in the International Variety Catalogue (Genres), but was rejected by genetic analysis (Jahnke et al. 2007).

Besides obvious utility in germplasm collections, detection of synonyms sometimes has great potential for significant savings and shortcuts in practical viticulture.

For instance, in 2002 using microsatellites Maletić et al. (2004) discovered extremely rare and almost eradicated old cultivar called Crljenak Kastelanski that proved to be synonym of Primitivo and Zinfandel. This information led to great interest of Croatian viticulturists to plant this grape. By getting planting

Table 2. Examples of synonymous cultivars discovered or confirmed by microsatellite analysis, with emphasis on cultivars shared by different winegrowing regions. International abbreviations are used for country names.

Cultivars sharing same genotype (synonyms) (alphabetic and chronological order)	Reference
Furmint (H), Moslavac (HR), Šipon (SLO) Brajdica crna (HR), Plavina (HR), Plavka (HR) Muškat Ruža Porečki (HR), Rosenmuskateller (A) bijeli Prosecco (I), Teran (HR)	Maletić et al. 1999
Blauer Portugiser (A), Portugues Azul (P)	Regner et al. 1999
Greco di Gerace (I), Malvasia delle Lipari (I), Malvasia di Sardegna (I), Malvasia de Sitges (E), Malvasia de La Palma (E, Canary islands), Malvasija dubrovačka (HR)	Crespan et al. 2006, Zerolo Hernandez et al. 2007
Crljenak kaštelanski (HR), Pribidrag (HR), Primitivo (I), Tribidrag (HR), Zinfandel (USA), Kratošija (Montenegro)	Maletić et al. 2004, Calò et al. 2008
Cipar (HR), Grec rouge (F), Grisa rousa (Piedmont, I)	Pejić and Maletić, unpublished, Schneider et al. 2001
Malvasia bianca lunga (I), Malvasia del Chianti (I), Maraština (HR), Pavlos (GR), Rukatac (HR)	Šimon et al. 2007
Malvasia (E), Malvasia Castellana (E), Dona Blanca (E)	Arranz et al. 2007
Moscato Bianco (I), Moscato (Cyprus), Tamyanka (BG) Lefkas (Cyprus), Verdzami (GR) Malaga (Cyprus), Muscat of Alexandria (Egypt)	Hvarleva et al. 2005
Garnachal (E), Tinto Basto (E) Machina (E), Tortosi (E), Rojal (E) Moravio (E), Bobal (E)	Fernandez-Gonzales et al. 2007
Afuz ali (Lebanon), Regina (I), Mennavacca (I)	Zulini et al. 2002
Babić (HR), Rogoznička (HR)	Zdunić et al. 2008
Pucalla (AL), Shesh I Bardhe (AL)	Ladoukakis et al. 2005
Plantscher (CH), Bordeaux blanc (F), Gros Bourgogne (F)	Vouillamoz et al. 2004
Black Kishmish (RU), Russian seedless (RU), Kishmish Chernyi (RU), Negrita (Chile) Sultanina, Thompson Seedless (USA), Keshmesh (Pakistan) "Quinn of the Vineyard", Scolokertek Kiralnoje (H)	Dangl et al. 2001
Moscato bianco (I), Moscatel fino (E), Muškat momjanski (HR), Muscat d'Alsace blanc (F), Muscat de Frontignan (F), ... + 22 additional synonyms Muscat of Alexandria (Egypt), Moscatel blanco (E), Moscatel de Malaga (E), Moscato francese (I)	Crespan and Milani 2001
Sangiovese (I), Guarnacciola (I), Tabernello (I), Toustain (Algeria) + 12 already supposed and confirmed synonyms	Di Vechi Staraz et al. 2007
Borsé (I), Cardin (I), Avaná (I), Hibou rouge (F), Canari (F), Luverdon (I), Chatus (F), Neiret (I), Nebbiolo di Dronero (I), Bian ver (I), Verdesse (F), Bianco (I), Listan (F), Biquet (I), Persan (F), Gouais blanc (F), Liseiret (I), Preveiral (I), Heunisch Weiss (A)	Schneider et al. 2001

material of Primitivo from Italy and Zinfandel from USA, more than 100,000 plants of this genotype have been planted in only three years Croatia. The regular and natural multiplication of Crljenak Kastelanski from only 25 vines detected so far would take much longer. Availability of high quality planting material of certain cultivar in one country can save time and resources in selection and propagation of its synonym in another one.

4. CLONAL LINES, SOMATIC MUTANTS AND CHIMERISM

While distinction of cultivars could be successfully achieved with microsatellite markers, microsatellite polymorphisms among the different clones of a cultivar are rarely observed (e.g. Techera et al. 2004). This observation testifies to the somatic stability of microsatellite alleles across long periods of vegetative reproduction of ancient grapevine cultivars (Zulini et al. 2005). Indeed, when 'clones' of a cultivar exhibit different microsatellite alleles at several of the analyzed loci, the cultivar is typically considered of polyclonal origin, i.e. composed of different cultivars, which may none the less be closely related (Vignani et al. 1996, Silvestroni et al. 1997, Cabezas et al. 2003, Zulini et al. 2005). However, somatic mutations of microsatellites do occur and produce polymorphism within a single cultivar. Occasionally, such mutations have been detected in reconstructions of grapevine pedigrees (Vouillamoz et al. 2003, 2004). More often, somatic mutations are detected by screening large numbers of loci and clones. For example, when 100 markers were genotyped in >20 clones each of Pinot noir and Chardonnay, intra-varietal polymorphism was detected at 17 markers (Riaz et al. 2002).

Interestingly, many of the variants were expressed as three alleles, a phenomenon which was observed in other studies as well (Franks et al. 2002, Bertsch et al. 2003, Hocquigny et al. 2004, Zulini et al. 2005, Moncada et al. 2006). In a diploid organism, the different number of alleles per locus is constrained to either one (in case of a homozygous genotype, where alleles at both homologous chromosomes are identical in size), or two (in case of a heterozygous genotype, where the alleles at the two homologous chromosomes differ in size). The presence of three alleles at a locus is due to chimerism: Cell layers giving rise to different plant tissues are developmentally independent from each other, and somatic mutations can produce a novel allele in one cell layer, while the other layer retains the two original alleles. Chimerism affects not only microsatellite alleles, but also genes underlying phenotypic traits. For example, the berry color of Pinot gris results from the loss of anthocyanin synthesis in the inner one of the two cell layers making up the berry skin, whereas anthocyanin is produced in the outer cell layer (Hocquingny et al. 2004).

In order to search for polymorphisms among clones, additional multilocus techniques have been applied. AFLP (Amplified Fragment Length Polymorphism) markers have become the most promising tool for clonal identification and differentiation (e.g. Cervera et al. 1998, 2000, 2002, Scott et al. 2000a, Imazio et al. 2002, Fanizza et al. 2003, Blaich et al. 2007).

5. PEDIGREE RECONSTRUCTION

5.1. Methodology

Most of the grapevine cultivars in existence today are centuries old and are thought to have arisen by several processes: domestication of wild vines, either in the early vine domestication sites south of the Middle East or later in the vine growing regions of Europe, spontaneous crosses between wild vines and cultivars and crosses between two cultivars. Due to the importance of grapevine cultivars both as a crop plant and as a cultural heritage, it is extremely interesting to understand the genetic events, which led to today's cultivar range. The longstanding and lively interest in the history of grape cultivars is reflected by a variety of speculations concerning the origin of popular cultivars such as Cabernet franc, Sylvaner and Traminer.

Ancient wild vines involved in the original crosses cannot be identified any more as they no longer exist, but parents, which are themselves cultivars, may still be cultivated or kept in collections. Using the methods of molecular analysis, such parent cultivars and their offspring can be recognised, and pedigrees describing the genetic history of grapevine cultivars can be reconstructed.

Microsatellites have proved to be the marker of choice for this purpose since they are transmitted in a codominant Mendelian manner. In a cross, each of the parents passes one allele per locus to the offspring (Table 3) and in consequence, each allele displayed by the offspring must also be present in at least one of the two parents. By examining the microsatellite allele composition of an individual and its two presumptive parents, it is possible to confirm or reject the proposed parentage. Microsatellite markers are used routinely in forensic investigations dealing with paternity disputes, and have recently found application in pedigree reconstruction in grapevines. Breeding records exist for a number of grapevine cultivars, e.g. the ones bred in the late 19th or the 20th century such as Müller Thurgau and Zweigelt, and the documented crosses can be verified (or falsified) by microsatellite analysis.

However, such prior assumptions on possible relationships seldom exist for grapevines, especially for the older cultivars. Without prior knowledge, the search for parent cultivars and their offspring cultivar in a cultivar collection is carried out by comparing microsatellite alleles within all possible sets of three

to identify pairs of cultivars that could have contributed the alleles of the third (offspring) cultivar. Furthermore, the chronological order of appearance of the grapevine cultivars remains widely unknown, providing no information on the generation sequence. This is why, when a compatible parent-offspring pair is identified by their microsatellite profiles, it is often impossible to determine which cultivar is the parent and which is the offspring. This question is resolved when triads, consisting of a parent pair and their offspring, can be reconstructed.

Table 3. Example for the codominant Mendelian inheritance of microsatellite alleles in the controlled cross St. Laurent x Blaufränkisch. At each locus, one allele per parent cultivar was transmitted to the offspring cultivar Zweigelt. Numbers represent the allele sizes in base pair. Data from Sefc et al. (1997).

Locus	St. Laurent	Zweigelt	Blaufränkisch
ssrVrZAG 7	157:157	155:157	155:155
ssrVrZAG 15	175:177	165:175	165:165
ssrVrZAG 21	200:206	202:206	202:206
ssrVrZAG 25	225:236	225:236	225:225
ssrVrZAG 30	149:151	147:151	147:149
ssrVrZAG 47	163:167	157:163	157:172
ssrVrZAG 64	139:163	139:159	139:159
ssrVrZAG 67	126:152	126:139	139:149
ssrVrZAG 79	238:246	236:238	236:250

Another consequence of the lack of prior information in pedigree reconstruction is the necessity to genotype a large number of highly polymorphic and unlinked markers in order to achieve high confidence in paternity cases derived from genotype matches (Vouillamoz and Grando 2006). Based on the frequency of the alleles that were passed on from the putative parent to its putative offspring, a likelihood value can be calculated, which indicates the statistical support for the putative relationship (e.g. Bowers and Meredith 1998, Vouillamoz et al. 2007b).

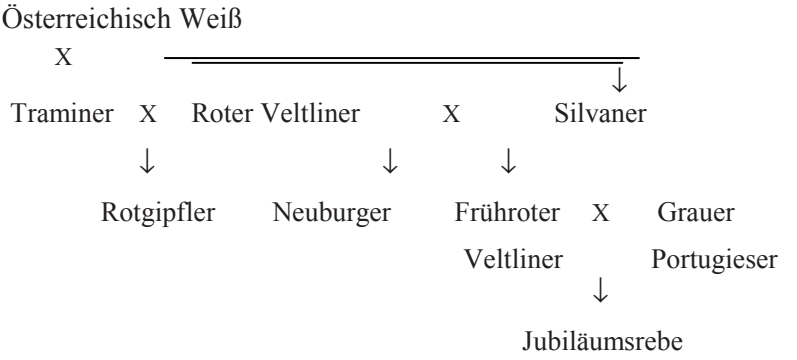
Pedigree reconstruction by nuclear markers can also be efficiently completed with the use of chloroplast microsatellite markers, which then provide information on the direction of the cross by identifying the female parent (see below: 7. Chloroplast SSR markers).

5.2. Reconstruction and verification of grapevine pedigrees

Among the first and most surprising results of parentage studies in grapevines was the discovery of the origin of the cultivar Cabernet Sauvignon (Bowers and Meredith 1997, Sefc et al. 1997). Cabernet Sauvignon was described as the world's most renowned grape variety for the production of fine red wine (Robinson 1994) and has been grown in France at least since the 17th century. A

close relationship between Cabernet Sauvignon and another French cultivar, Cabernet franc, had been suspected from the morphological similarity of the two cultivars. Microsatellite profile comparisons among central European grapevine cultivars confirmed Cabernet franc as one parent of Cabernet Sauvignon. The identification of the white wine cultivar Sauvignon blanc as the second parent of the famous red vine variety, however, was a surprise.

The further search for relatives among French grapevines led to another unexpected finding: 16 grapevine cultivars grown in north-eastern France turned out to be the progeny of a single pair of parents, Gouais blanc and Pinot (Bowers et al. 1999a). Among the progeny are prominent varieties as Chardonnay, Gamay noir, Aligoté, Auxerrois and Melon. While it seemed plausible that the highly valued Pinot should have given rise to a number of successful offspring cultivars, the contribution of Gouais blanc, as a parent of quality wine cultivars, was entirely unexpected. The variety is believed to have been introduced to France from eastern Europe and was widespread in the Middle Ages. However, it was so mediocre that, at various times and regions, it has been banned from vineyards and is no longer planted in France.



A comparison of the genetic profiles of cultivars from central Europe resulted in the reconstruction of a four generation-pedigree illustrating the close relationship between the cultivars Österreichisch Weiß, Traminer, Roter Veltliner, Silvaner, Rotgipfler, Neuburger, Frühroter Veltliner, Grauer Portugieser and Jubiläumsrebe (Sefc et al. 1998c). Genetic profiles showed that Silvaner is most likely the offspring of a cross between Traminer and Österreichisch Weiß, and thus rejected the hypothesis that Silvaner was selected from a wild vine on the banks of the river Danube. Traminer was mentioned first in 1349 and is nowadays widely distributed, whereas Österreichisch Weiß is an ancient variety that has no economic importance today. Since the growing of Österreichisch Weiß was rather restricted to the eastern part of Austria, the descendance of Silvaner also indicates its geographic origin from that region.

A second cross including the cultivar Traminer, Traminer x Roter Veltliner, led to the Austrian variety Rotgipfler. Roter Veltliner was also involved in two further crosses leading to the cultivars Neuburger and Frühroter Veltliner. Neuburger was previously supposed to descend from a natural cross between Pinot blanc and Silvaner. Microsatellite data, however, excluded the parentage of Pinot and suggested a cross between Silvaner and Roter Veltliner. Surprisingly, the same parents gave rise to another cultivar, Frühroter Veltliner, which is also known under the name Malvasier. Breeding reports of Jubiläumsrebe, a white wine variety for the production of dessert wine selected in the 1920's, denoted the cultivar as a cross between the red wine cultivars Blauer Portugieser and Blaufränkisch. However, microsatellite analysis demonstrated that, while the Portugieser genotype was consistent with its parentage of Jubiläumsrebe, the second parent is actually Frühroter Veltliner. The color variants of the Portugieser cultivars cannot be distinguished by microsatellite markers, but breeding records of the institution document that Grauer Portugieser was used in crosses with Frühroter Veltliner.

A prominent example of incorrect pedigree documentation is that of Müller Thurgau, also known as Riesling-Silvaner, which is widely grown in Germany, Switzerland and Austria. The cultivar was believed to originate from a cross, which Prof. Hermann Müller, born in the Thurgau province in Switzerland, carried out around 1890 between Riesling and Silvaner - hence the different names of the cultivar. However, molecular analyses confirmed Rielsing as a parent, but showed that Silvaner is not related to Müller Thurgau (e.g. Thomas et al. 1994, Sefc et al. 1998). A long search for the second parent identified the second parent as Madeleine Royal (Dettweiler et al. 2000), another cultivar Prof. Müller experimented with.

A search for possible parent-offspring combinations among the microsatellite profiles of grapevines from a Portuguese collection revealed the origin of the cultivar Boal Ratinho, a white vine from the Carcavelos region near Lisbon, as the progeny of a cross between Malvasia fina and Síria. Both the offspring and the parent cultivars have been grown in Portugal since ancient times. Various synonyms are used for the two parental cultivars, of which Boal Cachudo and Boal da Madeira for Malvasia fina and Roupeiro and Crato Branco for Síria have been confirmed in the same microsatellite study (Lopes et al. 1999).

In a survey of grapevines known as Petite Syrah in California, it was shown that most of the accessions grown under this name are identical to the cultivar Durif (Meredith et al. 1999). Durif is morphologically very similar to the cultivar Peloursin, and is described as either a seedling or a selection of Peloursin produced in 1880 in France. Microsatellite studies confirmed the former possibility, and identified the cultivar Syrah as a likely parent of Durif.

In recent years, many more grapevine pedigrees have been discovered,

confirmed or rejected with the help of microsatellite data (Table 4, Table 5).

Table 4. Grapevine crosses reconstructed and confirmed by microsatellite analysis.

Offspring cultivar(s)	Discovered or confirmed cross	Reference
Alicante Bouschet	Garnacha x Petit Bouschet	Cabezas et al. 2003
Aligoté, Aubin vert, Auxerrois, Bachet noir, Beaunoir, Chardonnay, Dameron, Franc noir Haute Saône, Gamay blanc Gloriod, Gamay noir, Knipperlé, Melon, Peurion, Romorantin, Roublot, Sacy	Gouais blanc (Heunisch) x Pinot	Bowers et al. 1999a
Arbi Abiadh	Khamri x Saouadi	Snoussi et al. 2004
Bacchus, Optima	(Silvaner x Riesling) x Müller Thurgau	Grando and Frisinghelli 1998
Blauburger	Blauer Portugieser x Blaufränkisch	Sefc et al. 1997
Boal Ratinho	Malvasia fina (= Boal Cachudo, Boal da Madeira) x Síria (= Roupeiro, Crato Branco)	Lopes et al. 1999
Cabernet Sauvignon	Cabernet franc x Sauvignon blanc	Bowers and Meredith 1997, Sefc et al. 1997
Castelo Branco (= Cruzado de Rabo de Ovelha)	Fernão Pires x Dialgaves	Magalhaes et al. 2003
Cayetana	Antão Vaz x Rabo de Ovelha	Lopes et al. 2006
Cercial	Malvasia Fina x Sercial	Lopes et al. 2006
Cornalin du Valais	Petit Rouge x Cornalin d'Aoste	Vouillamoz et al. 2003
Criolla San Juanina, Vina Antigua Negra, Torontel, Cereza, Uva del Padre, Criolla Mediana, Rosa del Perú, Huasquina Pisquera, Torontel, Torrontés Riojano,	País (= Listán Prieto, Criolla chica) x Muscat of Alexandria	Milla Tapia et al. 2007 Agüero et al. 2003

Torrontés Sanjuanino, Moscatel Amarillo		
Djebba	M'guergueb x Blanc3	Snoussi et al. 2004
Durif (= Petite Syrah)	Peloursin x Syrah	Meredith et al. 1999
Italia	Bicane x Muscat of Hamburg	Crespan et al. 1999
Jubiläumsrebe	(Grauer) Portugieser x Frühroter Veltliner	Sefc et al. 1998
Lafetschna	Completer x Humagne Blanche	Vouillamoz et al. 2004
Lusitano	Castelão Francês x Alicante Henri Bouschet	Lopes et al. 2006
Marroo Seedless	Carolina Blackrose x Ruby Seedless	Thomas et al. 1994
Matilde	Italia x Cardinal	Zulini et al. 2002
Müller Thurgau	Riesling x Madeleine Royale	Dettweiler et al. 2000
Muscat of Hamburg	Schiava Grossa x Muscat of Alexandria	Crespan 2003
Neuburger, Frühroter Veltliner	Silvaner x Roter Veltliner	Sefc et al. 1998
Plavac mali	Zinfandel x Dobricic	Maletic et al. 2004
Plavina	Primitivo (Zinfandel) x Verdeca	Lacombe et al. 2007
Posip bijeli	Zlatarica blatska bijeli x Bratkovina bijeli	Piljac et al. 2002
Rieslaner	Silvaner x Riesling	Grando and Frisinghelli 1998
Rotgipfler	Traminer x Roter Veltliner	Sefc et al. 1998
Ruby Seedless	Emperor x Sultana Moscata	Thomas et al. 1994
Sangiovese	Ciliegiolo x Calabrese di Montenuovo	Vouillamoz et al. 2007a
Silvaner	Traminer x Österreichisch weiß	Sefc et al. 1998
Sultana Moscata	Thompson Seedless x Muscat of Alexandria	Dangl et al. 2001
Syrah	Dureza x Mondeuse Blanche	Bowers et al. 2000
Tarrango	Touriga x Sultana	Thomas et al. 1994
Tinto Madrid (Garnacho)	Graciano (= Moratel) x Petit Bouschet	Cabezas et al. 2003
Victoria	Afus ali (= Regina) x Cardinal	Zulini et al. 2002
Vitouska	Malvasia bianca lunga (= Malvasia del Chianti) x Prosecco tondo	Crespan et al. 2007
Zweigelt	Blaufränkisch x St. Laurent	Sefc et al. 1997

Table 5. Pairs of cultivars, for which a parent-offspring relationship was inferred by microsatellite analysis. Without knowledge of the age of each cultivar, and without knowing the second parent, it cannot be determined which of the two cultivars is the parent and which the offspring.

Cultivar pair with putative parent-offspring relationship	Reference
Gibi – Pedro Ximenes	Vargas et al. 2007
Rèze – Cascarolo Bianco Rèze – Arvine Grande Rèze – Nosiola Rèze – Groppello di Revò	Vouillamoz et al. 2007b
Valbom – Alicante Henri Bouschet Tinto de Pegões - Castelão Francês Alfrocheiro - Castelão Francês Gouveio Estimado – Cayetana Gouveio Estimado – Gouveio Roxo Gouveio Estimado – Síria Síria – Trincadeira Preta	Lopes et al. 2006
Torrontés mendocino – Muscat of Alexandria	Agüero et al. 2003
Italiona - Muscat of Alexandria	Milla Tapia et al. 2007
Teroldego – Lagrein Teroldego – Marzemino	Vouillamoz and Grando 2006
Zinfandel (Primitivo, Crljenak k.) – Grk, Vranac, Crljenak viški	Maletic et al. 2004
Fortana – Lambrusco Maestri Uva tosca – Lambrusco Montericco	Boccacci et al. 2005
Plantscher – Furmint Himbertscha – Humagne Blanc	Vouillamoz et al. 2004
Goron – Cornalin du Valais Petit Rouge - Cornalin du Valais Mayolet - Cornalin du Valais Cornalin d'Aoste - Cornalin du Valais	Vouillamoz et al. 2003
Moscato bianco – Muscat of Alexandria	Crespan and Milani 2001
Ehrenfelser - Riesling	Grando and Frisinghelli 1998
Scheurebe - Riesling	Grando and Frisinghelli 1998

6. GENETIC STUDIES OF THE ORIGIN AND DIVERSITY OF EUROPEAN CULTIVARS

Viticulture has a longstanding tradition in southern and central Europe (reviewed by This et al. 2006). As early as 2300 BC, the Neolithic populations of the Minoan and Cycladic cultures adopted viticultural practices from their eastern neighbors (Marangou 1991), who domesticated grapevine and elaborated viticulture and wine techniques as soon as 6000 BC (McGovern et al. 1995, 1996). From 800 BC, Greeks and Phoenicians spread viticultural practices further west in the course of their colonization of the Mediterranean basin.

Likewise, it is widely supported by ancient texts that the Romans carried viticulture northwards to their colonies in the now French, German and Austrian regions. But it is not unlikely that a less elaborate viticulture could have existed in these regions before the Romans and the Greeks exported their know-how there. Wild vines were abundant in Southern and Central Europe at this time and may have served for the selection and domestication of locally cultivated grapevine varieties.

In addition, cultivated varieties were introduced to new growing areas, and later on, exchange of grapevine cultivars between regions may have been substantial. Genetic analysis of archaeological grape pips could shed some light on the history of European viticulture and grapevine cultivars (Manen et al. 2003). Nowadays, regionally typical sets of grapevine cultivars are established in the grape growing regions of Europe, while at the same time, some particularly successful cultivars such as Cabernet Sauvignon, Chardonnay, Pinot noir, Rheinriesling, Sauvignon blanc and Syrah have been included in the programs of virtually all major wine producing areas.

The domestication history of the extant grapevine cultivars, some of which may date back to Roman times (Vouillamoz et al. 2007b), is still contentious. The more traditional view envisions a restricted origin of domesticated grapes, in that current European grapevine cultivars have a common origin in Transcaucasia, the site of earliest grape domestication, from where grape cuttings were transplanted first into the Mediterranean basin and then to Central Europe (Olmo 1976). This 'Noah hypothesis' (McGovern 2003) is well supported by archaeobotanical, archaeological, cultural and historical data pointing to a putative single domestication centre in the South Caucasian and Middle-East regions. The alternative 'multiple-origin' hypothesis claims regionally independent domestication of grapevine from the wild *Vitis vinifera* spp. *sylvestris* throughout its distribution range (Mullins et al. 1992).

A study of European grapevine cultivars from Greece to Portugal with nuclear and chloroplast microsatellite markers (see also below) detected genetic structure between regional cultivar gene pools, and furthermore found that the genetic similarity between grapevine gene pools decreased with increasing geographical distance between regions (Sefc et al. 2003). This is not expected when all cultivars have a common origin in Transcaucasia, or when all cultivars were distributed across Europe by man, which would be largely independent of geographic distance. Hence, the data were interpreted to indicate either in situ domestication of local wild vines in multiple European wine regions, or that the extant cultivars result from hybridization between ancient introduced cultivars and local wild vines (Sefc et al. 2003).

Recently, the multiple-origin hypothesis received further support from the study of chloroplast genetic structure among wild and domesticated grapevine

cultivars from Europe and the Near East (Arroyo-García et al. 2006, Imazio et al. 2006; see below). Moreover, close genetic relationship between wild and cultivated grapes in Sardinia suggested local domestication (Grassi et al. 2003a). Similarly, French cultivars were found to be related to wild vines from south-western France and Tunisia (Aradhya et al. 2003). In contrast, no close relationship was found between cultivated and wild grapevines in Tunisia, suggesting that Tunisian cultivars could have been introduced from other regions (Snoussi et al. 2004).

A comparative study of grapevine cultivars typical for European grape growing regions ranging from Greece to Portugal (Sefc et al. 2000) showed a uniformly high genetic variability in all of the investigated grapevine gene pools (see also Aradhya et al. 2006). The high number of heterozygous individuals within the gene pools exceeded the expected degree of heterozygosity based on a random combination of alleles. Prior to domestication, grapevine plants were dioecious and attained a high level of heterozygosity by outbreeding. As a side effect, deleterious recessive traits accumulated in the genome (Olmo 1976), and a certain level of heterozygosity became a vital condition for the plants. Later on, the selection of successful plants in the course of domestication further favored heterozygous plants, and the observed excess of heterozygosity is probably a consequence of both natural and human selection against homozygosity in grape plants.

7. CHLOROPLAST SSR MARKERS

Microsatellites in the chloroplast genome typically consist of repeats of a single nucleotide (Table 6). Unlike the diploid nuclear genome, which displays two microsatellite alleles - one maternal and one paternal - per locus and individual (see Table 1), the chloroplast genome is haploid, with only one allele per locus in each individual. Furthermore, since the chloroplast genome does not recombine during sexual reproduction, all alleles on the chloroplast are linked and therefore inherited together.

The first assessment of cpSSR diversity in grapevine was by Lefort et al. (2000). Five universal microsatellites primers developed for angiosperms by Weising and Gardner (1999) were tested in 77 grapevine cultivars from Greece and other geographic origins. The 5 cp SSR loci yielded a total of 13 alleles whose combinations yielded 17 chloroplast haplotypes (chlorotypes). The lack of genetic structure among the Greek cultivars was interpreted as a consequence of extensive exchange of cultivars between Greek regions. In the same study, maternal inheritance of the chloroplast genome, which is typical for angiosperm plants, was supported by the cp-microsatellite analysis of the documented cross between cvs Bicane as the female and Muscat de Hambourg as the male par-

ent,which gave rise to the cv Italia (Table 4).

Arroyo-García et al. (2002) confirmed the maternal inheritance of grapevine chloroplasts by analysis of 20 progeny from a cross between cultivars Dominga (used a female) and Autumn seedless (used as male). Hence, genotyping with cp SSR allows to determine the direction of spontaneous crosses, such as in the case of Cabernet Sauvignon, where Sauvignon blanc was identified as the female partner in the cross with Cabernet franc (Bowers and Meredith 1997). Parentage studies and cultivar identification can be strengthened by the combined use of nSSR and cpSSR as exemplified by the case of Muscat of Hamburg, found to be a progeny of Schiava Grossa x Muscat of Alexandria, with the use 30 nSSR nuclear and 5 cpSSR (Crespan 2003). More recently, Akkak et al. (2007) used nuclear and chloroplast microsatellites to show that Cardinal cannot be the progeny of the cross Flame Tokay (mutant of Ahmer Bou Amer) x Ribier (syn. 'Alphonse Lavallee') carried out in 1939 at the Horticulture Field Station of Fresno, California, as reported in various grapevine literature. One of the parents in the cross was probably mislabelled as Flame Tokay.

Furthermore, cpSSR markers were successfully employed for profiling of must samples, in particular since the respective proportion of cultivars in blends could be inferred on the basis of the relative signal intensities of the alleles recorded by an automatic sequencer (Baleiras-Couto and Eiras-Dias 2006).

Chloroplast markers have also been used for genetic characterization of regional cultivar gene pools, and to address the domestication history of the grapevine and the origin of certain cultivars. Doulaty Baneh et al. (2007) conducted a study of wild grapevine and grapevine cultivars in Iran with two polymorphic cpSSR markers, ccmp 3 and ccmp10 (Weising and Gardner 1999) in combination with nuclear markers. In the wild vines, ccmp10 was monomorphic, but ccmp3 displayed 2 alleles. Interestingly, the seedless cultivar Sultani (synonym Sultanina in Greece) harbored a chlorotype absent from Iranian wild vines, although it has long been told to originate from the city of Sultanieh (Iran). Rossoni et al. (2003) combined AFLP and cpSSR markers to genotype local cultivars of Oltrepò pavese (Pavia, Italy), and concluded that local cultivars of this region were in fact from diverse origins.

In different *Vitis* species (*Vitis vinifera*, *Vitis berlandieri, Vitis riparia, Vitis rupestris* and interspecific hybrids), three of ten analysed loci (cpSSR3, cpSSR5 and cpSSR10; Weising and Gardner 1999) revealed intra and interspecific length variation (Arroyo-García et al. 2002) and the distribution of chlorotype frequencies in Spanish and Greek cultivars suggested that domesti cation happened independently in these two regions. The three cpSSR loci were also used in a study of Tunisian grapevine germplasm (Snoussi et al. 2004), where the same four chlorotypes were found as in the Spanish and Greek cultivars, albeit at different frequencies, in both the wild and cultivated grapevines.

Table 6. Polymorphic chloroplast microsatellite primers tested in *Vitis vinifera*. Primer pairs were initially designed for *Nicotiana tabacum* L. chloroplast genome [[a] Chung and Staub (2003), [b] Weising and Gardner (1999) and [c] Bryan et al. (1999)]. Information compiled from Arroyo et al. (2006). [d]Allele size observed in species of the genus *Vitis* other than *Vitis vinifera.*

Name	Forward (F), Reverse (R) primers (5' to 3')	SSR motif	Gene names	Allele sizes in *Vitis* ssp. (bp)[d]	Expected size in tobacco (bp)
ccSSR-5 [a]	F: TCTGATAAAAAACGAGCAGTTCT R: GAGAAGGTTCCATCGGAACAA	$(T)_{10}$	rps2-rpoC2	254,255	270
ccSSR-9 [a]	F: GAGGATACACGACAGARGGARTTG R: CCTATTACAGAGATGGTGYGATTT	$(A)_{13}$	Ycf3	165,166	173
ccSSR-14 [a]	F: GGGTATAATGGTAGATGCCC R: GCCGTAGTAAATAGGAGAGAAA	$(T)_{14}$	rps19-rpl2	201,202,203,204[d]	200
ccSSR-23 [a]	F: AYGGRGGTGGTGAAGGGAG R: TCAATTCCCGTCGTTCGCC	$(A)_{14}$	rp12-TrnH	279[d],280,281,282	217
cpSSR3 [b]	F: CAGACCAAAAGCTGACATAG R: GTTTCATTCGGCTCCTTTAT	$(T)_{11}$	trnG intron	106,107	112
cpSSR5 [b]	F: TGTTCCAATATCTTCTTGTCATTT R: AGGTTCCATCGGAACAATTAT	$(C)_7(T)_{10}(T)_5C(A)_{11}$	3´to rps2	105,104	121
cpSSR10 [b]	F: TTTTTTTTTAGTGAACGTGTCA R: TTCGTCGDCGTAGTAAATAG	$(T)_{14}$	rpl2-rps19	114,115,116	103
NTCP8 [c]	F: ATATTGTTTTAGCTCGGTGG R: TCATTCGGCTCCTTTATG	$(T)_{11}$	trnG intron	248,249	251
NTCP12 [c]	F: CCTCCATCATCTCTTCCAA R: ATTTATTTCAGTTCAGGGTTCC	$(T)_{10}(A)_{13}$	Rps2/RF862	118,119	126

Imazio et al (2003) profiled six Italian and four Spanish populations of wild grapevine with six nuclear and eight chloroplast microsatellites, a study extended to eight Italian and six Spanish populations by Grassi et al. (2003b).These studies showed a high diversity in Italian wild vine, in congruence with the fact that Italy has repeatedly served as ice age refuge. Spanish wild vines were less diverse and genetically distant from Italian ones. Similarly, in the wild vines studied by Arroyo-García et al. (2006) and Grassi et al. (2006), chlorotype diversity was higher in central Mediterranean and Eastern locations than in the western locations, which agrees with the proposition, based on phenotypic variation, that the Anatolian Peninsula and Transcaucasia represent the diversity centre of *V. vinifera*.

The most extensive genetic study on the European wild and domesticated grapevine gene pool included 513 grapevine cultivars and 688 wild grapes collected from 130 locations spanning from the Iberian Peninsula to the Middle East (Arroyo-García et al. 2006). The chloroplast marker set from Arroyo- García et al. (2002) selected from Weising and Gardner (1999) for their polymorphism in *Vitis,* was supplemented with cpSSRs from tobacco (Bryan et al. 1999, Chung and Staub 2003) to obtain nine polymorphic marker loci (Table 6). The distribution of chlorotypes in the cultivated grapes, and the genetic relationships between grapevines and wild vines from different regions, led to the inference of two domestication centers for cultivated grapevine, one in the Near East and one in the Western Mediterranean. Similar conclusions were reached by other authors (Aradhya et al. 2003, Grassi et al. 2003, Imazio et al. 2006). Since certain chlorotypes were found to be restricted to certain regions, controversial origins of some grapevine cultivars could perhaps be resolved by analysis of their chloroplast genome.

8. GENETIC DATABASES OF SSR PROFILES

A database of grapevine genetic identity profiles at 6 nuclear microsatellite loci was reported in 1994 for more than 200 cultivars, with the initial aim to offer it to the public research community (Thomas et al. 1994). Not long after the publication, commercial interest in using the database as the basis of a commercial grapevine DNA identification service at the Australian Wine Research Institute (AWRI) resulted in restricted access (Scott et al. 1996). The AWRI service has operated since 1996 and has received from customers a diverse range of samples including wood tissue, berry clusters, leaf tissue and canes for a multitude of purposes. Information contained in the database includes detailed information about the plant used as the source of the cultivar DNA profile with all plants sourced from national or state germplasm collections.

A similar methodology inspired a second database service released under the name Greek Vitis Database (Lefort and Roubelakis-Angelakis 2000, Lefort et al. 2002b, Lefort 2003). This kind of database is aimed not only to make widely available the genetic information about Greek cultivars but also and especially to facilitate germplasm management of Greek grapevine resources in Greece, disseminated in several collections. This database service is in fact a cluster of multimedia databases, offering information, genetic profiles at nine nuclear microsatellite loci for 298 cultivars, mainly of Greek origin, as well as genetic profiles obtained with 5 chloroplast microsatellite loci and an image database of 240 cultivars. Allele size calling has been brought in agreement with other laboratories according to the results published in a study of European grapevines (Sefc et al. 2000) in order to make results useable and comparable between different regions of the world.

A similar database was developed for Bulgarian genetic resources and included a microsatellite profile database of grapevine cultivars (Russanov et al. 2003), based on the use of the same set of nuclear microsatellite loci. The Institute for Grapevine Breeding of the Julius Kühn-Institut (Federal Research Centre for Cultivated Plants, JKI), in Geilweilerhof hosts the European Vitis Database established in 1997 by the European Network for Grapevine Genetic Resources Conservation and Characterization (GENRES081) and the Vitis International Variety Catalogue. Only the latter proposes a few microsatellite profiles as a complement to ampelographic data. A few other databases are available through the web and some others are still not available though their existence is known (Table 7). One might expect the release of more information to the scientific community in the future.

As foreseen by Thomas et al. (1994) long time ago, international collaboration should result in a hopefully near future in an international database of SSR profiles of cultivars from all collections worldwide, supporting the management of ampelographic collections as well as a microsatellite-based certification system. The ECPGR Working Group on Vitis has been promoting such a tool since 2003 with the objective that the European Vitis Database becomes that international database.

9. CONCLUSIONS

Microsatellite markers have proved extremely useful for accurate cultivar identification, uncovering synonyms, determining genetic relatedness and for genetic mapping. What is not certain, however, is whether microsatellite markers will remain the preferred marker type of the future given the rapid pace of DNA marker technology. Recently, the sequencing of two grapevine genomes has been completed (Jaillon et al. 2007, Velasco et al. 2007), which opens the

Table 7. Existing public and unpublished databases of grapevine SSR profiles.

Database name	Physical address	Internet Address of public databases	Number of genotypes (if known)
European *Vitis* Database	IRZ, Siebeldingen, Germany	http://www.genres.de/eccdb/vitis/	in preparation
Grape Microsatellite Collection (GMC)	IASMA, San Michele, Italy	not public	
Grape SSR database	Australian Wine Research Institute (AWRI)	not public	
International *Vitis* Variety Catalogue	IRZ, Siebeldingen, Germany	http://www.vivc.bafz.de/index.php	46
SSR profiles (not searchable)	BOKU, Vienna, Austria	http://www.boku.ac.at/zag/forsch/grapeSSR2.htm	162
The Bulgarian Plant Genomics Database	Agrobioinstitute, Sofia, Bulgaria	http://bulgenom.abi.bg/AgroBioInstitute%20Selected.htm	76
The Greek *Vitis* Database	University of Crete, Heraklion, Greece	http://gvd.biology.uoc.gr/gvd/index.htm	298
The Swiss Vitis Microsatellite Database	University of Neuchâtel, Switzerland	http://hydra.unine.ch/svmd/	170
Ukrainian, Moldovan and Russian *Vitis* Database	Magarach Institute, Yalta, Ukraine	not public	104
Vitis SSR database	University of California, Davis, USA	not public	
Vitis SSR database	INRA Montpellier, France	not public	

door to the development and deployment of additional marker techniques such as SNP (single nucleotide polymorphism) genotyping. But it is obvious from this review that no other molecular marker or technology, including ampelography, has provided so much useful information in such a short period of time for grapevine genetics.

The standardization capacity of microsatellites is relatively high in comparison to other genetic identification tools which makes this technology even more effective. A large 4-year project 'Management & Conservation of Grapevine Genetic Resources' (GrapeGene06) (http://www.montpellier.inra.fr/ grape gen06/) is under the action between 25 partners from 17 countries with the aim of standardizing microsatellite protocols and database of majority of the grapevine cultivars.

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